

Evolution of ‘maleness’ and outcrossing in a population of the self-fertilizing killifish, *Kryptolebias marmoratus*

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ABSTRACT

Question: Does the persistently high frequency of males in the Twin Cays population of *Kryptolebias* (formerly *Rivulus*) *marmoratus* (Pisces: Rivulidae), a self-fertilizing, androdioecious species, result from ecophenotypic effects or genetic divergence from other populations?

Hypothesis: Because males are easily produced in the laboratory by temperature manipulations of embryos or juveniles, their frequency in this population is environmentally induced.

Methods: Common garden experiment, two generations.

Conclusions: Genetic differences exist between the Twin Cays population and other populations in the tendency to produce males. Since males likely induce androdioecious outcrossing, and the Twin Cays population is not ancestral to others, this genetic difference may indicate a shift from predominant selfing to outcrossing, a direction not predicted by current theory.

Keywords: Atherinomorpha, clonal reproduction, Cyprinodontiformes, hermaphroditism, mating system, Rivulidae, *Rivulus*.

INTRODUCTION

Organisms with reproductive strategies that involve both hermaphroditic and gonochoristic components (see ‘Glossary of terms’ at the end of the paper) are informative models for analysing the factors that promote or maintain both outcrossing and self-fertilization in natural populations. The mangrove killifish, *Kryptolebias* (formerly *Rivulus*) *marmoratus*, displays such a mixed reproductive strategy: it is the only vertebrate known that propagates largely by self-fertilization. Most natural populations consist almost entirely of internally

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self-fertilizing hermaphrodites. At the same time, it is the only vertebrate with an androdioecious mating system. Formerly, all available evidence suggested that most populations of this species consisted solely of obligate selfers, and that their genetic structure was one of homozygous clonal lineages. The considerable inter-individual variation of these populations was attributed to *de novo* mutation and migration among sites. Recent extensive surveys of variation at microsatellite loci in Floridian populations have shown that while many populations do contain individuals that are (nearly) homozygous, and some consist entirely of apparent homozygotes, of mostly different genotypes, other populations also contain individuals that are heterozygous at variable numbers of loci. The pattern of variation suggests that self-fertilization is mixed or episodically alternated with (generally) low levels of outcrossing (Mackiewicz *et al.*, 2006b). Matings between hermaphrodites are unknown in this species and are unlikely to be productive, for sperm is believed to be in limiting supply and probably does not leave the ovotestis (Harrington, 1963). Therefore, outcrossing (and consequent heterozygosity) most likely stems from matings between hermaphrodites (acting as 'pseudofemales') and rare gonochoristic males. Unlike hermaphrodites, males produce abundant sperm. Very old hermaphrodites can sometimes preferentially lose testicular but not ovarian function, and one such individual was successfully crossed with a male from another strain early in the study of the biology of this species (Harrington and Kallman, 1968). Functional hermaphrodites sometimes emit viable but unfertilized eggs along with those fertilized internally (Harrington, 1963), and these could be available for fertilization by males (Atz, 1964). Successful male \times functional hermaphrodite crosses have recently been documented in the laboratory (Mackiewicz *et al.*, 2006a). However, factors that influence the frequency of outcrossing in natural populations are unknown, and many basic questions remain unanswered. The generally low but variable levels of outcrossing in Floridian populations have implications for the use of this organism as an experimental animal: the progenies of one individual collected from a natural population might have a relationship that is essentially clonal, but progenies of others from the same site might differ at variable levels that could even approach those of outcrossing species.

Twin Cays, a tiny mangrove island on the Belize barrier reef, supports an abundant and virtually unique population of *K. marmoratus* that has contained about 20% males at least since it was first sampled in 1989 (Davis *et al.*, 1990; Turner *et al.*, 1992a; D.S. Taylor, unpublished). In an equally compelling distinction from other natural populations, every hermaphrodite sampled on the island proved to be highly heterozygous by direct progeny test using multi-locus DNA fingerprinting (Lubinski *et al.*, 1995), and this level of heterozygosity has also persisted (Taylor *et al.*, 2001). These observations have been extended by Mackiewicz *et al.* (2006c), who used heterozygosity of microsatellite loci in this population to calculate an outcrossing rate of 0.55 to 0.58 (versus 0.1 to 0.2 in Floridian populations), a rate that appears to have been stable for at least 14 years. The data suggest that, on average, no individual in this population is removed from an outcrossing event by more than one or two generations of selfing.

