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Evolutionary consequence of a change in life cycle complexity: A link between precocious development and evolution toward female-biased sex allocation in a hermaphroditic parasite

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The evolutionary consequences of changes in the complex life cycles of parasites are not limited to the traits that directly affect transmission. For instance, mating systems that are altered due to precocious sexual maturation in what is typically regarded as an intermediate host may impact opportunities for outcrossing. In turn, reproductive traits may evolve to optimize sex allocation. Here, we test the hypothesis that sex allocation evolved toward a more female-biased function in populations of the hermaphroditic digenean trematode *Alloglossidium progeneticum* that can precociously reproduce in their second hosts. In these precocious populations, parasites are forced to self-fertilize as they remain encysted in their second hosts. In contrast, parasites in obligate three-host populations have more opportunities to outcross in their third host. We found strong support that in populations with precocious development, allocation to male resources was greatly reduced. We also identified a potential phenotypically plastic response in a body size sex allocation relationship that may be driven by the competition for mates. These results emphasize how changes in life cycle patterns that alter mating systems can impact the evolution of reproductive traits in parasites.

KEY WORDS: ***Alloglossidium progeneticum*, local sperm competition, phenotypic plasticity, progenesis, Trematoda.**

The complexity of parasite life cycles, which often involve diverse routes of infection and multiple host species to complete development, has long garnered interest among biologists. Theoretical studies have largely focused on the causes that led to the evolution of complex life cycles (Choisy et al. 2003; Parker et al. 2003; Gandon 2004). Empirical work has mainly focused on how parasite life-history traits, such as timing of reproduction, optimal growth patterns, or host manipulation, may evolve as a response to changes in complex life cycle patterns (Kozlowski 1992; Gemmill et al. 1999; Hammerschmidt et al. 2009; Parker et al. 2009; Benesh et al. 2012; Benesh et al. 2013). Some studies have proposed that complex life cycles of hermaphroditic parasites may have evolved (or are maintained) as a need to encounter mates to promote outcrossing (Brown et al. 2001) or to avoid mating among identical clones in the case of trematodes (Rauch et al. 2005). However, a change in life cycle pattern itself may alter the parasite mating

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system and thus have downstream evolutionary consequences. In particular, among digenean trematodes some species may have altered mating systems due to the early onset of sexual maturation in an intermediate host (Lefebvre and Poulin 2005).

The most common digenean life cycle follows an obligate three-host pattern. Miracidia that develop from eggs infect a molluscan first intermediate host. Asexual reproduction within the mollusc leads to the release of larval cercariae that subsequently infect a second intermediate host and encyst as metacercariae (a juvenile stage that is not yet sexually mature). Completion of the life cycle is attained upon ingestion of the second intermediate host by a definitive host (typically a vertebrate) wherein the parasite becomes sexually mature (Fig. S1). However, there are notable variations in this life cycle pattern. In particular, there are digenean species that precociously reproduce (historically termed progenetic) in what is typically regarded as the second intermediate host (Lefebvre and Poulin 2005). For example, in the genus *Alloglossidium*, some species have a typical three-host pattern with ictalurid catfishes as definitive hosts (where within the intestine, adult worms are free to outcross), whereas others have two-host patterns with a crustacean or leech definitive host (Smythe and Font 2001). Of particular interest is *Alloglossidium progeneticum*, which has a facultative two- or three-host life cycle. In this species, the mating system is significantly altered by the precocious life cycle change because within its crayfish second host, worms become sexually mature within their cyst (Fig. S1). Thus, this developmental change leads to forced self-fertilization. This drastic change in the mating system (increased selfing) may then have downstream evolutionary consequences such as inbreeding depression or changes to reproductive traits themselves.

In this study, we focus on a potential evolutionary change in sex allocation as a consequence of a change in life cycle complexity that alters the parasite's mating system. In self-compatible hermaphroditic species, local sperm competition (competition between related sperm) is a concept that provides predictions for sex allocation in relation to the mating system (reviewed in Schärer 2009). In particular, with a high selfing rate or a small mating group size, sex allocation should favor a female-biased allocation, as an individual should only produce enough sperm to fertilize the available eggs. Schärer (2009) notes that the evolution of sex allocation may occur in two ways: (1) It may occur as a fixed (or average within population) evolutionary response optimized over generations. We refer to this as a genetically based response among populations. And (2) sex allocation may occur as a phenotypically plastic response (individuals adjust sex allocation in response to current conditions). The few empirical studies on sex allocation in parasitic flatworms have focused exclusively on the latter (Trouve et al. 1999a; Schärer et al. 2001; Schjørring 2009; Al-Jahdali 2012).

Here, we provide a test of an among population genetically based response in sex allocation by comparing populations of *A. progeneticum* that vary in their life cycle pattern (obligate three-host vs. facultative precocious two- or three-host life cycle) while controlling for potential environmental confounding factors. Specifically, we test the hypothesis that sex allocation evolved toward a more female-biased function in the derived facultative precocious populations. This study was made possible due to recent survey efforts that discovered life-history variation among populations. Thus, we first provide a brief summary of the newly discovered life history, geographic, and genetic variation in the trematode *A. progeneticum*. We then present statistical analyses of the reproductive morphology that show strong support for reduced allocation to male resources in populations with precocious development. These results emphasize how changes in life cycle patterns that alter mating systems can impact the evolution of reproductive traits in parasites.

Methods

STUDY SPECIES

The digenetic trematode *A. progeneticum* is a simultaneous hermaphrodite that can facultatively use either two or three hosts to complete development (Fig. S1). Originally described from gravid specimens encysted within the antennal glands of the crayfish *Procambarus spiculifer* from Calls Creek, GA (Sullivan and Heard 1969), a subsequent study of *A. progeneticum* in Calls Creek found adults free in the intestines of brown bullheads, *Ameiurus nebulosus* (Font and Corkum 1975). We note, however, our surveys only found snail bullheads, *Ameiurus brunneus*, in Calls Creek (Table S1). The presence of gravid specimens (thus sexually mature) within both what is typically considered the second intermediate host (crayfish) and definitive third host (ictalurids) is to date the only such occurrence within the genus *Alloglossidium* (Smythe and Font 2001; Kasl et al. 2014). Prior to this study, *A. progeneticum* had only been reported from Calls Creek (Sullivan and Heard 1969; Font and Corkum 1975). Our recent survey efforts, reported herein, identified geographic variation in the ability to precociously develop, with most populations exhibiting an obligate three-host life cycle pattern (Fig. 1, Table S1).

COLLECTIONS

From June 2011 through May 2014 ictalurid catfishes and crayfish hosts were sampled across the southern United States (Texas, Oklahoma, Arkansas, Louisiana, Mississippi, Alabama, Georgia; Fig. 1, Table S1). All parasites taken from the intestines of catfishes were heat-fixed (passing the wet-mount over the flame of an alcohol burner) underneath a cover slip without pressure to

