Testing the Mating System Model of Parasite Complex Life Cycle Evolution Reveals Demographically Driven Mixed Mating

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ABSTRACT: Many parasite species use multiple host species to complete development; however, empirical tests of models that seek to understand factors impacting evolutionary changes or maintenance of host number in parasite life cycles are scarce. Specifically, one model incorporating parasite mating systems that posits that multihost life cycles are an adaptation to prevent inbreeding in hermaphroditic parasites and thus preclude inbreeding depression remains untested. The model assumes that loss of a host results in parasite inbreeding and predicts that host loss can evolve only if there is no parasite inbreeding depression. We provide the first empirical tests of this model using a novel approach we developed for assessing inbreeding depression from field-collected parasite samples. The method compares genetically based selfing rate estimates to a demographic-based selfing rate, which was derived from the closed mating system experienced by endoparasites. Results from the hermaphroditic trematode *Alloglossidium renale*, which has a derived two-host life cycle, supported both the assumption and the prediction of the mating system model, as this highly inbred species had no indication of inbreeding depression. Additionally, comparisons of genetic and demographic selfing rates revealed a mixed mating system that could be explained completely by the parasite's demography (i.e., its infection intensities).

Keywords: selfing rate, inbreeding depression, trematode, hermaphrodite, precocious development, infection intensity.

Introduction

Many parasite species have complex life cycles that require sequential movements among hosts to complete their life cycle. In particular, parasite complex life cycles entailing trophic transmission (i.e., predator becomes infected by eating infected prey) have evolved independently across several phyla, including nematodes, platyhelminths, pentastomes, and acanthacephalans (Parker et al. 2015*a*, 2015*b*). Commonly, this trophic transmission involves a juvenile parasite stage in an intermediate host (i.e., the prey) and a sexually reproducing parasite stage in the definitive/final host (i.e., the predator). In reviews on the evolutionary origin or maintenance of helminth trophic transmission, Parker et al. (2015*a*, 2015*b*) highlight how changes in the number of hosts used in a parasite life cycle can impact various aspects of parasite fitness, such as transmission (Choisy et al. 2003; Parker et al. 2003), growth and fecundity (Parker et al. 2003; Benesh et al. 2013), and mate availability (Brown et al. 2001). Moreover, maintenance of a complex life cycle has potential fitness costs, such as the cost of generalism, defined as the ability to exploit different hosts such as the prey and predator hosts (Parker et al. 2015*a*; Benesh et al. 2022), or the risk of not reproducing if not transmitted to the definitive host (e.g., ingestion of infected prey by nonhost predator or the intermediate host dies before being consumed by predator host). These maintenance costs, in part, led Parker et al. (2015*a*, p. 268) to ask, "Why should a parasite suppress its growth and reproduction in an earlier host, making successive exploitation of more than one-host species an obligatory (rather than facultative) feature of the life cycle? Why reproduce sexually only in the definitive host?"

One model that addresses this question directly is that of Brown et al. (2001), which we refer to as the mating system model of parasite complex life cycle evolution. The mating system model is based on the premise that trophically transmitted hermaphroditic parasites become increasingly concentrated in hosts as transmission progresses up the food chain, resulting in more outcrossing opportunities. Prey hosts typically have lower infection intensities (the number of parasites in each host) than predator hosts (Brown et al. 2001). Because predators eat many infected prey, there will be more potential mates in a predator

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host. The model, therefore, implicitly assumes that parasites sexually reproducing within the prey hosts experience more inbreeding because of the presence of fewer potential mates (i.e., there is increased selfing). Having both prey and predator hosts in the life cycle may also reduce inbreeding because transmission across multiple hosts likely creates mixing of unrelated parasites either via larval dispersal or via intermediate host dispersal (Rauch et al. 2005; Criscione and Blouin 2006). Such mixing may reduce the chance for kin mating (Criscione et al. 2022). With more inbreeding, the fitness cost imposed in the model is inbreeding depression (i.e., fitness of an inbred individual is less than that of an outcrossed individual; Charlesworth and Willis 2009). The model predicts a predator host would be lost only if there is no resulting parasite inbreeding depression. In light of the assumption and prediction of the Brown et al. (2001) model, the question of Parker et al. (2015*a*) is addressed in that parasites should not sexually reproduce in their prey host, as this could lead to inbreeding (via increased selfing or kin mating) and hence inbreeding depression, a hypothesis that dates to Grabda-Kazubska (1976) and Font (1980). Consequently, a parasite complex life cycle in the form of trophic transmission is maintained, in part, because inbreeding depression represents one possible cost to truncation of the predator host. The model is applicable to the species-rich trematodes and cestodes (more than 97,000 estimated species, most being hermaphroditic; Strona and Fattorini 2014); however, selfing rates and inbreeding depression data are hard to obtain from these parasites because of difficulties in laboratory maintenance of multihost life cycles (Criscione 2016; Detwiler et al. 2017; Caballero and Criscione 2019). As such, the tenets of Brown et al. (2001) remain untested.

Our primary goals were to test both the assumption and the prediction of Brown et al. (2001) in the trematode *Alloglossidium renale*. We first highlight that among trematodes (flukes), there is diversity in the number of developmental stages and/or hosts required for life cycle completion (Roberts et al. 2009). The most common trematode life cycle requires three hosts (Olson et al. 2003). A mollusc first intermediate host gets infected by a larval miracidium (direct penetration or consumes egg with miracidium). In the mollusc, there is obligate asexual reproduction producing genetically identical (clonemates) cercariae. These cercariae leave the first host to penetrate and encyst as metacercariae (juvenile stage) in a second intermediate host, which is ingested by a third host (i.e., the definitive host), where flukes sexually reproduce to produce a larval miracidium in an egg. Eggs are released into the external environment, often with host feces. The latter two hosts represent the trophic transmission stages that are the focus of our study. One deviation from an obligate three-host life cycle that can be used to test the Brown et al. (2001) model is the evolution of precociousness (historically termed "progenesis") where sexual maturation occurs in what is typically regarded as the second intermediate host (i.e., the prey host; Lefebvre and Poulin 2005*c*). Precocious development can lead to life cycle truncation via the elimination of the ancestral third host (i.e., the predator host). A meta-analysis and a few intraspecific studies have not found any fitness costs (estimated via body size, egg size, and/or fecundity) associated with precocious development (Lefebvre and Poulin 2005*a*, 2005*b*; Oliva and Alvarez 2011; Kasl et al. 2015; Villa and Lagrue 2019). Given the likely fitness costs to generalism (Parker et al. 2003, 2015*a*; Benesh et al. 2022), Lefebvre and Poulin (2005*b*, p. 47) raised a point similar to the questions of Parker et al. (2015*a*; i.e., "why facultative progenesis is not more widespread remains a mystery"). One possible answer to this "mystery" is again provided by Brown et al. (2001) in that precocious life cycles are prone to inbreeding and thereby susceptible to inbreeding depression (Grabda-Kazubska 1976; Font 1980).