In plants, phenotypic plasticity is frequently responsible for intraspecific breeding system variation and can mediate functional (but non-evolutionary) transitions between self-fertilization and outcrossing. For example, many largely cleistogamous species also have chasmogamous flowers (see 'Glossary of terms'), and natural populations of several ostensibly self-fertilizing species can include predominantly outcrossing as well as predominantly selfing individuals. Both phenomena often have significant environmental and/or demographic components (e.g. Waller, 1980; Schemske and Lande, 1985; LeCorff, 1993;

Redbo-Torstensson and Berg, 1995; Diaz and Macnair, 1998; Porras and Munoz, 2000; Culley, 2002; Sigrist and Sazima, 2002). Such environmentally mediated transitions avoid constraints that might be imposed on genetically based shifts by the ‘cost of meiosis’ or other putative barriers.

In *K. marmoratus*, functional gonochoristic males, despite their rarity in most natural populations, are readily induced in the laboratory by manipulation of environmental temperature during development (Fig. 1). [Note: Historically, *K. marmoratus* provided the

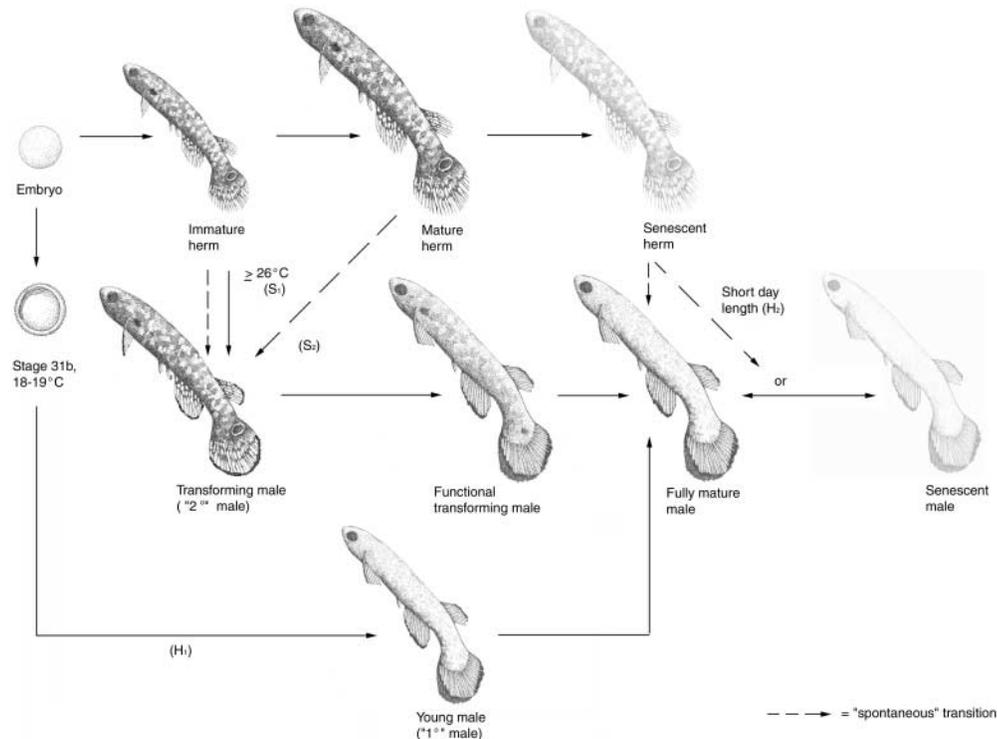


Fig. 1. Current knowledge of the thermolabile hermaphrodite-to-male transition (‘gender inversion’) in *K. marmoratus*. Low or high temperatures can induce males at different developmental stages. Pathway H₁ (‘H’ = Harrington, ‘S’ = secondary), which results in ‘primary’ males, is experimentally quite consistent and has received most attention. However, temperatures that induce it are generally lower than those regularly encountered by this species in its natural environment and are so low that hermaphrodites usually do not oviposit. Many embryos incubated at this temperature cannot hatch successfully unless they are artificially dechorionated. Pathways S₁ and S₂ can be induced by rearing/maintaining hermaphrodites at higher temperatures similar to those encountered in natural habitats and at which oviposition and natural hatching routinely occur. The ‘secondary’ males that result from this pathway are therefore more likely relevant ecologically than primary males. This pathway is less consistent experimentally than pathway H₁. Pathway S₂, the transition from a functioning and fertile adult hermaphrodite (rather than a juvenile, immature, or senescent one) to a male, is reported here for the first time and has been encountered only in some field-collected Twin Cays specimens. Pathway H₂, the transition of senescent hermaphrodites to (usually senescent) males, seems to be mediated mostly by photoperiod rather than thermal factors. In our hands, it has proven extremely inconsistent. This diagram is based on Harrington (1967, 1968, 1971, 1975) and laboratory observations made over a 15-year period.