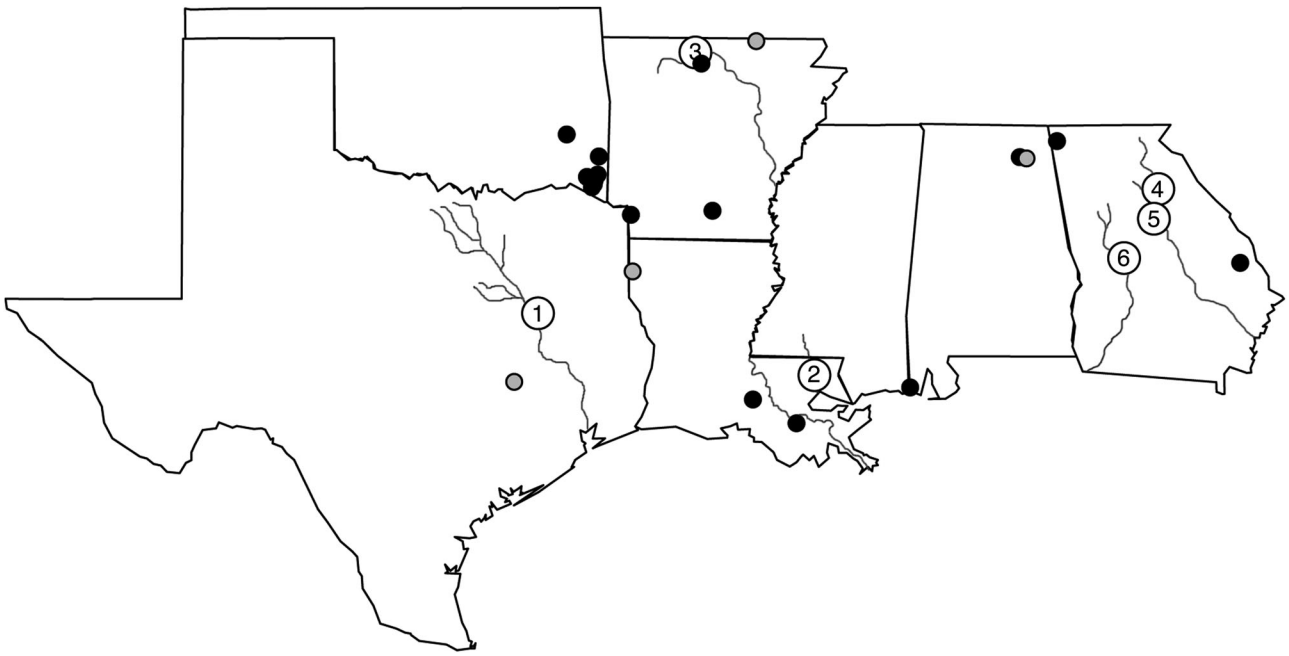

Figure 1. Sites positive for *Alloglossidium progeneticum* in the southern United States confirmed with sequence data. Black dots represent sites where nongravid metacercariae were recovered from crayfish hosts, thus indicating nonprecocious development. Gray dots represent sites where only adult worms were collected from fish, thus precocious life history cannot be ascertained. Sites used for comparative analyses are indicated by circles with numbers. Sites 1 (Gus Engeling), 2 (Chappapeela Creek), and 3 (Crooked Creek) are obligate three-host populations. Sites 4 (Calls Creek, type locality), 5 (Big Indian Creek), and 6 (Richland Creek) are the only identified facultative precocious populations in our survey.

ventrodorsally orient the worms in preparation of morphological analyses (Font 1994). Cysts taken from crayfish hosts were examined for eggs under a compound microscope and classified as either encysted metacercariae (i.e., not gravid) or encysted adults (i.e., gravid). Some gravid cysts were mechanically excysted and heat-fixed as above to allow for morphological analyses. All parasites were stored in 70% ethanol to allow for potential future molecular analyses.

EXTRACTION, DNA AMPLIFICATION, AND SEQUENCING

DNA was extracted by placing individual worms in 200 μl of 5% chelex containing 0.2 mg/ml of proteinase K, incubated for 2 h at 56°C and boiled at 100°C for 8 min. Two markers were used for *A. progeneticum* identification and analysis of genetic diversity across its expanded range: an 839 base pair ribosomal DNA (rDNA) fragment spanning the 3′ end of the 18s and the first internal transcribed spacer region (ITS1), and a 679 base pair region of the mitochondrial NADH-dehydrogenase subunit 1 gene (ND1). Polymerase chain reaction (PCR) amplifications were performed using 25 μL reactions. The nuclear ribosomal sequence was obtained using a reaction consisting of 3 μL of template extract, 16.25 μL water, 2.5 μL 10× buffer, 1.5 μL $MgCl_2$ (25 mM), 0.5 μL dNTP (10 mM/each), 0.5 μL of each primer (20 μM), and 0.25 μL of Taq polymerase (Omega Bio-Tek, Inc., Norcross, GA; 5 units/μL), and a thermocycling profile of 95°C for 3 min, once; 94°C for 45 sec; 60°C for 30 sec; 72°C for 60 sec, 35 times; 72°C for 7 min, once. For the rDNA region we used the forward primer s18 (5′-TAACAGGTCTGTGATGCC-3′; located toward the 3′ end of the 18s rDNA) and reverse primer 5.8s1 (5′-GCTGCGCTCTTCATCGACA-3′; located within the 5.8s gene; Jousson et al. 2000). The s18 primer was used for sequencing. PCR for the ND1 region was the same with the exceptions that 15.25 μl water and 2.5 μl $MgCl_2$ (25 mM) were used. The thermocycler profile for the ND1 PCR is described in Criscione and Blouin (2004). The forward primer MB352 (5′-CGTAAGGGKCCTAAYAAG-3′; Criscione and Blouin 2004) and reverse primer CC28 (5′-CWTCTCAARGTTAACAGCCT-3′; anchored in the asparagine tRNA) were used for the PCR of the ND1 region. In addition to MB352, an *A. progeneticum* specific internal reverse primer CC57 (5′-CCCATAATCTATGTGTGCTAAC-3′) was used for sequencing. PCR products were purified using the Ultra Clean PCR clean-up Kit (MO BIO Laboratories, Inc., Solana Beach, California) and sent to the DNA Analysis Facility on Science Hill at Yale University (New Haven, CT) for sequencing. Contiguous sequences from individuals were assembled using Sequencher[tm] (GeneCodes Corp., version 4.1.4, Ann Arbor, MI, USA), and submitted to

GenBank under accession numbers KT455707-KT455827. Both nuclear and mitochondrial sequences were aligned using Clustal W within the BioEdit program, version 7.1.8 (Hall, 1999).

MOLECULAR IDENTIFICATION OF SPECIMENS AS *A. progeneticum*

For the survey work, at least one worm per host species was sequenced at each site positive for specimens of *Alloglossidium* (Table S1). Due to the subtle morphological differences among species of *Alloglossidium* known to infect catfishes (Kasl et al. 2014), molecular sequences from new sites were compared to those taken from the type locality (Calls Creek, GA) for positive identification of *A. progeneticum*. We also compared the rDNA region to sequences of other species in the genus *Alloglossidium* including Genbank sequences (Tkach and Mills 2011: JF440783.1, *A. corti*; JF440765.1, *A. fonti*; JF440771.1, *A. geminum*; JF440808.1, *A. kenti*; Kasl et al. 2014: KC812276.1, *A. floridense*) and sequences from additional known or cryptic species obtained as part of our ongoing survey work (to be published elsewhere as part of a phylogenetic study of the genus). For the ND1 sequences, TCS version 1.21 (Clement et al. 2000) was used to construct a haplotype network. This network is not intended to be a final representation of the phylogeographic patterns as sampling was haphazard and opportunistic. Rather the purpose is to support the status of the specimens as *A. progeneticum* and to provide initial data on intraspecific variation especially in the six focal populations used for the morphological analyses (discussed below). ND1 haplotype (H_d) and nucleotide diversities (π) within these six focal populations were calculated in DnaSP version 5.10 (Librado and Rozas, 2009).

MORPHOLOGICAL ANALYSES TO TEST FOR SEX ALLOCATION

Based on populations with suitable sample sizes, the molecular confirmation of populations as being *A. progeneticum*, and the presence of encysted metacercariae or encysted gravid adults, we chose six representative populations for downstream analyses. Calls Creek, Big Indian Creek, and Richland Creek (Fig. 1; sites 4, 5, and 6, respectively) are classified as facultative precocious (two- or three-host life cycle) populations due to the presence of encysted gravid worms in crayfish and gravid worms in bullheads. More detail on our survey findings is given in the Results, but basically all encysted worms in crayfish from these populations are gravid. Additionally, Richland Creek is a different river drainage (Flint River system) than that of Calls Creek and Big Indian Creek (Oconee River system). Gus Engeling, Chappapeela Creek, and Crooked Creek (Fig. 1; sites 1, 2, and 3, respectively) are classified as obligate three-host populations as encysted metacercariae in crayfish from these locations were not gravid. The obligate three-host populations represent 3 nonconnected river systems.