Alloglossidium renale provides an excellent system to test both the assumption (i.e., increased inbreeding in a "prey-only" life cycle) and the prediction (i.e., no inbreeding depression in a truncated life cycle) of Brown et al. (2001) because, in part, phylogenetic analysis indicated that *A. renale* has a derived truncated life cycle where trophic transmission from the second host to the third host was lost (Kasl et al. 2018). Specifically, *A. renale*, has a two-host life cycle with sexual reproduction in the paired antennal glands of the Mississippi grass shrimp *Palaemon kadiakensis* (figs. 1, S1; figs. S1, S2 are available online) and asexual reproduction in a presumed aquatic snail first host (Font and Corkum 1975). The antennal glands are discrete mating boundaries, as the adult flukes cannot move between them. Phylogenetic reconstruction showed an ancestral three-host life cycle in the genus *Alloglossidium* where a sister species to *A. renale*, *Alloglossidium progeneticum*, also has a three-host life cycle (asexual reproduction in a snail, metacercariae encyst in the antennal glands of crayfish, adults sexually reproduce in catfishes). The *A. renale* system is also ideal to test the Brown et al. (2001) model because the ancestral mating system of *A. renale* was likely one of no inbreeding. Specifically, populations of *A. progeneticum* with an obligate three-host life cycle have little to no evidence of selfing (J. M. Hulke and C. D. Criscione, unpublished data), a result consistent with other trematodes that have fully aquatic or aquatic to terrestrial/bird three-host life cycles (Agatsuma and Habe 1986; Criscione and Blouin 2006; Keeney et al. 2007; Louhi et al. 2010; Criscione et al. 2011, 2020; Namsanor et al. 2020). A study on the distribution of *A. renale* between glands within hosts revealed that 29% of 381 glands had single infections with gravid individuals (Hulke et al. 2021). Hence,

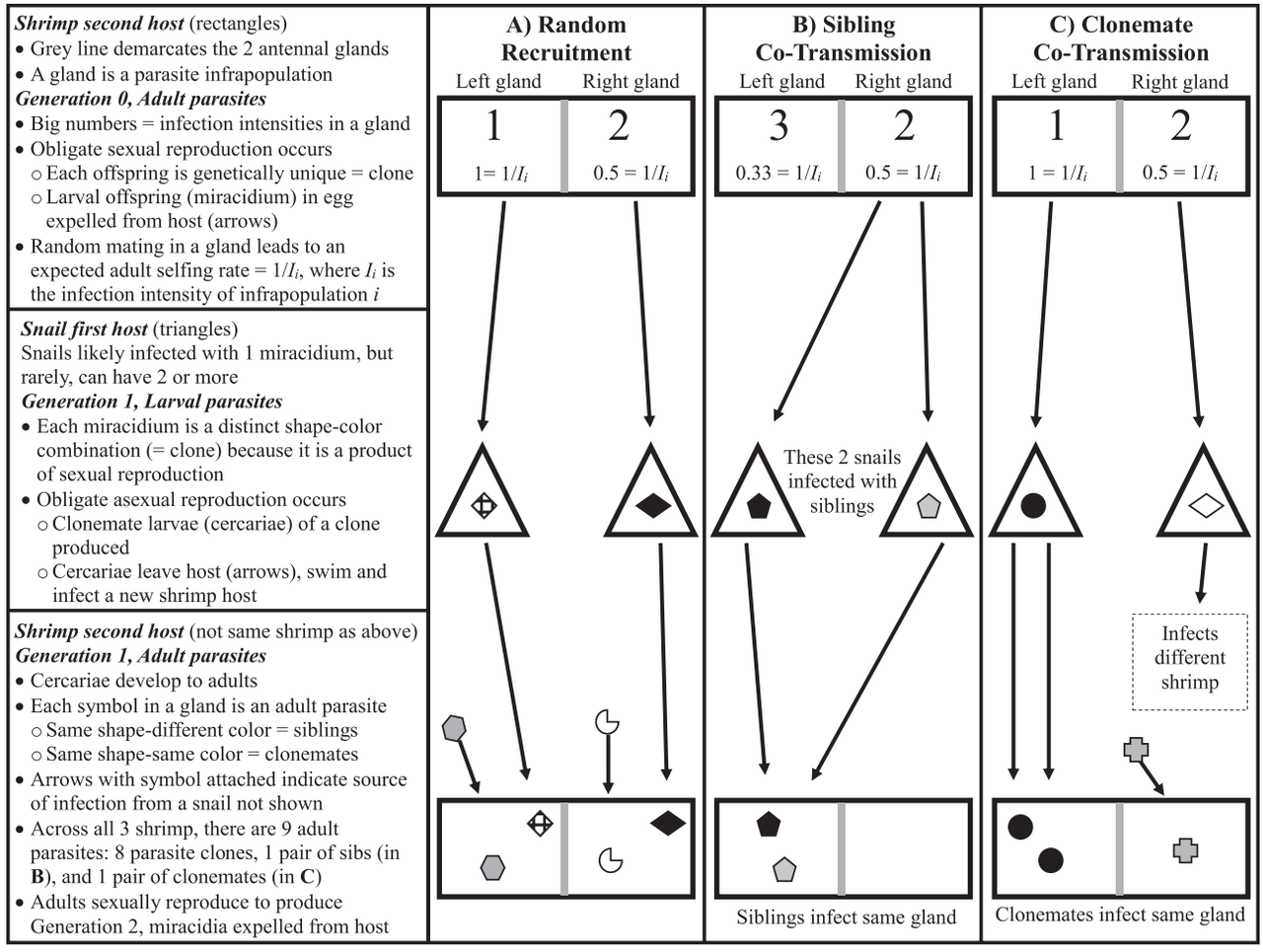

Figure 1: The two-host life cycle of *Alloglossidium renale*. The legend on the left describes the life cycle and meanings of the various shapes or numbers displayed. *A* illustrates random recruitment (with respect to clone, i.e., genetically unique entity that was the product of sexual reproduction) of generation 1 adult parasites into shrimp hosts. *B* illustrates a situation of sibling cotransmission, and *C* shows a situation of clonemate cotransmission. It is not shown, but clonemate and sibling cotransmission into one gland could happen as well (e.g., imagine another black pentagon in the gland shown in *B*).

there is evidence that *A. renale* can self-mate, and thus has potential for inbreeding, in natural populations.

In testing the Brown et al. (2001) model, we also provide a novel means to test for inbreeding depression in nature by comparing genetic selfing rate (s_G) estimates to demographic selfing rate (s_D) estimates (see "Methods"). Using the feature that endoparasites exist in closed mating systems (i.e., individuals cannot mate with parasites in another host), Detwiler et al. (2017) showed how infection intensities can be used to calculate population-level s_D estimates. The distribution of infection intensities is a key descriptor of parasite demography, hence our use of the term "demographic" to label s_D. For *A. renale*, parasites in a host are further isolated into mating groups between the two antennal glands, so intensity per gland is used in estimating s_D (see "Methods").

Methods

Field Collections

Details on sampling and collection locations are given in Hulke et al. (2021). In short, we had four sample collections from three locations: Whisky Bay, Louisiana, in 2019 (LA-2019); Gus Engeling Wildlife Management Area in Texas in 2015 (TX-2015); and a standing water body in Lefore County, Mississippi, in 2014 (MS-2014) and again in 2018 (MS-2018). Parasites were preserved in 70% ethanol until DNA extraction.

An infrapopulation typically refers to all the conspecific parasites within a host individual to denote the boundary of intraspecific interactions (Bush et al. 1997). Because the mating boundary of *Alloglossidium renale* is an antennal gland (henceforth, "gland"), we make the distinction

between a host and gland infrapopulation throughout the article. Likewise, we make the distinction of intensity per host and intensity per gland as the number of parasites per infected host or gland, respectively. The component population refers to all the conspecific parasites in a host population at a given place and time (Bush et al. 1997).

In all four collections, we could estimate s_D because we kept track of each host and gland (left vs. right) a parasite came from. For MS-2018 and LA-2019, preserved parasites were stored by gland; thus, samples could be used to test for cotransmission of related individuals by gland (see below). For MS-2014, preserved individuals were stored in vials separated by host only; thus, we could test for cotransmission of related parasites only at the host level. Most parasites from TX-2015 were put into a single vial, so it was not possible to test for cotransmission of related parasites.