first known example of 'environmental sex determination' in vertebrates (see Harrington, 1967).] In the light of this observation, and with the plant literature also in mind, we hypothesized that the high proportion of males on Twin Cays, which we term its 'maleness', would prove to have a largely ecophenotypic basis. Put another way, its breeding system, with unequivocally higher levels of (male-dependent) outcrossing, would be based upon the plasticity in gender phenotype characteristic of the species in general. The results of the common garden experiment reported here show that the 'phenotypic plasticity' hypothesis is incomplete or untenable. The production of males at high frequencies in the Twin Cays population seems to have, at least in part, a genetic basis. That is, maleness, a critical requisite for outcrossing, has apparently evolved in this population and is not entirely the result of ecophenotypic induction. This has some interesting implications for the evolution of androdioecious breeding systems.

MATERIALS AND METHODS

Rationale

If maleness in the Twin Cays population results solely from environmental effects, then, when lines derived from that population are propagated alongside other stocks in a common environment, the frequency of males should drop to levels characteristic of the other populations. Thus, the 'phenotypic plasticity' hypothesis predicts that male frequencies in laboratory lines derived from Twin Cays hermaphrodites will return to normal values. If maleness has a genetic basis on Twin Cays, then elevated levels of male production should persist in culture. [Note: The males produced in the experiments discussed here are the result of 'spontaneous transitions' at ordinary maintenance temperatures (22–24°C) and not of temperature manipulations.] In our hands, low levels of transition from immature or juvenile hermaphrodites to ('secondary') males occur in most laboratory lines. The frequencies of these transitions can sometimes vary even among the progenies of co-clonal organisms raised and held in identical environments (M.T. Fisher, unpublished).

Stocks

Dan2k

Progenitors were collected with traps and nets at the edge of rain-flooded mangrove forest, 10.25 km south of the central town marker, Dangriga, Belize, on 24 and 25 January 2000. Approximately 58 hermaphrodites and no males were collected. This mainland collection was not different from most others made of this species. These specimens were not analysed for genetic variation. However, in nearly all collections of this species, the number of detectable genotypes approaches the number of individuals in the sample (Turner *et al.*, 1990, 1992b), so it is likely that most of the individuals used were genetically distinct. This population was chosen for comparison because it was geographically proximate to the Twin Cays population but was composed completely of hermaphrodites.

TC2k

Progenitors were mostly collected with traps from crab holes on Twin Cays, an island approximately 12 km from the Belize mainland (see Davis *et al.*, 1990) and adjacent to Carrie Bow

Cay, where the STRI laboratory is located, on 26–28 January 2000. Approximately 54 apparent hermaphrodites and 15 obvious males at various stages of transition or sexual maturity were collected. Twelve of the presumptive hermaphrodites subsequently developed into males during the next 6 months in the laboratory, at least three of which had oviposited within 45 days before the beginning of transition and none were obviously senescent.

Maintenance and egg collection

All animals were housed in an environmental chamber held at $24 \pm 1^\circ\text{C}$ with a 12 h light/12 h dark photoperiod and with artificial lighting (Vitalite). For egg collection, all hermaphrodites (including the field-collected progenitors) were held singly in stackable glass 'finger bowls' (10 cm diameter) with removable plastic screen (2.5 mm) false bottoms (approximately 5–7 mm clearance from the bottom of the bowl). Salinity was 23 parts per thousand (artificial marine salts). Water was changed approximately every 7 days, when eggs were also collected. Fish were fed daily with *Artemia* nauplii supplemented at least twice per week with dried krill, floating pellets, white worms, or chopped red worms.