All specimens used for morphological analyses were stained using Semichon's acetic carmine, dehydrated in a graded ethanol series, cleared using xylene, and mounted on slides using either Damar gum or Canada balsam. Because the morphology of *A. progeneticum* has not been described from adult specimens originating from fish hosts, especially from obligate three-host populations, we provide a list of morphological measurements for traits commonly used in trematode taxonomy in Table S2.

To assess if the life cycle change leading to precocious development ultimately leads to an evolutionary change in sex allocation, we compared reproductive traits within and between the six representative populations discussed above. Ideally, we would use volume measurements of the reproductive organs. However, the staining and mounting methods, which were necessary for specimen identification, did not permit measurement of volume. As surrogates of volume, we measured the length and width of the ovary and both testes. Length was the longest axis going from anterior to posterior and width was the longest axis perpendicular to length (see Fig. 2). An exploratory principle components analysis (PCA with Varimax rotation in SYSTAT version 12; SYSTAT Software, Inc., Chicago, IL) of all measured worms revealed that the six measures of the reproductive organs fell out as two factors. One factor was composed of the ovary length and width and the other factor was composed of the lengths and widths of both testes (Table S3). Based on this PCA, we created summated scores to represent female function [(ovary length + ovary width)] or male function [(anterior testis length + anterior testis width + posterior testis length + posterior testis width)]. Summated scales are an appropriate way to summarize correlated variables of the same trait (Hair et al. 1998). Sex allocation was estimated as a ratio: male function/(male function + female function) (Janicke and Schärer 2009; Janicke et al. 2013). As noted by Janicke et al. (2013), this estimator is a relative measure of sex allocation where higher values indicate more male-biased sex allocation. This estimator also carries the assumption that testis and ovary size are reasonable proxies for investment into male and female sex function, respectively. Additional discussion of appropriate means to measure sex allocation can be found in Schärer (2009).

In the following statistical analyses, we employed generalized linear mixed models (GLMMs). For all analyses, the sex allocation ratio of male function/(male function + female function) was used as the dependent variable. Following the recommendations of Schärer (2009), if significance was observed, we repeated the same analyses on male or female function separately to determine if changes in one or both of these traits were driving sex allocation. All statistical analyses were performed using the package lme4 (to fit restricted maximum likelihood models [REMLs]; Bates and Maechler, 2009) and lmerTest (to obtain *P* values using the Satterthwaite approximation; Kuznetsova et al. 2015) in the program R (R Core Team, 2015). Residuals from

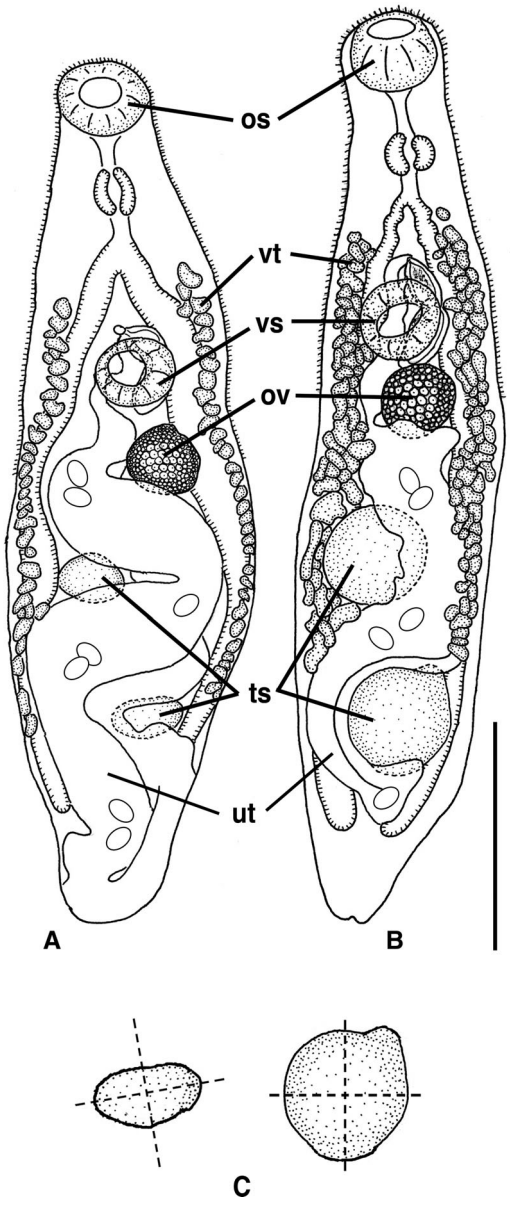

Figure 2. Ventral view of adult *Alloglossidium progeneticum* specimens from fish. (A) Worm from a precocious population (Calls Creek, GA) and (B) worm from a nonprecocious population (Gus Engeling, TX). Locations of oral sucker (os), vitellaria (vt), ventral sucker (vs), ovary (ov), testes (ts), and uterus (ut) are indicated. For simplicity, the uterus shows a few representative eggs, but is typically completely filled with eggs. Scale bar = 400 μm. Note testes are much smaller in the specimen from the facultative precocious population (A) than the obligate three-host population (B). (C) Panel showing how length (vertical dotted line) and width (horizontal dotted line) were measured.

all of the tests were visually examined to see if the data met the assumptions of normality and homogeneity of variance.

Because selection on sex allocation may favor a phenotypically plastic response to current conditions (Schärer 2009), an analysis that tests for a genetically based evolutionary response among populations can be confounded if specimens are collected from different conditions (e.g., high vs. low intensity of infection). Thus, we first conducted statistical tests within the obligate three-host populations and within the facultative precocious populations to look for evidence of plastic responses. Within the obligate three-host populations Gus Engeling and Crooked Creek, we had sufficient samples from fish to test for the influence of intensity of infection (number of worms per infected host) on sex allocation. The premise here is that with a lower intensity in fish, local sperm competition will be higher (i.e., there is a smaller social group size; Schärer 2009). Thus, our estimate of sex allocation should be greater under higher intensities. Parasites were separated into low intensity (i.e., originating from a fish with <20 worms) or high intensity (i.e., originating from a fish with >20 worms) categories to evaluate the plastic adjustment of sex allocation. This threshold was chosen to correspond to the highest observed intensity from the precocious populations (see below). We measured at least 10 worms for each category from each of the two populations (total $N = 45$). Worms came from at least two different host individuals in each category per population. The model included the fixed effect of intensity, body size (total length of the worm) as a covariate, and the interaction between the two. The random effects included population, the interaction between population and intensity, and host individual nested within the population by intensity interaction. We did not include body width along with total length of the worm as summated score of body size because we found body width to be unreliable due to the fact that heavily gravid worms are expanded in width.

Within all three of the facultative precocious populations, we only had adult worms from fish that had infection intensities <20. However, adults are also present as encysted worms within crayfish. Thus, we could test for the influence of an overall outcrossing opportunity on sex allocation by comparing worms from fish, which are freely able to outcross, and crayfish, wherein encysted worms are forced to self-mate. The idea here is that with forced self-fertilization, sex allocation would shift toward a female-biased function because there are no additional mates besides oneself (i.e., there is high local sperm competition). From each of the three populations, we measured at least 10 worms for each of two categories: forced self-fertilization in crayfish versus opportunity to outcross in fish (total $N = 63$). For simplicity, we refer to this classification as the mating opportunity fixed effect. The model included the fixed effect of mating opportunity, body size as a covariate, and the interaction between the two. The random effects included population, the interaction between population and mating opportunity, and host individual nested within the population by mating opportunity interaction.