DNA Extraction and Microsatellite Genotyping

The anterior portion of a fluke (<0.5 mm of tissue) was removed for DNA extraction to prevent the possibility of extracting sperm from an outcrossing event. Methods for DNA extraction, whole genome amplification, polymerase chain reaction, and genotyping of 19 loci (ALRE_r17074, ALRE_8921, ALRE_5947, ALRE_6037, ALRE_11453, ALRE_3134, ALRE_4875, ALRE_2190, ALRE_2125, ALRE_2951, ALRE_r25337, ALRE_200, ALRE_4802, ALRE_5576, ALRE_5709, ALRE_5926, ALRE_3690, ALRE_156, and ALRE_2616) are given in Hulke and Criscione (2023).

Clonemate and Sibling Identification

Following the terminology of Arnaud-Haond et al. (2007), "clone" represents a unique genetic entity. In the case of trematodes, a clone is an individual that is the product of sexual reproduction. In the mollusc first host, a clone, via obligate asexual reproduction, produces many genetically identical individuals, which we refer to as "clonemates."

As s_G estimates reflect the previous generation(s) of mating, and as asexual reproduction occurred in the parasite's larval stage before the sampled adult stage (fig. 1), clonemates need to be identified for downstream analyses (Prugnolle et al. 2005). To statistically determine whether repeated multilocus genotypes (MLGs) were clonemates, we used COLONY (ver. 2.0.6.7; Jones and Wang 2010). COLONY uses a maximum likelihood method to assign sibships (full siblings, half-siblings, and unrelated) among sampled individuals and then assesses whether full-sibling relationships are possible clonemate relationships.

COLONY was run separately for each sample collection with the following settings: monoecious, diploid, polygamy, inbreeding, and clonal inference. We did three runs with the full-likelihood method, set run length to very long with very high precision, and used allele frequencies estimated from the data and updated during the run. No a priori sibships were designated. False allele and allelic dropout rates were set to 0.005 to account for possible mutation and/or technical scoring error, respectively. Clonemate and full-sibling relationships were identified using the output files bestClones and BestFSFamily, respectively. Relationships were deemed significant if the probability of inclusion was ≥90%.

Tests of Full-Sibling and Clonemate Cotransmission

Along with selfing, both clonemate and sib mating can contribute to the total inbreeding. Criscione et al. (2022) described how clonemate mating has a genetic inbreeding signature identical to that of selfing and used the proportion of clonemate dyads (P_C) within hosts to make inferences on the potential contribution of clonemate mating to inbreeding. Similarly, Detwiler and Criscione (2017) used the proportion of sibling dyads (P_K) within hosts to draw on the potential for biparental inbreeding. Both P_C and P_K are ecological metrics of transmission (i.e., they are probabilities of co-occurring clonemates or full siblings, respectively, within infrapopulations) and evolutionary metrics of potential inbreeding (i.e., they can be used to infer potential clonemate or full-sibling mating rates, respectively). Using the COLONY clonemate and full-sibling relationships among adult flukes, we estimated P_C and P_K with average values (weighted by sample size) across gland infrapopulations (for infection intensities > 1) as $w\overline{P_C}$ and $w\overline{P_K}$, respectively (Detwiler and Criscione 2017; Criscione et al. 2022). To test whether there is nonrandom transmission of clonemates or full siblings into glands and thus whether there is the potential for clonemate or full-sibling mating to contribute to the total inbreeding, $w\overline{P_C}$ and $w\overline{P_K}$ were compared with the component population proportion of clonemate dyads (P_{EC}) and full-sibling dyads (P_{EK}), respectively. The terms P_{EC} and P_{EK} represent the random chance expectations of P_C and P_K, respectively (Detwiler and Criscione 2017; Criscione et al. 2022). We conducted randomization tests whereby all individual flukes were shuffled among all infected glands (including single infections) while keeping gland intensities constant. After each randomization (10,000 times), $w\overline{P_C}$ and $w\overline{P_K}$ were calculated. If observed values of $w\overline{P_C}$ or $w\overline{P_K}$ fell within the 95% confidence intervals (CIs) of their respective simulated distributions, then the values were considered to not significantly differ from that expected under random allocation of parasites among glands. To check that the null distributions accurately reflected the random chance expectations of P_C and P_K, we verified that

the means of the simulated $w\overline{P_C}$ and $w\overline{P_K}$ values matched the observed P_{EC} and P_{EK} values, respectively. An R function (function.kin.distribution.R) to calculate $w\overline{P_C}$ and $w\overline{P_K}$ and code to conduct the simulations (Kin.mating.simulations) are available at GitHub (https://github.com/JennaHulke/Parasite.Distribution/tree/main). Simulations were run separately for MS-2018 and LA-2019. For reasons noted above, analysis for MS-2014 was conducted at the level of the host, whereas cotransmission of related parasites could not be tested for TX-2015.

Tests of Inbreeding, Selfing Rates, and Genotypic Disequilibrium

We found little to no evidence for clonemate cotransmission and no evidence for full-sibling cotransmission (see "Results"). Thus, inbreeding and s_G estimates were conducted across the parasite component populations. As asexual reproduction occurs after sexual reproduction (fig. 1), clonemates must be reduced to one representative for each clone to assess the mating system stemming from sexual reproduction (Prugnolle et al. 2005).

Although we used COLONY to test whether repeated MLGs were clonemates, we wanted to evaluate result robustness in terms of sensitivity to clonemate identification. Thus, we estimated s_G from three datasets within each of the four collections. The first dataset was based on the COLONY results; clonemates were reduced to one representative. If COLONY classified individuals with different MLGs as clonemates (see "Results"), we retained the MLG with the highest probability in the output Off-Genotype as the representative of that clone. The next two datasets represent extremes of possibilities. The second dataset retained all genotyped individuals and thus assumes that all repeated MLGs are the product of sexual reproduction. In highly inbred populations, repeated MLGs that are products of sexual reproduction are common (e.g., Bomblies et al. 2010). The third dataset treated all repeated MLGs as clonemates and thus was reduced to a single representative of each MLG. GenALEx (ver. 6.503) was used to identify repeated MLGs (Peakall and Smouse 2012).

In each of the three datasets within each of the four collections, we used five s_G estimates of component populations. The first used deviations from Hardy-Weinberg equilibrium as quantified by F_{IS}, which was estimated in GENETIX (ver. 4.05; Weir and Cockerham 1984; Belkhir et al. 2004) with significance determined by 10,000 randomizations of alleles among individuals and CIs obtained by 10,000 bootstraps over individuals. Selfing rates (designated s_{GF}) were calculated from the inbreeding equilibrium relationship $s_{GF} = 2F_{IS}/(1 + F_{IS})$ (Jarne and David 2008). The second method (s_{GG}) used identity disequilibrium (i.e., correlated heterozygosity between loci), as measured by the g_2 statistic, which quantifies the relative excess of genotypes heterozygous at two loci and can be equated to a selfing rate (see eq. [9] in David et al. 2007). We used the R package inbreedR (Stoffel et al. 2016) to estimate s_{GG}, test statistical significance with 10,000 permutations of single-locus data among individuals, and produce CIs by 10,000 bootstraps over individuals. The next two estimators were from the Bayesian model–based approaches implemented in the software BES (Redelings et al. 2015) and INSTRUCT (Gao et al. 2007). BES models coalescence events while accounting for identity disequilibrium to estimate the likelihood of selfing rates. In BES, we set the generic script parameter of f_other to 0. Three independent chains of 100,000 iterations were run to infer the selfing rate (s_{GB}). The statreport command was used to obtain the median estimate and credible interval of s_{GB}. The potential scale reduction factors for the s_{GB} estimates of LA-2019, MS-2018, MS-2014, and TX-2015 were all approximately 1, indicating that the chains had similar posterior distributions. INSTRUCT uses information on homozygosity and allele frequencies to estimate selfing rates. In INSTRUCT, the selfing rate (s_{GI}) and its credible interval were estimated using the best run (the highest posterior median log likelihood) of three independent chains. We assumed a single population (K set to 1) and used mode 2 (infer population selfing rates). Each chain had 1,000,000 iterations in total with a burn-in of 500,000. The Gelman-Rubin statistics for convergence were good, with medians and credible intervals from each chain being nearly identical. The preceding four s_G estimates account for the cumulative effect of inbreeding over multiple generations. The fifth selfing rate (s_{GC}) and its CI were obtained from COLONY in the output file Inbreeding. COLONY uses pedigree analysis to estimate whether individuals are the product of a selfing event. Thus, s_{GC} represents the proportion of sampled individuals that are classified as the product of a selfing event from the previous mating generation (i.e., it is a single-generation estimate).