Incubation of ova and rearing of fry

Eggs were incubated in the dark at the same temperature and salinity at which the adults were maintained. Infertile or dead eggs were removed every 2 or 3 days. Some embryos reach full development but then enter a diapause and do not hatch. These 'stubborn eggs' were induced to hatch with physical agitation, application of peat extract, or by making the incubation water cloudy by the addition of dry fish food. A few extreme cases were manually dechorionated. Fry were housed in groups of no more than 8–10 in small Tupperware containers at the same salinity and temperature as the adults. They were fed *Artemia* nauplii exclusively and raised in these containers until their caudal ocelli were evident. They were then placed individually in finger bowls or small Tupperware containers and raised to adulthood. In our hands, overall embryo and fry mortality averages 25–30%. Much of the fry mortality is apparently due to cannibalism, for if growth rates are even slightly uneven, larger fry devour smaller ones.

Breeding design and progeny terminology

The first-generation progeny of an isolated, single field-caught hermaphrodite are designated as an 'F₁ brood'. The progeny directly derived from one or more hermaphrodites in an F₁ brood are collectively designated as an 'F₂ brood'. One to four different full-sib F₁ hermaphrodites were used as the parent of each F₂ brood, and their fertilized ova and resultant fry were pooled without reference to a particular individual parent. A 'line' refers to all the descendants of a field-collected hermaphrodite, regardless of generation or gender. [Note: The F₂ data from Twin Cays were limited by an often critical shortage of hermaphrodites in the F₁ broods, sometimes resulting in the termination of lines in the first generation, and by lower fecundity of many of the hermaphrodites that did appear.] Two previous attempts to compare the Twin Cays population with others in a common environment (1989–1991 and 1995–1996) were ruined by a similar paucity or absence of hermaphrodites in the F₁.

Gender determination

In laboratory-reared broods, gender was scored at an age of 10–12 months; many hermaphrodites mature within 2 or 3 months and oviposit at a minimum size of about 30 mm. Males were determined by the gradual loss of the caudal ocellus (and other dark body markings, if they were initially present) and the development of orange-pink body colour, which is highly correlated with testicular development (Soto and Noakes, 1994). In our hands, these traits have been reliable indicators of male gender, but in some instances they were absent from Twin Cays progeny when they were first scored, but appeared later, presumably indicating variability in the rate/extent of testicular development. Consequently, with the Twin Cays progeny, gender was re-scored at about 18 months. The orange-pink colour was used to score males in field collections from Twin Cays. In all cases, individuals that displayed even a trace of that colour in the field eventually developed the full suite of male secondary sex characteristics in the laboratory. As noted above, some Twin Cays specimens lacked orange colour and were considered to be hermaphrodites when examined in the field, but eventually developed full male characteristics in the laboratory; some of these had oviposited before gender transition was noted. This phenomenon could indicate protogynous hermaphroditism in this population, and warrants further study.

Statistical analysis

Contingency chi-square (χ^2) tests were used to compare the proportions of males and hermaphrodites (Sokal and Rohlf, 1995). Yates's correction for continuity ($\chi^2_{(Y)}$) was used where appropriate. Initially, proportions were compared among broods within generations, separately for the Dangriga and Twin Cays lines. If that comparison revealed no significant heterogeneity, then the data for the entire generation–locality were pooled for comparisons with other generations and localities. Thus, for example, F₁ brood data from 16 Dangriga lines were compared in a single contingency table (degrees of freedom = 15) and, since this comparison was not significant, the data were then pooled in comparisons with pooled data from F₂ Dangriga broods, and so on.

RESULTS

Brood data and gender ratios are given in Table 1.

Dangriga lines

The average frequency of 12% males in the F₁ generation is close to the overall average cumulative frequency of about 7–10% for all lines maintained in our laboratory. This frequency is higher than that encountered in most natural populations and also higher than that usually obtained in other laboratories. The temperature and salinity at which the fish are maintained in the laboratory are well within the ranges encountered by this species in nature, and we have no explanation for the higher frequency of males in our hands. Line Dan2k15, with 45% males, is an obvious outlier. However, no males were produced by this line in the F₂ generation. This result obviously suggests that all the factors that can influence gender in this species have not been identified. This brood aside, the Dangriga results are in