Based on the results of the within population analyses above, we limited the among-population analysis (i.e., the test for a genetically based evolutionary response in sex allocation as a result

of the change in the life cycle) to a comparison of adult worms obtained from low intensity fish infections (originating from a fish with <20 worms). The goal here was to test the hypothesis that the increased self-fertilization (i.e., an increase in local sperm competition) in the facultative precocious populations would lead to a female-biased shift in sex allocation. The analysis included all six of the representative populations and included 10 worms from each population. Worms came from at least two different fish in each population (total $N = 62$). The model included the fixed effect of life history (obligate three-host vs. facultative precocious), body size as a covariate, and the interaction between the two. The random effects included population nested within life history and host individual nested within population within life history.

Results

SURVEY RESULTS: LIFE HISTORY, MORPHOLOGICAL, AND MOLECULAR VARIATION

In total, 28 locations (including the type locality of Calls Creek) were found to have *A. progeneticum* (verified with sequence data, discussed below). Encysted adults in crayfish were found only in three sites in Georgia (Calls Creek, Big Indian Creek, and Richland Creek; sites 4, 5, 6 in Fig. 1, respectively). In these sites, all encysted worms are gravid except for the very small ones, which likely indicate a recent infection where the worm has not yet had time to mature. Moreover, gravid worms were also recovered from the intestines of ictalurids in sites 4, 5, and 6 (Fig. 1, Table S1). Thus, these three populations are considered facultative precocious populations. From four populations, we only recovered worms from fish hosts, thus we cannot definitively classify the life history of these populations (Fig. 1, Table S1). Of the other 21 sites where infected crayfish were found ($N = 151$ infected crayfish), we recovered 1352 encysted metacercariae that were of comparable size to those found in sites 4, 5, and 6 discussed above. None of these metacercariae were gravid (Table S1). Thus, we classify these latter 21 sites as obligate three-host populations as a fish host would be necessary for the worms to mature.

Comparisons of rDNA sequences obtained through survey efforts ($N = 119$) to those taken from the type locality (Calls Creek; site 4, Fig. 1) led to the identification of 27 new locations of *A. progeneticum*, expanding the known range across six states (Fig. 1; Table S1). Moreover, nine new species of crayfish and five new ictalurid species were identified as intermediate and final hosts, respectively (Table S1). Within the ITS1 region of the rDNA sequence, a 14-nucleotide insert (5′-TTATCCTAAAAGGT-3′) is diagnostic compared to this region of the five other described species of *Alloglossidium* that infect fish (Kasl et al. 2014). Among the *A. progeneticum* specimens, there was a single variable site that had no association with geography, host species, or life history. As this site occurred at the end of a mononucleotide repeat (eight or nine repeats of A), its validity remains suspect. At ND1 ($N = 119$), 37 unique haplotypes were identified (Fig. 3; Table S1). No premature stop codons were found after translation of the sequences (using amino acid translation code 9 on GenBank). Maximum *p*-distance between any two ND1 haplotypes was 1.6%, which is well within the range typically found for intraspecific mtDNA variation among parasitic flatworms (Vilas et al. 2005).

Of the 37 haplotypes identified, only three corresponded to the facultative precocious populations. Of these, two haplotypes (haplotype 13 and 14) were shared between sites within the same river drainage (Calls Creek and Big Indian Creek, Oconee River system), while the third (haplotype 18) was unique to the Richland Creek site, located within the Flint River drainage (Fig. 3; Table S1). Hence, haplotype (H_d) and nucleotide diversities (π) are low within these facultative precocious populations ($H_d = 0.2$, $\pi = 0.0003$; $H_d = 0.33$, $\pi = 0.0005$; $H_d = 0$, $\pi = 0$; Calls Creek, Big Indian Creek, and Richland Creek, respectively). In general, the obligate three-host populations had greater haplotype and nucleotide diversities ($H_d = 0.8$, $\pi = 0.0042$; $H_d = 0.64$, $\pi = 0.0021$; $H_d = 0.2$, $\pi = 0.0009$; Chappepeela Creek, Gus Engeling, and Crooked Creek, respectively).

INFECTION INTENSITY AND PLASTICITY IN SEX ALLOCATION

For populations with the obligate three-host life cycle, when treating sex allocation as the response variable, the interaction between intensity and body size (total length of the worm) was not significant ($F_{1,41} = 0.75$, $P = 0.39$). Thus, the interaction was pooled. After pooling, the covariate, total worm length, had a significant positive relationship with sex allocation (i.e., the larger the worm, the more allocation to the male function; $F_{1,42} = 13.58$, $P < 0.001$; Fig. 4A). Intensity of infection was also significant where the high intensity category corresponded to higher male-biased sex allocation ($F_{1,42} = 9.59$, $P = 0.003$; Fig. 4B). In the analyses of the individual summated scales of male and female function, interactions between intensity and body size were not significant (male function: $F_{1,41} = 2.94$, $P = 0.09$, female function: $F_{1,41} = 1.85$, $P = 0.18$). After pooling interactions, body size had a significant positive association to both male ($F_{1,42} = 176.72$, $P < 0.001$) and female ($F_{1,42} = 63.04$, $P < 0.001$) functions. However, infection intensity was not significant for either male ($F_{1,42} = 0.38$, $P = 0.54$) or female ($F_{1,42} = 2.57$, $P = 0.12$) functions. Despite the lack of significance with infection intensity to male or female function alone, there was a general trend of slightly higher summated scales for female function in cases of low intensity and slightly higher summated scales for male function in cases of high intensity (Fig. 4C). Thus, it appears that the combined changes in male and female function are driving the overall sex allocation in the direction of