For each of the datasets in each of the four collections, allelic richness (rarefied to the smallest collection sample size) and gene diversities were calculated using FSTAT (ver. 2.9.4; Goudet 1995), and genotypic disequilibrium was tested using GENEPOP (ver. 4.7.5; Rousset 2008). For the latter, Markov chain settings were 1,000 dememorization, 100 batches, and 1,000 iterations per batch. To globally determine whether the number of significant pairwise loci associations was greater than expected by chance, we used the exact binomial test ($\alpha = .05$).

Tests of Inbreeding Depression

Ritland's (1990) theory provides a means to test for inbreeding depression by comparing a progeny array selfing

rate (s_P) to an inbreeding equilibrium estimate of F. Specifically, the relative fitness of a selfed individual is $w = 2[(1 - s_P)F/(s_P(1 - F)]$ (eq. [3] and fig. 1*c* in Ritland 1990). The premise is that with inbreeding depression, a portion of selfed individuals do not survive to adulthood. Hence, F estimated from an adult-stage sample (i.e., F_{IS}) is lower than predicted from the inbreeding equilibrium formula $F = s_P/(2 - s_P)$ (Jarne and David 2008).

Our method is a modification of Ritland's (1990) method. Instead of using s_P, we describe below two s_D estimates. The potential for selfing is estimated, so s_D carries the assumption that selfing occurs in nature. We return to this assumption in "Discussion." In simple terms, s_D is compared with s_G. As the s_G estimates s_{GF}, s_{GG}, s_{GB}, and s_{GI} reflect the cumulative effect of inbreeding over multiple generations (i.e., using F, in the case of s_{GF}, or analogous to using F in the cases of s_{GG}, s_{GB}, and s_{GI}), the relative fitness of selfing can be rewritten as $w = [s_G(1 - s_D)/(s_D(1 - s_G)]$ (modified variables but the same as eq. [8] in Ritland 1990). We did not use s_{GC} in this context because being a single-generation estimate, it does not test the same hypothesis as the four multiple-generation estimates. In general, if $s_G < s_D$, then one would infer inbreeding depression, as inbred individuals are not surviving to become adults; $s_G = s_D$ indicates no inbreeding depression; and $s_G > s_D$ indicates outbreeding depression.

Our two s_D estimates follow the premise that with random union of gametes (i.e., random mating for a hermaphrodite) in a population of N monoecious individuals, the proportion of selfing is $1/N$ (Wright 1943). Specifically, if there is random mating within an infrapopulation, an individual parasite's selfing rate or an average infrapopulation selfing rate is $1/I_i$, where I_i is the infection intensity of infrapopulation i (Detwiler et al. 2017). For example, an intensity of 2 has an average selfing rate of 0.5, an intensity of 3 has an average selfing rate of 0.33, and so on (fig. 1). Detwiler et al. (2017) presented theory (their eq. [5]) showing that if hermaphroditic parasites mate randomly within infrapopulations and there is random reproductive success in the component population, then the component population selfing rate is simply the inverse of the mean infection intensity; we refer to this demographic estimate as s_{DR}. For *A. renale*, s_{DR} is the inverse of the mean infection intensity per gland. However, among parasitic helminths, especially flatworm parasites, density-dependent growth and fecundity are common ecological phenomena that result in large inequalities in reproductive success (also known as the crowding effect; Read 1951; Poulin 2011). With such crowding, the total number of parasite offspring originating from each infrapopulation may be similar no matter the level of intensity (Dobson 1986). The effect is to increase the component population selfing rate (Detwiler et al. 2017). To generate s_D when there is random mating within infrapopulations and density-dependent fecundity, the sum of the average selfing rates of infrapopulations ($1/I_i$) is divided by the number of infrapopulations (Detwiler et al. 2017). We refer to the latter estimate as s_{DC}. We note that the random and density-dependent reproductive successes modeled within s_{DR} and s_{DC}, respectively, represent the ends of a likely continuum. To obtain CIs for both s_{DR} and s_{DC}, we bootstrapped (10,000 times) infected hosts separately for each collection. After each resampling of infected hosts with replacement, s_{DR} and s_{DC} were recalculated as described above.

In a hierarchical framework, we assessed whether s_D significantly differed from s_G. Within each sample collection, we compared s_D with s_G 84% CIs (or credible intervals for the Bayesian estimates). Nonoverlapping 84% CIs approximate a significant difference at $p = .05$ (MacGregor-Fors and Payton 2013). If two or more of the four s_G estimates (s_{GF}, s_{GG}, s_{GB}, and s_{GI}) did not have overlapping 84% CIs with s_D, then we deemed s_D and s_G significantly different within that sample collection as $p = .014$ with an exact binomial at $\alpha = .05$ (one difference has $p = .185$). Next, to address whether s_{DR} or s_{DC} could explain s_G overall, we asked how many of the four sample collections had a significant difference between s_D and s_G. If two or more of the four sample collections had a significant deviation, then overall, s_D was deemed different from s_G: $s_G < s_D$ would be interpreted as inbreeding depression, and $s_G > s_D$ would be interpreted as outbreeding depression.

Results

Demographic Summary

Prevalence, mean abundance, and mean intensity per host were summarized in Hulke et al. (2021). Table 1 provides the total number of infected hosts sampled, mean intensity per infected gland, and the percentage of parasites in single infections for each sampling location.

Identification and Cotransmission Tests of Clonemates and Full Siblings

Table 2 summarizes genotyping information and statistics for clonemate and full-sibling identification. Note that the number of unique MLGs can be greater than the number of identified clones, as similar MLGs might be statistically determined to be clonemates. Also, the number of unique MLGs can be fewer than the number of identified clones because repeated MLGs may not be statistically rejected as the product of sexual reproduction. Such a situation might occur when marker information is low and/or when there is high inbreeding. In LA-2019, which had only 10 polymorphic loci, marker information was too low to

Table 1: Demographic summary statistics

Location	No. infected hosts	No. parasites observed	Mean intensity per infected gland	Parasites in single infections (%)
Louisiana 2019	45	107	1.65	35.5
Mississippi 2018	96	205	1.46	41.9
Mississippi 2014	33	80	1.77	33.8
Texas 2015	39	72	1.31	58.3

distinguish more unique MLGs (fig. S2). However, clonemate statistical testing identified nearly all repeated MLGs in LA-2019 as the product of sex, so clonemate overestimation was not an issue. Also, comparisons of F_{IS} values between datasets with and without repeated MLGs suggest that inbreeding resulted in repeated MLGs (see below).

Only one sample collection had support for clonemate cotransmission. Of the 196 individuals genotyped from MS-2018, eight clones had significant clonemates; $P_{EC} = 0.0006$. Only one pair of clonemates was found in the same gland leading to $w\overline{P_C} = 0.0183$ (marginally significant as just outside the simulated 95% CI: 0–0.00917). We could not test P_C in TX-2015 (discussed above), but only two clones of 61 genotyped parasites had one clonemate each (see supplemental_S1.xlsx, available online). We did not find any evidence of full-sibling cotransmission in the three populations that could be analyzed (table 2).