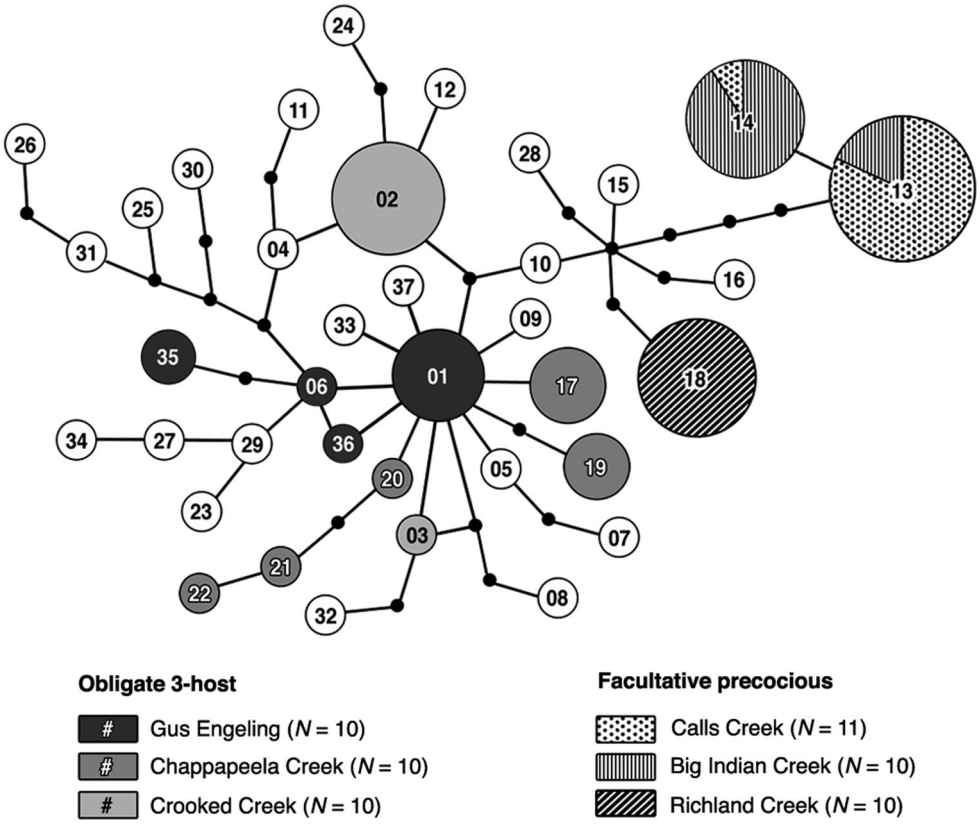

Figure 3. **Statistical parsimony network of ND1 mtDNA haplotypes. Numbers represent haplotype ID (Table S1). Each connection is a single mutational step with small black circles representing inferred haplotypes. The network is intended to show the relationships and diversity of haplotypes collected from the six populations used for the morphological analyses. Haplotypes from these six focal populations are shown as shaded (obligate three-host) or patterned (facultative precocious) circles and are scaled according to the number of individuals with that haplotype (e.g., one individual from Gus Engeling had haplotype 6). The network is not intended to be a final representation of phylogeographic patterns due to opportunistic surveys resulting in uneven sampling. White haplotypes are those not represented in the six focal populations and are not scaled. We note haplotypes from the obligate three-host populations may be shared with other nonfocal sampling locations, but this is not shown in the figure (see Table S1). However, haplotypes from the facultative precocious populations are not shared with any of the nonfocal sites nor the obligate three-host populations.**

greater male-biased function under higher intensities. Random effects did not account for a significant portion of the variance in any of the above three models (Table S4).

MATING OPPORTUNITY AND PLASTICITY IN SEX ALLOCATION

For the test of mating opportunity (as a function of host type) on sex allocation within populations with a facultative precocious life cycle pattern, the interaction between mating opportunity and body length was significant ($F_{1,55} = 11.25$, $P = 0.001$, Fig. 5A). We conducted a post hoc analysis (using R package lsmeans) to see where along the gradient of body size we started to see a statistical difference in sex allocation between worms from fish and crayfish. This occurs at a body size of 1696 μm (df = 2.21, t-ratio = −3.93, $P = 0.05$), so worms from fish with body sizes greater than 1696 μm have higher sex allocation than worms from crayfish. In addition, we conducted post hoc analyses to determine if the slopes for worms from fish and crayfish were different than 0. A significant positive relationship between body size and sex allocation was observed for worms collected from fish ($\beta = 5.37 \times 10^{-5}$, $F_{1,30} = 19.69$, $P < 0.001$), whereas worms encysted in crayfish had a nonsignificant negative relationship between body size and sex allocation ($\beta = -1.21 \times 10^{-5}$, $F_{1,28} = 0.61$, $P = 0.44$; Fig. 5A). When conducting the analyses on the female summated scales, the interaction was not significant ($F_{1,54} = 1.54$, $P = 0.22$). After pooling, body size had a significant positive association with female function ($F_{1,56} = 71.27$, $P < 0.001$), but mating opportunity was not significant with female function ($F_{1,19} = 0.99$, $P = 0.33$). In contrast, male function showed a significant interaction between mating opportunity and body size ($F_{1,56} = 39.19$, $P < 0.001$; Fig. 5B). As this result was similar to that found for sex allocation, it appears that changes in the male function are largely driving the significance found for sex allocation. In general the random effects did not account for

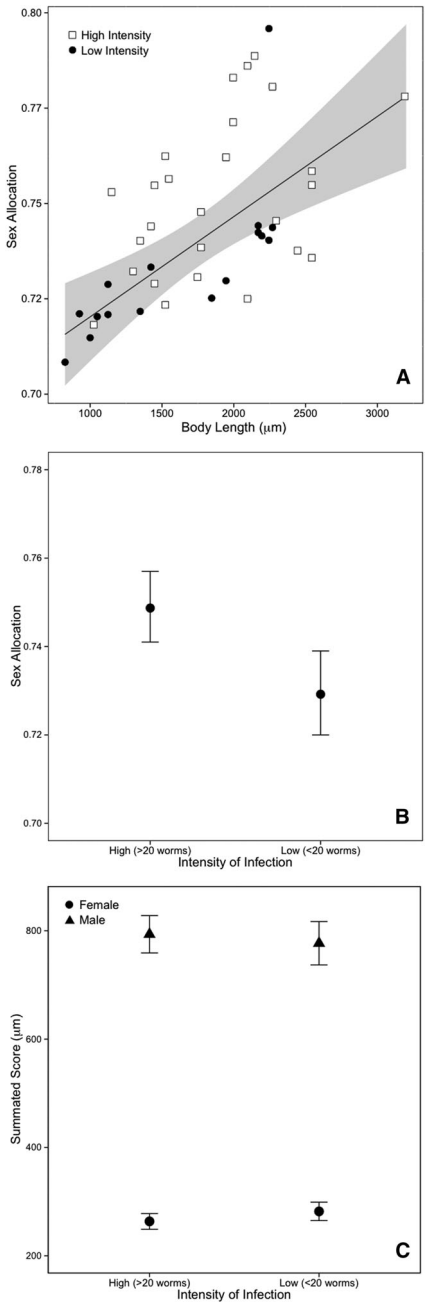

Figure 4. Linear mixed model results for the fixed effect of infection intensity for worms from two of the obligate three-host populations of *Alloglossidium progeneticum* (Gus Engeling and Crooked Creek). (A) Relationship of body size (μm) to sex allocation. Sex allocation is expressed as the ratio of the male summated scores to the combined male and female summated scores. Shaded regions represent 95% confidence intervals based on the model SEs. The raw data points (i.e., not model adjusted) are superimposed over the model prediction. (B) Least square means plot showing the relationship between intensity of infection and sex allocation. (C) Least square means plot showing the relationship between intensity of infection and individual female (ovary) and male (combined testes) summated scores (μm). Error bars in (B) and (C) represent 95% confidence intervals.

a significant portion of the variance in the above three models (Table S5).

LIFE HISTORY AND GENETICALLY-BASED RESPONSE IN SEX ALLOCATION

Because the above results suggest a plastic response of sex allocation to the current conditions worms experience (e.g., infection intensity or mating opportunity), the tests for a genetically based evolutionary response among populations with different life histories were restricted to adult worms obtained from fish with low intensities of infection (originating from a fish with <20 worms). A significant interaction was found between life history and body length of the worm ($F_{1,56} = 5.17$; $P = 0.02$, Fig. 6A). Post hoc analysis of sex allocation across the range of body sizes showed that there was a difference in sex allocation between life histories (greater in the obligate three-host populations) until a size of 2224 μm, at which point there was no longer a difference (df = 5.85, t-ratio = 2.46, $P > 0.05$). At the maximum-recorded size of 2500 μm, nonsignificance was marginal (df = 8.37, t-ratio = 1.77, $P = 0.11$). Thus, over most of the range of body size, the obligate three-host life history has a more male-biased sex allocation compared to the facultative precocious life history. With the latter in mind, the main fixed effects still could be interpreted. Body size had a significant positive association to sex allocation ($F_{1,56} = 11.43$, $P = 0.001$). Life history was significant where the facultative precocious life history showed more of a female-biased sex allocation relative to the obligate three-host life history ($F_{1,19} = 17.5$, $P < 0.001$; Fig. 6A). Subsequent analyses of the female summated scores showed a nonsignificant interaction between life history and body size ($F_{1,23} = 0.46$, $P = 0.51$). After pooling, body size had a significant positive relationship with female function ($F_{1,26} = 70.4$, $P < 0.001$). Life history was not significant ($F_{1,4} = 2.74$, $P = 0.18$) though the trend was for greater female function in the facultative precocious life history (Fig. 6B). Male function also had a nonsignificant interaction ($F_{1,55} = 0.59$, $P = 0.45$). After pooling, both a positive relationship with body size ($F_{1,58} = 150.4$, $P < 0.001$) and life history ($F_{1,4} = 10.87$, $P = 0.03$) were significant. Male function was greater in the obligate three-host life history (Fig. 6A). As with the mating opportunity analysis above, most of the change in sex allocation in the life-history analysis appears to be driven by changes in male function. In the models testing sex allocation and male summated scores, random effects accounted for a significant portion of the variance (Table S6).

Discussion

SUMMARY OF MAIN RESULTS

1. Survey data verified with sequence data expanded the geographic distribution (across six states in the southern United States), host species distribution (five final ictalurid hosts and

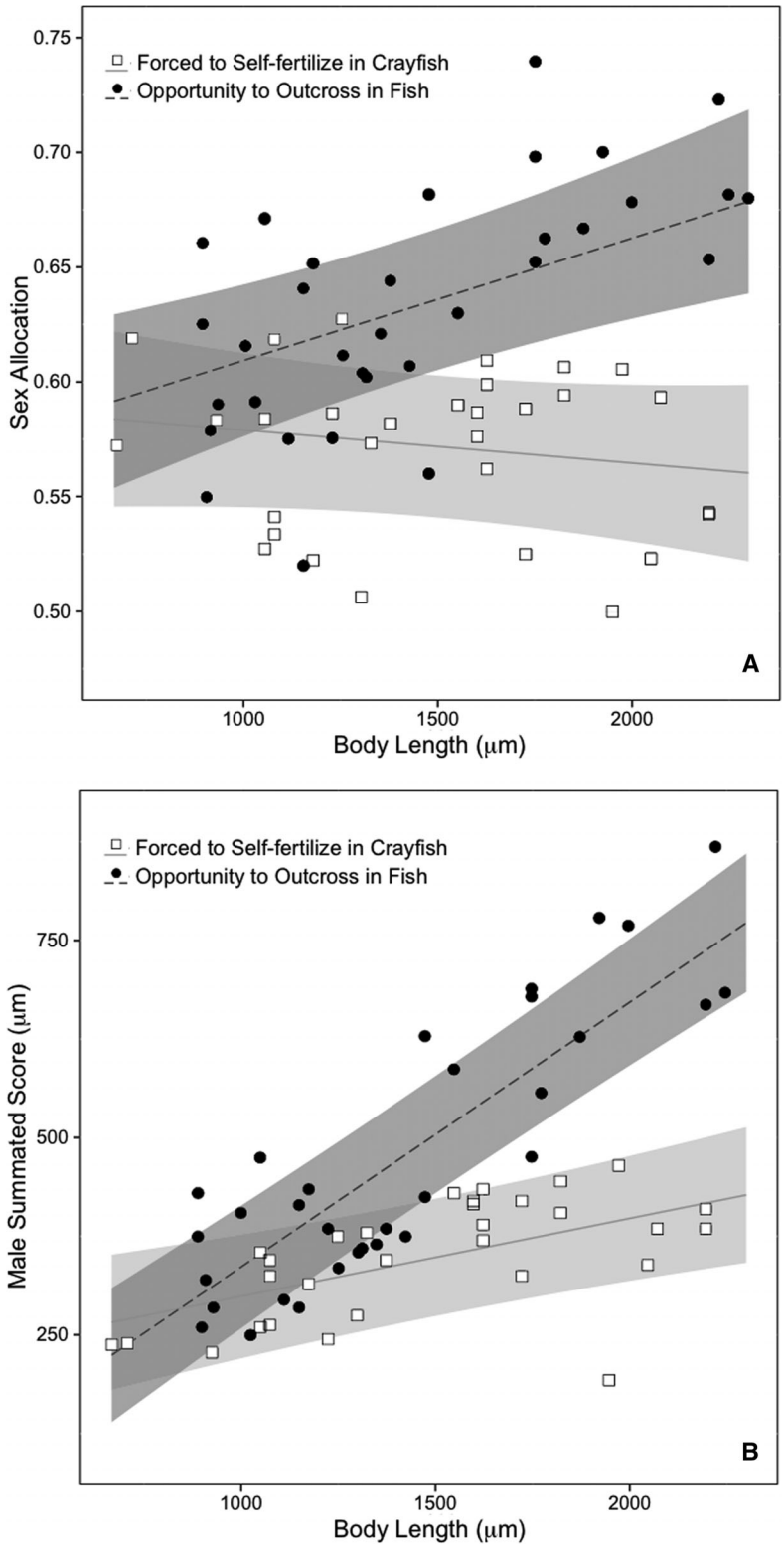

Figure 5. Linear mixed model plot showing the interaction between total worm length (μm) and mating opportunity (opportunity to outcross in fish vs. forced self-fertilization in crayfish) on sex allocation (A) or on male summated scores (B) in sites with facultative precocious development (Calls Creek, Big Indian Creek, Richland Creek). Note, in (A) sex allocation does not scale with body size when there are no potential mates other than oneself (i.e., encysted in crayfish host). Sex allocation is expressed as the ratio of the male summated score to the combined male and female summated scores. Shaded regions represent 95% confidence intervals based on the model SEs. The raw datapoints (i.e., not model adjusted) are superimposed over the model predictions.