Inbreeding, Selfing Rates, and Genotypic Disequilibrium

The s_G estimates from all three datasets within each of the four collections were largely congruent, indicating that the results were robust to clonemate inferences (supplemental_S1.xlsx). This congruence may be because there were few "true" clonemates in the dataset (table 2). The presence of clonemates drives down F_{IS}, so reducing true clonemates to one representative should increase F_{IS} (Prugnolle et al. 2005). However, in comparing the two extreme datasets (all genotyped individuals vs. one representative of each MLG), the opposite effect is observed (supplemental_S1.xlsx). This opposite result suggests that many of the repeated MLGs are indeed the product of sexual reproduction, as the removal of sexual inbred individuals would act to reduce homozygosity and thus decrease F_{IS}. Regardless, all three datasets had similar results. Therefore, we

Table 2: Summary genotyping information and statistics for clonemate and full-sibling identification

	Louisiana 2019	Mississippi 2018	Mississippi 2014	Texas 2015
No. polymorphic loci	10	16	17	18
No. genotyped parasites	99	196	79	61
No. unique MLGs	75	191	76	58
No. clones[a]	98	186	75	59
No. clones with clonemates[b]	1 (2)	8 (18)	4 (8)	2 (4)
$w\overline{P_C}$[c]	0	**.0183**	.0077	NA
P_{EC}[d]	.0002	.0006	.0013	NA
No. full-sib families[e]	10 (26)	12 (27)[f]	5 (10)	6 (13)
$w\overline{P_K}$[c]	0	0	.0044	NA
P_{EK}[d]	.0054	.0016	.0029	NA

Note: MLG = multilocus genotype; NA = analyses not conducted.

[a] Clones (each clone is the product of sexual reproduction) statistically identified using COLONY.

[b] Number of clones with one or more clonemates. In parentheses are the total number of individuals that belong to these clone families.

[c] Average proportion (weighted by sample size) of clonemate dyads ($w\overline{P_C}$) or full-sibling dyads ($w\overline{P_K}$) within glands (or hosts for Mississippi 2014). Values in bold indicate that the observed value fell outside the simulated 95% confidence interval of randomly allocating parasites among glands (or hosts for Mississippi 2014).

[d] The component population proportion of clonemate dyads (P_{EC}) and full-sibling dyads (P_{EK}), which represent the random chance expectations of P_C and P_K, respectively.

[e] In parentheses are the total number of individuals that belong to these full-sib families.

[f] In one family of three individuals, two were clonemates, and these had a full-sib relationship to the third individual. All three are included in this total.

henceforth present results stemming from the reduced dataset based on the COLONY-identified clonemates.

Within each sample collection, the five s_G estimates were largely similar in showing a mixed mating system with high levels of selfing. With one exception (see below), all s_G estimates were greater than 0.66 (fig. 2). For s_G estimates, MS-2014 ranged from 0.665 to 0.776, MS-2018 ranged from 0.816 to 0.883, LA-2019 ranged from 0.568 to 0.823, and TX-2015 ranged from 0.773 to 0.808. Among the four estimators that account for cumulative inbreeding over multiple generations, s_{GF} was always the highest, and s_{GG} was always the lowest with the two Bayesian estimators in between. The elevated s_{GF} might be expected, as F_{IS} is subject to technical factors such as null alleles (Jarne and David 2008). Nonetheless, the five genetic estimates, including the contemporary s_{GC} estimates, were reasonably consistent within each collection. The sole exception was the s_{GG} estimate (0.57) for LA-2019, where the 95% CI was 0.0–0.717. Considering polymorphic loci only, LA-2019 had allelic richness and gene diversities similar to those of the other collections (table 3; see individual locus results in supplemental_S1.xlsx). Thus, beyond the fact that LA-2019 had the fewest polymorphic loci (table 3), we do not have an explanation for the broad CI of this s_{GG} estimate. Genotypic disequilibrium was significant in 22 of 120 pairwise comparisons for MS-2018 ($p < .0001$), 31 of 136 for MS-2014 ($p < .0001$), 5 of 45 for LA-2019 ($p = .073$), and 36 of 153 for TX-2015 ($p < .0001$).

Inbreeding Depression

In all sample collections, all s_G point estimates, except s_{GG} in LA-2019 and s_{GC} in TX-2015, were above their respective s_{DR} point estimate (fig. 2). In comparing s_{DR} with the cumulative inbreeding s_G estimates (s_{GF}, s_{GG}, s_{GB}, and s_{GI}), one of four differed significantly in MS-2014 ($p = .185$), four of four differed significantly in MS-2018 ($p < .0001$), three of four differed significantly in LA-2019 ($p = .0005$), and none were significant in TX-2015 ($p = 1$). Therefore, two of the four sample collections showed $s_G > s_{DR}$, leading to an overall conclusion of outbreeding depression ($p = .014$). In comparing s_{DC} with the four cumulative inbreeding s_G estimates, none were significant in MS-2014 ($p = 1$), one of four differed significantly in MS-2018 ($p = .185$), one of four differed significantly in LA-2019 ($p = .185$), and none were significant in TX-2015 ($p = 1$). None of the four sample collections showed a difference between s_G and s_{DC} ($p = 1$); hence, there is no field-based evidence of inbreeding depression.

Discussion

We had three key findings that were consistent across three geographic locations and a temporal sample. First, there were few clonemates or full siblings. Importantly, there was no full-sibling cotransmission and little to no evidence of clonemate cotransmission. Hence, sibling or clonemate mating has very little to no role in shaping the inbreeding of *Alloglossidium renale*. Second, all four collections had high levels of inbreeding with F_{IS} ranging from 0.634 to 0.790 (table 3). As kin mating was deemed inconsequential, the inbreeding must result from self-fertilization. Estimates of s_G, including those influenced by cumulative inbreeding (s_{GF}, s_{GG}, s_{GB}, and s_{GI}) and a contemporary estimate (s_{GC}), were consistent within locations and showed that *A. renale* has a mixed mating system with high selfing rates ranging from 0.66 to 0.88. Third, comparisons of s_D to s_G provided no evidence for inbreeding depression. With the s_{DR} estimates, outbreeding depression would need to be invoked to explain the s_G estimates. However, s_{DC} could account overall for the s_G estimates, and thus the s_{DC} estimates lead to the conclusion of no inbreeding depression. With the above results, we find that *A. renale* meets both the assumption (i.e., parasites with a truncated life cycle are more likely to experience inbreeding) and the prediction (i.e., a truncated life cycle with loss of a predator host is favored only when there is no inbreeding depression) of the mating system model of parasite complex life cycle evolution. We discuss our findings in relation to the hermaphroditic mating systems of other parasitic flatworms and the broader context of mating system evolution itself.

Few Repeated MLGs and Little to No Clonemate or Full-Sibling Cotransmission

We found few repeated MLGs in all four collections. For *A. renale*, it might be expected to find repeated MLGs for two reasons: (1) obligate asexual reproduction in the mollusc first host could result in clonemates, and (2) albeit still sexual reproduction, high inbreeding could result in repeated MLGs via the perpetuation of inbred lines (e.g., Siol et al. 2008; Bomblies et al. 2010; Detwiler et al. 2017). Except for two individuals from MS-2014 that matched one individual in MS-2018, there were no repeated MLGs across datasets. Given the high selfing rates in the Mississippi location (fig. 2) and that these MLGs were completely homozygous across 17 loci, such a finding may represent the continuation of an inbred line. Overall, the finding of so few repeated MLGs is surprising given both the asexual life cycle stage and high inbreeding of *A. renale*.