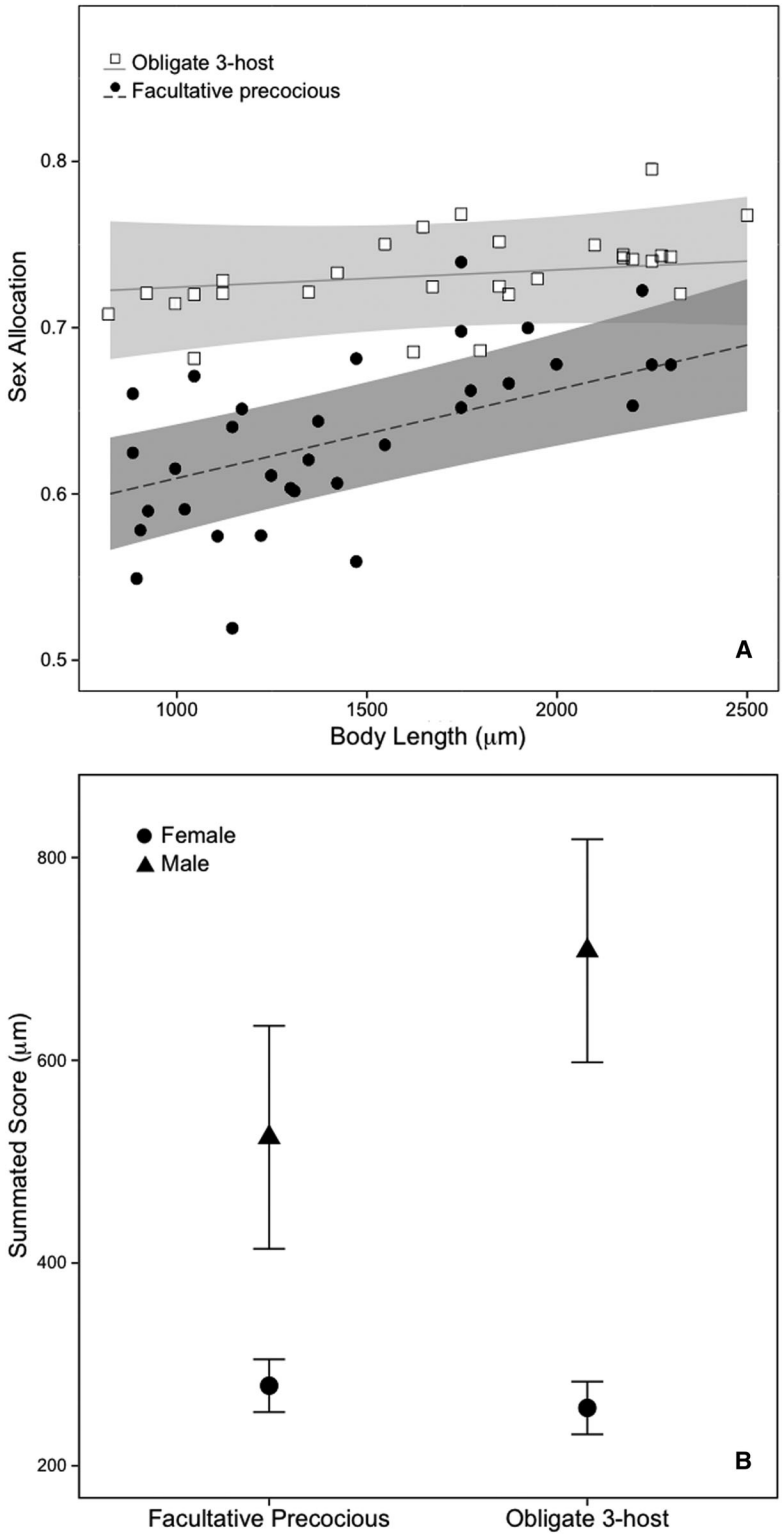

Figure 6. Results of the life history (facultative precocious vs. obligate three-host life cycle pattern) linear mixed models. (A) The interaction between life history and body size on sex allocation. Sex allocation is expressed as the ratio of the male summated score to the combined male and female summated scores. Shaded regions represent 95% confidence intervals based on the model SEs. The raw datapoints (i.e., not model adjusted) are superimposed over the model prediction. Note, up to a body size of 2224 μm the obligate three-host worms have significantly greater male-biased sex allocation relative to the facultative precocious individuals (discussed in text). (B) Least square means plot showing the relationship between life history and female (ovary) and male (combined testes) summated scores (μm). Error bars represent 95% confidence intervals.

nine intermediate crayfish hosts), and life-history variation (obligate three-host and facultative precocious) *of A. progeneticum*.

2. ND1 sequence data support the intraspecific status of specimens across locations and life history. However, haplotypes associated with facultative precocious populations were not shared with those found in obligate three-host populations. Additionally, haplotypes were not shared between the facultative precocious populations in different river drainages.
3. Within obligate three-host populations, worms taken from fish with a high intensity of infection had higher male-biased sex allocation than worms recovered from fish with low intensities. Worm body size also had a significant positive relationship with sex allocation.
4. Within facultative precocious populations, there was a significant interaction between worm body size and mating opportunity. In particular, there was a positive relationship between worm body size and sex allocation in worms from fish hosts (i.e., opportunity to outcross), but this relationship was absent in encysted adult worms from crayfish hosts (i.e., forced self-fertilization).
5. The test for a genetically based response of sex allocation showed that the populations with the facultative precocious life cycle evolved toward a female-biased sex allocation compared to the populations with an obligate three-host life cycle. This pattern was primarily driven by a decrease in male function in the facultative precocious populations.

ELUCIDATING THE BIOLOGY OF *A. progeneticum*

Prior to this study, the only known location of *A. progeneticum* was Calls Creek and the only known life history was that of a facultative precocious life cycle (Sullivan and Heard 1969; Font and Corkum 1975). Our survey and sequence data provide several key insights into the ecology and evolutionary history of *A. progeneticum*. First, *A. progeneticum* is much more widespread than originally thought (Fig. 1). Second, there is variation among populations in the developmental patterns of its life history and third, several ictalurid and crayfish species can serve as hosts. Because of the subtle morphological differences among the species of *Alloglossidium* that infect ictalurids, it is likely that *A. progeneticum* specimens were lumped under the umbrella classification of other *Alloglossidium* species (especially *A. corti*) in studies prior to the use of molecular data (Tkach and Mills 2011, Kasl et al. 2014). This lumping was also likely compounded by the fact that the variation in life cycle pattern went unrecognized. For example, reports of nongravid, encysted metacercariae in crayfish were assumed to be *A. corti* (McAllister et al. 2011) based on historical life cycle work (McCoy 1928). Indeed, sequence data have since verified that the encysted metacercariae from crayfish that were identified as *A. corti* in McAllister et al. (2011) are in fact, *A. progeneticum*. Adult specimens from slender madtoms (*Noturus exilis*) reported in McAllister et al. (2015) are also *A. progeneticum*. The measurements provided in Table S2, along with the extent of the vitellaria (extending from just below the cecal bifurcation to the anterior margin of the posterior testis; Fig. 2), can be used as a means to help with species identification in the absence of sequence availability (Kasl et al. 2014). Nonetheless, sequence data were, and will continue to be, imperative in correctly identifying new populations of *A. progeneticum*.

The identification of *A. progeneticum* from the other *Alloglossidium* species that infect fish is clearly discernable from the 14-nucleotide insert in the ITS1. The intraspecific status of our samples of *A. progeneticum* is supported by the lack of variation across this rDNA region and the low divergence at the mtDNA ND1 region. We will be addressing the phylogeny of the genus in a future manuscript. However, it is important to note here that preliminary findings based on these sequence data indicate the obligate three-host life cycle is the ancestral trait and the facultative precocious life cycle is the derived life history in *A. progeneticum* (E. Kasl et al., unpubl. data).

The ND1 data are also suggestive of two other interesting features of *A. progeneticum* evolutionary history that will warrant further investigation. First, haplotype and nucleotide diversities tend to be much lower in the facultative precocious populations (sites 4, 5, and 6; Figs. 1 and 3) compared to the populations with an obligate three-host life cycle (sites 1, 2, and 3; Figs.1 and 3). Because all encysted worms in the facultative precocious populations are forced to self-mate, inbreeding is assumed to be higher in these populations. Although inbreeding reduces the effective size (and hence measures of genetic diversity) at an autosomal locus, the mating system should not impact the genetic diversity at a haploid locus like the ND1 (Graustein et al. 2002, Charlesworth 2003). Nevertheless, the biology of self-maters (e.g., a single individual may colonize a population) may promote bottleneck/founder events that will result in low genetic diversity (Charlesworth 2003). It may also be that a selfing-gene (i.e., a mutation that enabled maturation while in the cyst) has swept through these populations resulting in a bottlenecking of a progenitor mtDNA haplotype. Additional data are needed to disentangle the causes of reduced genetic diversity in the facultative precocious populations. Second, no haplotypes are shared between the Flint River drainage (haplotype 18 in Richland Creek) and Oconee River drainage in Georgia (haplotypes 13 and 14 in Calls Creek and Big Indian Creek; Fig. 3). Moreover, the ND1 network (Fig. 2) suggests two possible origins of the evolution of facultative precocious development. For example, haplotype 18, which was only in Richland Creek, has less divergence to haplotypes 15 and 16 than to 13, which was in Calls and Big Indian Creeks (Fig. 2). Haplotypes 15 and 16 were from nongravid metacercariae (i.e., obligate three-host life history) collected from a nearby location

in Georgia (Jackson Branch, Table S1). Additional loci will be needed to confirm if facultative precocious development has independently evolved among populations of *A. progeneticum*. If indeed, these data reflect independent origins, then the evolution toward female-biased sex allocation in the facultative precocious populations (as discussed below) would also reflect independent evolutionary events in these two drainages.

PHENOTYPIC PLASTICITY OF SEX ALLOCATION

The effect of intensity of infection on sex allocation, in which worms from the high intensity category (i.e., larger social group size) had higher male-biased sex allocation, is in line with previous experimental findings on the plastic responses of sex allocation in the trematode *Echinostoma caproni* (Trouve et al. 1999a) and the free-living flatworm *Macrostomum lignano* (Janicke et al. 2013). For instance, Trouve et al. (1999a) found that an increase in the number of unique clones within a host increased allocation to male function and decreased allocation to female function. The above studies focused on the laboratory manipulation of mating group size. From field-based collections of the trematode *Gyliauchen volubilis*, Al-Jahdali (2012) reported greater male-biased sex allocation from higher intensity infections. Here, we also find support for mating group size (assumed here to correlate to social group size as measured by infection intensity) as a driver of a plastic response in sex allocation in a natural environment. We recognize that a caveat of our study is that we do not know how many unique clones (resulting from the asexual phase in the snail, Fig. S1) are present within an individual host even at high intensities. Nonetheless, population genetic studies on trematodes with similar life cycle patterns (e.g., obligate aquatic three-host life cycles) have shown that very few repeated clones are present within the final fish host (Criscione and Blouin 2006; Criscione et al. 2011; Gorton et al. 2012). Thus, we reason it is a safe assumption that the number of genetically unique individuals, and hence social group size, increases with the intensity of infection.

With regards to the overall opportunity to outcross (free in a fish gut vs. encysted in a crayfish), the interaction between body size and mating opportunity is an intriguing result. We observed a positive relationship between body size and sex allocation from worms in fish (i.e., there was greater allocation to the male function in larger individuals; Fig. 5A). This is concordant with the positive relationship of body size and sex allocation observed in the infection intensity analysis where all worms were from fish (see results section on the relationship between intensity of infection and sex allocation, Fig. 4A). In contrast, when worms were forced to self-mate, there was no relationship between body size and sex allocation (Fig. 5A). Thus, in the facultative precocious populations, there appears to be a plastic response for the size-dependent relationship to sex allocation that depends on the mating opportunity (forced self-fertilization in crayfish vs. opportunity to outcross in fish).

Size-dependent sex allocation has been reported in simultaneous hermaphroditic organisms (see Schärer 2009). However, the relationship may be positive or negative. Theoretical work shows that a negative relationship would result when small individuals with few resources should invest in male function due to a steep increase in male fitness gain. As individuals get larger, sex allocation would become female biased because fitness from male function has diminishing returns (Schärer 2009). A positive relationship may result if there is a competitive advantage to gain more mates with a larger body size. With more mates, larger individuals would have more male-biased sex allocation (Schärer et al. 2001; Schärer 2009). In our case, we observed a positive relationship. If competitive ability is the explanation, then one would expect that if competition is removed, then the positive relationship would no longer hold. Indeed, we see no body size relationship with sex allocation for the encysted adult worms recovered from crayfish hosts (i.e., there is no mate competition with forced self-mating) (Fig. 5A). Regardless of the explanation for the observed positive relationship, the interaction highlights another potential evolutionary consequence for a change in the mating system. That is, sex allocation itself may not be the only trait that evolves, but a size-dependent relationship (or lack thereof) with sex allocation may also evolve.

LIFE HISTORY AND SEX ALLOCATION

We found strong support that the change in life history from obligate three-host to facultative precocious development, which led to a change in mating system with potentially higher inbreeding, led to sex allocation that was more female-biased (Fig. 6A). This change largely resulted from a reduction in male function (Fig. 6B). The reduction of the testes size in the facultative precocious specimen is even clearly discernable in a line drawing comparison (Fig. 2). Even though the ovary size tended to be larger in the facultative precocious life history, there was no significant difference (Fig. 6B). We note, however, that other reproductive traits such as the vitellaria, which provide nourishment to the embryos, may also be important in terms of female allocation. The three dimensional overlapping and fragmentation of the vitellaria precluded an accurate measurement with our current staining and mounting methods.

To control for the potential plastic responses in sex allocation noted in the prior section, we limited our analysis on the genetically based evolutionary response of sex allocation to adult worms sampled from fish hosts with low intensities of infection. Because we controlled for these potential environmental influences, our results should reflect a genetically based response in the evolution of a more female-biased sex allocation in the facultative precocious life history.

A potential caveat has to deal with the actual mating system itself. Although we can be certain that there is forced-self mating in the crayfish hosts of the facultative precocious populations, we do not yet know if worms from fish in the obligate three-host populations are outcrossing. However, population genetic studies on trematodes with similar life cycle patterns (e.g., aquatic obligate three-host life cycles) have been shown to repeatedly have Hardy–Weinberg Equilibrium, and thus, are necessarily outcrossing (Criscione and Blouin 2006; Criscione et al. 2011; Gorton et al. 2012). In addition, cross-experiments have repeatedly shown that when trematodes are in the presence of other conspecifics they outcross (Agatsuma and Habe 1985; Nollen 1999; Trouve et al. 1999b). Thus, we consider it is a safe assumption that worms from fish in the obligate three-host populations at least have a higher level of outcrossing than compared to worms in the facultative precocious populations. Nonetheless, a direct test of the levels of inbreeding is warranted as it represents another potential consequence of a change in a parasite's life cycle pattern.

To our knowledge, our study represents the first to examine for a genetically based among-population evolutionary response in sex allocation in parasitic flatworms. The change in sex allocation is clearly associated with a change in the parasite's mating system. In comparison to some other studies that have examined among-population variation in sex allocation in animals, we find support that changes in mating systems or modes of reproduction can impact the evolution of sex allocation among populations. For example, Johnston et al. (1998) found an association between the mating system of a self-compatible hermaphroditic snail and sex allocation. Individuals from populations with higher selfing rates (estimated from a single locus) devoted a lower proportion of reproductive tissue to sperm production. In the free-living flatworm *Schmidtea polychroa*, populations with a greater frequency of parthenogenesis as a mode of reproduction had reduced sperm production (Weinzierl et al. 1998).

Overall, we believe that the significance of the results reported herein lies in the fact that a change in the life cycle pattern can alter the mating system and thus have consequences for the evolution of parasite reproductive traits. Specifically, forced selfing versus opportunities for outcrossing can alter allocation to male or female function. There are several other species of digenean trematodes that have independently evolved life cycles that deviate from the three-host pattern as a result of precocious development within what is typically regarded as an intermediate host (Lefebvre and Poulin 2005). Some of these species are also forced to self-fertilize as a result of maturation within a cyst. For instance, cryptic trematode species within *Stegodexamene anguillae* show among lineage variation in the frequency of precocious maturation while encysted in an intermediate host (Herrmann et al. 2014). It would be interesting to see if sex allocation toward female-biased function correlates to the frequency of forced self-fertilization in these closely related parasites. In a broader context, it will be worthwhile to compare across the digenean phylogeny to test if sex allocation toward female-biased function evolves repeatedly in these different lineages where precocious life cycle changes have occurred.

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DATA ARCHIVING

The doi for our data is doi:10.5061/dryad.g52j4. Molecular sequence data have been submitted to GenBank (accession numbers KT455707-KT455827).

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's website:

Figure S1. Diagram of the life cycle patterns of Alloglossidium progeneticum.
Table S1. Locality, host type, life stage (M = metacercariae, E = encysted adult [i.e., a gravid worm], A = adult), prevalence (%), and mean intensity (±1 SD, range in parentheses) for Alloglossidium progeneticum collected across sites spanning the southeastern United States (see Fig. 1).
Table S2. Comparative morphometrics with mean values ± SD (range) of adult Alloglossidium progeneticum specimens across the southeastern United States.
Table S3. Alloglossidium progeneticum: variable factor loadings, factor eigenvalues, and percent total variance accounted for by each factor from the Varimax rotated correlation matrix of all sampled worms.
Table S4. Infection intensity linear mixed model analyses using restricted maximum likelihood (REML) in R.
Table S5. Mating opportunity linear mixed model analyses using restricted maximum likelihood (REML) in R.
Table S6. Life history linear mixed model analyses using restricted maximum likelihood (REML) in R.