The distribution of trematode clonemates within and among hosts has been studied in relatively few systems, but the available studies suggest that clonemate abundance distributions are tied to life cycle peculiarities, including external environments that the hosts inhabit. Specifically, trematode species with life cycle phases in

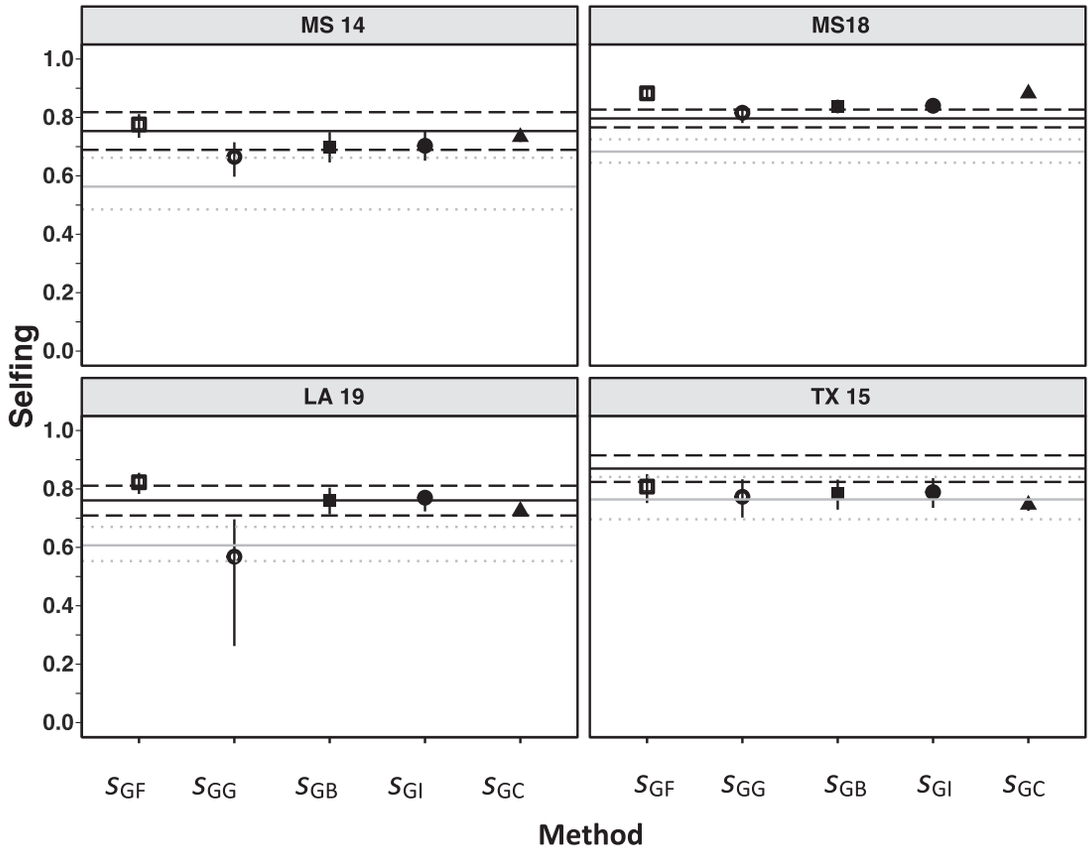

Figure 2: Selfing rates of *Alloglossidium renale* for the four sample collections. Genetic selfing rate point estimates are indicated by the shapes: open square (s_{GF}), open circle (s_{GG}), closed square (s_{GB}), closed circle (s_{GI}), and closed triangle (s_{GC}). The 84% confidence intervals (CIs; or Bayesian credible intervals) are shown for each. Demographic selfing rate estimates are shown by horizontal lines. Solid gray lines are based on random mating within glands and random reproductive success (s_{DR}); dotted gray lines are their 84% CIs. Solid black lines are based on random mating within glands and density-dependent variation in reproductive success; dashed black lines are their 84% CIs.

aquatic environments have yielded few clonemates, whereas those in semiterrestrial or terrestrial environments have found higher clonemate proportions (Criscione et al. 2020, 2022; Keeney et al. 2023). A three-host (aquatic snail to aquatic snail to bird) trematode, *Cotylurus* sp., studied by Keeney et al. (2023) was found to deviate from this "environmental" association in that a high proportion of clonemates co-occurred within aquatic snail second hosts (cercariae of an aquatic snail first host leave and encyst in a different snail individual). The habitat of this *Cotylurus* sp. is "pond-like" and near shore, which may lead to snails in proximity and thus may facilitate clonemate cotransmission.

Table 3: Allelic richness, F_{IS}, and gene diversity (H_S) from COLONY-based datasets (reduced to a single representative of each clone)

			Allelic richness[c]		H_S[d]	
Location	No. polymorphic loci[a]	F_{IS}[b]	All	Polymorphic	All	Polymorphic
Louisiana 2019	10	.699	2.16	3.4	.144	.274
Mississippi 2018	16	.790	2.26	2.88	.283	.335
Mississippi 2014	17	.634	3.14	3.47	.278	.311
Texas 2015	18	.678	2.63	2.72	.244	.254

[a] Of the 19 genotyped loci, the number that were polymorphic in each collection.
[b] F_{IS} is the multilocus estimate.
[c] Average across all 19 loci or just the polymorphic loci and rarefied to the smallest sample size of 59.
[d] H_S is the mean gene diversity across all 19 loci or just the polymorphic loci.

The *A. renale* habitats we sampled are also pond-like and near shore, but we found a low abundance of clonemates and little evidence for clonemate cotransmission (table 2). The difference may lie in that grass shrimp are more mobile than snails in being able to swim in the water column and therefore may not sit immediately adjacent to a snail first host. Hence, cercariae become mixed in the water column as they swim or as shrimp themselves swim.

We emphasize that the clonemate cotransmission observed in MS-2018 ($P_C = 0.0183$) would have little contribution to the inbreeding level, as illustrated by the following equations from Criscione et al. (2022). Both the clonemate mating rate (t_C) and true selfing rate (s) contribute to the apparent selfing rate (s_a) following the equation $t_C + s = s_a$. If we use an average of the four genetic estimates with cumulative inbreeding (s_{GF}, s_{GG}, s_{GB}, and s_{GI}) from MS-2018 (fig. 2) as an estimate of s_a ($=0.844$) in the formula $s = (s_a - P_C)/(1 - P_C)$, then $s = 0.841$ and $t_C = 0.003$. Hence, inbreeding equilibrium F based on selfing alone would be 0.726, whereas the combined effect of selfing and clonemate mating increases to only 0.73.

The high inbreeding observed for *A. renale* (table 3) is on par with that observed in the tapeworm *Oochristica javaensis* (table 1 in Detwiler and Criscione 2017). In contrast though, high levels of sibling cotransmission and a large proportion of repeated MLGs were observed for *O. javaensis*, which has no asexual development (Detwiler and Criscione 2017). In addition to selfing being driven by low intensities (Detwiler et al. 2017), the life cycle of *O. javaensis* has a high potential for sibling cotransmission and consequent kin mating in that tapeworm proglottids, which have hundreds of sibling larvae, are released in gecko host feces as intact packets. Subsequently, a beetle that consumes this packet is eaten by a gecko. In contrast, *A. renale*, as in many trematode species, releases individual eggs into the environment before the miracidial infection of a mollusc host (fig. 1), which often harbor one miracidium to few miracidia (Louhi et al. 2013). Hence, sibling cotransmission might be limited in trematode systems in general (Criscione et al. 2022; Keeney et al. 2023). Overall, for *A. renale*, the pattern of high inbreeding from selfing and few repeated MLGs may indicate that *A. renale* has large effective population sizes at each of these locations. In addition, with little to no evidence of significant clonemate or sibling cotransmission, it appears that the transmission of *A. renale* is conducive to mixing of unrelated individuals into its shrimp second host.

Relationships between Mating Systems and Life Cycle Truncation

Brown et al. (2001) envisioned higher parasite inbreeding in life cycles with "prey-only" hosts because a life cycle with prey and predator hosts would help concentrate potential parasite mates. Only three studies (including this study) have estimated inbreeding of precocious trematodes. *Coitoceacum parvum* has a facultative precocious life cycle with a snail first host, amphipod second host, and fish definitive host. While encysted in the amphipod, *C. parvum* can either sexually reproduce or remain in its juvenile state until consumed by a fish (MacFarlane 1939; Holton 1984). Lagrue et al. (2009) reported from a single location high inbreeding with F_{IS} ranging from 0.726 to 0.985 across 12 microsatellite loci (excludes two potentially duplicated loci; see Detwiler and Criscione 2011). Fitting the scenario of Brown et al. (2001), mean infection intensities of *C. parvum* are lower within amphipods (1.68) compared with fish (9.48; Lefebvre and Poulin 2005*a*). However, the selfing rate of *C. parvum* is likely driven by the forced selfing that occurs within the encysted stage in amphipods rather than low infrapopulation intensities per se.

Proctoeces cf. *lintoni* has a facultative precocious life cycle consisting of a snail first host, limpet second host, and clingfish facultative third host. Like *C. parvum*, *P. lintoni* can sexually reproduce within the second host. However, *P. lintoni* does not form a cyst enabling it to outcross within limpets. At a single location, *P. lintoni* did not deviate from Hardy-Weinberg equilibrium across different limpet species and the fish host (Valdivia et al. 2014). Mean intensities within limpets range from 6 to 14 and in fish average around 9 (Valdivia et al. 2010). As intensities between the second and third hosts are not qualitatively different, *P. lintoni* does not fit the premise of Brown et al. (2001). Coupled with the opportunity to outcross in the limpet second hosts, no inbreeding is manifested.

In contrast to the above two precocious species, *A. renale* has an obligate two-host life cycle. But like *P. lintoni*, adults within the second host are not encysted and free to outcross. Mean intensities per host are low (ranging 1.85–2.58; Hulke et al. 2021), but more importantly, the mean intensities per mating unit (i.e., gland) drop to a range of 1.31–1.77 (table 1). Moreover, the percentage of individuals in single gland infections is high (ranging 33.8%–58.3%; table 1); this percentage represents the lower-bound selfing rate as individuals in single infections are forced to self mate. The truncated life cycle of *A. renale* has high inbreeding and thus meets the assumption of the Brown et al. (2001) model. However, the additional partitioning of potential mates due to the within-host site habitat (i.e., glands) precludes outcrossing opportunities to drive the selfing rather than the host trophic level per se, as envisioned by Brown et al. (2001). There is a need for more studies on the mating systems of species with precocious life cycles to determine the relationship between life cycle truncation and mating system changes.

Comparison of s_G to s_D Does Not Support Inbreeding Depression

Our inbreeding depression test builds on the theory of Ritland (1990) but provides a novel means to test parasite systems when progeny array data are intractable. While progeny array data are often accessible in plant and some mollusc systems (Goodwillie et al. 2005; Jarne and Auld 2006), such data for parasite systems remain difficult to obtain. However, parasites exist in closed mating systems, and as such, s_D can be estimated because the total number of possible mates exist in a well-defined space (i.e., the host individual or site within a host in the case of *A. renale*).

Our method is not without caveats. First, Ritland's (1990) method assumes that selfing is the only cause of inbreeding. However, as discussed above, we could rule out clonemate and sibling mating in contributing to inbreeding. Second, a primary assumption in our s_D estimate is that selfing occurs. If selfing does not occur, then one would falsely conclude inbreeding depression. While most trematodes are hermaphroditic, there is evidence that selfing may not occur for some species. For instance, *Philophthalmus hegeneri* reared in single infections compared with intensities >1 do not produce fertilized eggs (Fried 1962), and radiolabeling experiments of transferred individuals showed no selfing (Moseley and Nollen 1973). Other trematodes such as *Paragonimus* spp. and *Fascioloides magna* are found only sexually mature when encapsulated as pairs, while unpaired individuals remain in a wandering juvenile state (Yokogawa 1965; Foreyt 1992). This developmental mechanism has been hypothesized to ensure cross-fertilization (Yokogawa et al. 1960) and could indicate these trematode species are unable to self-fertilize. In the case of *A. renale*, we are confident that selfing occurs, because all four sample collections had high rates of single gland infections (33.8%–58.3%; table 1), and in all cases, these individuals were gravid.

We estimated s_D under two models of reproductive success: random reproductive success (s_{DR}) and density-dependent reproductive success (s_{DC}). For either, all s_G estimates were not significantly different from or greater than the s_D estimates (fig. 2). Hence, results of both lead to the conclusion of no inbreeding depression and thereby support the prediction of the Brown et al. (2001) model. Nonetheless, it is interesting to ponder which form of variation in reproductive success might best reflect the biology of *A. renale*.

Using s_{DR}, two sample collections had $s_G > s_D$, a result that leads to the inference of outbreeding depression. Outbreeding depression is typically found in the context of interpopulation crossings that result in reduced fitness due to resulting genetic incompatibilities (e.g., disruption of local adaptive loci or coadapted gene complexes; Schierup and Christiansen 1996; Dolgin et al 2007; Escobar et al. 2008; Sletvold et al. 2012). Grindeland (2008) observed outbreeding depression in crosses of the polyploid *Digitalis purpurea* at 30 m. However, this plant has low pollen and seed dispersal, which leads to strong local genetic structuring and potential for microhabitat local adaptation (Grindeland 2008). At the scale we sampled (<100 m^2 in each location), such fine local structuring is hard to envision for *A. renale* because the shrimp host is mobile at this scale, and we observed high mixing of both clonemates and siblings across hosts. Also, microhabitat local adaptation seems implausible given that each shrimp host harbors only a single generation of parasite breeders (contrast with a plant where seeds may germinate next to their maternal parent).

In contrast, all sample collections had an s_{DC} estimate that did not globally deviate from the s_G estimates. In this sense, the hypothesis that the selfing rate is influenced by density-dependent rather than random reproductive success has a higher likelihood to explain the observed selfing rate estimates from the genetic data. While we do not have data on whether *A. renale* experiences crowding, density-dependent growth and fecundity have been observed in many cestode and trematode species (Nollen 1983; Valero et al. 2006; Poulin 2011 and references therein). Given that *A. renale* inhabits a small, confined site of infection (see the relative size of antennal gland to fluke size in fig. S1), intraspecific competition for resources and resulting density-dependent fecundity remains plausible in this system.

The only other precocious species for which inbreeding depression was tested was *C. parvum*; no inbreeding depression was observed (Lagrue and Poulin 2009; Villa and Lagrue 2019). So although not explicitly tested, the body of work on *C. parvum* also supports the prediction of the Brown et al. (2001) model.

Despite species estimates upward of 130,000 (Strona and Fattorini 2014), knowledge of inbreeding depression in the predominantly hermaphroditic parasitic flatworms (Neodermata: trematodes, cestodes, and monogenes) lags far behind that of molluscs, the other major hermaphroditic animal clade (species estimates around 117,000; Jarne and Auld 2006; Escobar et al. 2011). Indeed, explicit inbreeding depression tests exist for only five neodermatan species: *C. parvum* (Lagrue and Poulin 2009; Villa and Lagrue 2019), *Schistocephalus solidus* (Milinski 2006; Benesh et al. 2014), *O. javaensis* (Caballero and Criscione 2019), *Dicrocoelium dendriticum* (Criscione et al. 2022), and *A. renale* (this study). This lack of inbreeding depression data in parasitic flatworms as well as the fact that the Brown et al. (2001) model has not been tested for 23 years reflects the fact that many parasite species are not amenable to experimental setups; the need to maintain multiple

hosts makes it difficult to stage crosses. Because the mating boundary (i.e., host individual) can be identified for the vast majority of neodermatans, our single-sample, field-based inbreeding depression test will help fill this data gap and as such will enable more studies to test the generality of the Brown et al. (2001) model. Moreover, our method is applicable to any hermaphroditic organism where mating boundaries can be defined, such as in experimental situations or where discrete units can be delimited in nature (e.g., possibly in some aquatic snail systems where snails exist in isolated seeps or in some cnidarian systems where brooding corals exist in restricted reef patches). Hence, in general, our method provides another tool to facilitate studies of inbreeding depression.

The Mating System Model in the Broader Context of Complex Life Cycle Evolution

Complex life cycles have been defined as large ecological and/or morphological shifts across an organism's lifespan (Moran 1994; Benesh 2016). Several hypothesized factors driving the evolution of complex life cycles have been put forth, such as stage-specific resource acquisition impacting population growth (e.g., Wilbur 1980) or individual fitness (Ebenman 1992) and decoupling genetic correlations in developmental pathways to allow efficient selection at different life stages (reviewed in Moran 1994). It is beyond our scope to cover all such hypotheses, so we refer readers to an excellent synthesis by Benesh (2016) that relates the adaptive decoupling hypothesis to both developmental and ecological traits found across helminth parasites with trophic transmission. Nonetheless, we wish to connect some of the concepts put forth in the free-living and parasite complex life cycle literature to illustrate how the Brown et al. (2001) model relates to other models and to highlight obstacles or facilitators to life cycle truncation at the definitive host stage.

Briefly, Parker et al. (2015*a*) summarized two other models to incorporate a host in the life cycle. Downward incorporation is ecologically driven and occurs when an intermediate host is added to facilitate transmission to the definitive host. Phylogenetic analysis of trematodes (Cribb et al. 2003) indicates likely downward incorporation in the Plagiorichiida clade, which contains the genus *Alloglossidium*. Reversal of this model would lead to loss of the prey and not predator, so it does not directly apply to our system. However, the model highlights transmission along with generalism as a cost to keeping a host. The upward incorporation model adds a predator host if parasite survival increases upon ingestion of a predator host followed by an increase in body size made affordable by greater resources in a larger host. The latter phase of increased body size carries similarity to life history hypotheses for free-living complex life cycles (reviewed in Moran 1994). Although upward incorporation is not consistent with the phylogenetic history in the Plagiorichiida clade, it highlights body size as a possible constraint to losing the definitive host. However, among precocious trematodes, including those in the genus *Alloglossidium*, body size does not appear to be a constraint to maintain a predator host, as body size does not differ between precocious species and those that keep their predator host (Lefebvre and Poulin 2005*b*; Kasl et al. 2018). Moran (1994) emphasized that fitness factors unrelated to growth or resource acquisition, such as mate location and dispersal, could drive phenotypes at different life stages and hence life cycle complexity itself. It is in this arena that the Brown et al. (2001) model lies where the underlying premise of their model is that of mate location via the passive mechanism of bioaccumulation as parasites progress from prey to predator host. In addition, avoidance of kin mating is a well-recognized driver for the evolution of dispersal (Clobert et al. 2004). As we noted in the introduction, complex life cycles also serve to disperse unrelated parasites. In this regard, the Brown et al. (2001) model integrates evolutionary theory on dispersal, mating systems, and complex life cycles. Simply phrased, a parasite complex life cycle could be maintained to avoid inbreeding depression. In the case of *A. renale*, lack of costs related to inbreeding depression or body size may have enabled life cycle truncation (i.e., loss of the predator host), especially if there are potential costs associated with generalism and transmission between a prey and predator host.

The Broader Context of Mixed Mating Evolution and the Role of Demography

It is clear that *A. renale* has a mixed mating system with high selfing rates. This conclusion is robust, as the different s_G estimates, which cue in on different genetic signatures, are largely similar within locations (fig. 2). When averaging the four genetic estimates with cumulative inbreeding (s_{GF}, s_{GG}, s_{GB}, and s_{GI}), the four collections had selfing rates of 0.730, 0.844, 0.711, and 0.789 for LA-2019, MS-2018, MS-2014, and TX-2015, respectively. There is variance in selfing rates among the sample collections, but among-population variance is common in plants and cnidarians (Whitehead et al. 2018; Olsen and Levitan 2023). Indeed, Whitehead et al. (2018) emphasized how a single selfing rate estimate does not encapsulate the mating system of a species, as genetic and ecological factors shaping mating systems can vary among populations. Whitehead et al. (2018, p. 1) expressed "the need for studies exploring variation in the relative influence of ecological and genetic factors on the mating system." Below, we describe how our data support an ecological

factor, specifically, the demography of parasite infections, as the primary, if not sole, driver of the mating system in all three populations of *A. renale*.

While theory predicts populations should evolve either to complete selfing because of the automatic advantage of selfing (Fisher 1941) or complete outcrossing in the presence of inbreeding depression greater than 50% (Lande and Schemske 1985), mixed mating systems are common in nature (Goodwillie et al. 2005; Jarne and Auld 2006). It is recognized that various genetic, ecological, and demographic factors can lead to mixed mating (Goodwillie et al. 2005). Nonetheless, more than 20 theoretical models on the maintenance of mixed mating invoke selection directly on the selfing rate or indirectly on associated reproductive traits by modeling inbreeding depression (reviewed in Goodwillie et al. 2005; Johnston et al. 2009). Several models do include a role for demography, such as population structure (e.g., Ronfort and Couvet 1995), in leading to a stable mixed mating system, although inbreeding depression is still modeled. Even in Holsinger's (1991) model that does not include inbreeding depression and where population density is a primary factor, selection on the discounting rate, which in turn affects the selfing rate, is incorporated.

Among empirical studies, the role of ecology or demography in shaping mixed mating systems has been shown via factors such as mate or pollinator availability (e.g., Kalisz and Vogler 2003; Tatarenkov et al. 2015; Noël et al. 2016), habitat fragmentation (e.g., Eckert et al. 2010), colonization events (e.g., Baker 1955), and population size/density (e.g., Barrett and Husband 1990; Spigler et al. 2010). Nonetheless, selection is still often invoked, for example, via reproductive assurance where selfing is favored under low mate/pollinator availability (e.g., Kalisz et al. 2004). While experimental (e.g., Karron et al. 1995; Eppley and Pannell 2007) or field-based (e.g., Detwiler et al. 2017) studies have shown correlations between selfing rates and population density/size or spatial distance, we are not aware of any field-based studies where s_G estimates can be fully explained by a sole null demography-based expectation of a selfing rate. Our results in comparing s_{DC} with the s_G estimates provide such an example (fig. 2).

Granted, a null demography-based selfing rate using a random rate of mating exposure in a free-living species may be difficult to derive, especially under situations of wind- or pollinator-dependent fertilization in plants or when potential mates can move in and out of a location. The closed mating systems experienced by parasites enabled us to derive a null demographic expectation following that of Wright (1943; i.e., the hermaphroditic selfing rate is $1/N$ with random mating) coupled with how variation in reproductive success could be manifested. As stated by Bromham (2009, p. 394), "a null model that fits observation does not prove the null model true." Thus, we recognize that our results do not rule out a role of selection in shaping the mating system. Nonetheless, the significance in our utilization of a simple null model and in the downstream results lies in the fact that the observed mixed mating itself along with the variation in selfing rates among populations could arise without natural selection driving the mating system.

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Statement of Authorship

C.D.C. and J.M.H. designed the research project. J.M.H. generated the genetic data. J.M.H. and C.D.C. performed the analyses and wrote the manuscript. The authors have no competing interests to declare.

Data and Code Availability

All data and R code used in this article are publicly available on Zenodo (https://doi.org/10.5281/zenodo.13007742; Hulke and Criscione 2024).

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