Table 1. Progeny gender data

Line	F ₁ broods				F ₂ broods ^d			
	Hermes	Males	Total	Ratio	Hermes	Males	Total	Ratio
Dangriga^a								
Dan2k01	19	0	19	0				
Dan2k02	9	2	11	0.18				
Dan2k03	9	0	9	0				
Dan2k04	14	3	17	0.18	7	0	7	0
Dan2k07	5	0	5	0				
Dan2k08	23	3	26	0.12				
Dan2k10	16	1	17	0.06				
Dan2k12	6	0	6	0				
Dan2k15	6	5	11	0.45 ^b	12	0	12	0
Dan2k18	15	2	17	0.12	14	0	14	0
Dan2k19	6	1	7	0.14				
Dan2k20	6	1	7	0.14	7	0	7	0
Dan2k21	10	1	11	0.09				
Dan2k22	6	0	6	0	6	0	6	0
Dan2k24	9	1	10	0.10				
Dan2k25	10	2	12	0.17				
<i>Totals</i>	169	22	191	0.12	46	0	46	0
Twin Cays^c								
TC2k01	0	21	21	1.0				
TC2k02	1	1	2	0.5	1	1	2	0.5
TC2k03	15	5	20	0.25	39	15	54	0.28
TC2k04	1	5	6	0.83	0	2	2	1.0
TC2k07	0	5	5	1.0				
TC2k09	0	14	14	1.0				
TC2k10	1	3	4	0.75	1	6	7	0.86
TC2k11	4	3	7	0.43				
TC2k12	1	3	4	0.75	0	1	1	1.0
TC2k13	0	3	3	1.0				
TC2k105	1	1	2	0.5	1	1	2	0.5
<i>Totals</i>	24	64	88	0.73	42	26	68	0.38

^a Dangriga, F₁ overall $\chi^2 = 20.15$, d.f. = 15, $0.25 > P > 0.1$, non-significant.

^b χ^2 (∞) F₁ line Dan2k15 vs. all other Dangriga lines (pooled) = 9.54, d.f. = 1, $P < 0.005$. χ^2 all F₁ Dangriga lines but Dan2k15 = 10.48, d.f. = 14, $0.75 > P > 0.5$, non-significant.

^c χ^2 (∞) F₁ = 34.02, d.f. = 6 (all broods less than 5 pooled), $P < 0.001$.

^d F₂ progeny from 1 to 4 breeders; F₁ progeny from single field-caught hermaphrodites.

all respects typical of most lines of this species maintained in our laboratory. No males were encountered in the F₂ broods. F₃ data for three lines are at hand: Dan2k04 (2 breeders), 22 hermaphrodites; Dan2k12 (one breeder), 14 hermaphrodites; and Dan2k18 (3 breeders), 39 hermaphrodites; again, no males were produced.

Twin Cays lines

The frequency of males in F_1 broods is heterogeneous, but in all cases but one is higher than in the Dangriga lines. The heterogeneity prevents a direct comparison of Dangriga and Twin Cays averages, but if we take the *lowest* value, 25% (TC2k03), and apply it to the entire sample, the difference between Dangriga (*including* the outlier Dan2k15) and Twin Cays F_1 broods is statistically significant. The difference in male production between the F_1 Dangriga and Twin Cays lines is striking. The F_2 data are more fragmentary, but the overall frequency of males in the F_2 (38%), while lower than the F_1 broods (73%), is obviously higher than both the F_1 (12%) and F_2 (0%) Dangriga averages.

The proportion of males in both the F_1 and F_2 Twin Cays broods is higher than those we have observed in the natural population (20–25%). This increased frequency of males could stem from the same (unknown) factors responsible for the higher frequency of males in our laboratory colony in general, perhaps coupled with enhanced susceptibility on the part of the Twin Cays population. In other words, some condition in our laboratory may amplify an inherent difference between the populations. On the other hand, we cannot completely discard the notion that males, which, when mature, are highly conspicuous, are selectively removed from the natural population by predation. Moreover, roughly 20% of the presumptive hermaphrodites collected on Twin Cays in 2000 matured into males in the laboratory within 4 months of collection, suggesting that field surveys may underestimate the proportion of males in that population.

DISCUSSION

The Twin Cays lines clearly produced a higher proportion of males than those from Dangriga, even though there was a drop in male frequency in F_2 versus F_1 lines from both localities. That is, in qualitative terms, the tendency towards higher male production of the Twin Cays population persists for at least two generations in the ‘common garden’ environment of culture in our laboratory. Therefore, this tendency (‘maleness’) likely has a genetic component and is not simply ecophenotypic, even though gender succession in *K. marmoratus* can also be influenced by environmental temperature. Plausibly, the Twin Cays population might contain specific genetic factors that lower the threshold of an environmentally mediated shift to the development of males rather than hermaphrodites. Our data certainly do not rule out the existence of ecophenotypic effects in addition to genetic factors in producing males in this population.

The data also do not allow us to assess the variance in strength of maleness among individuals. In the atherinid fish *Menidia menida*, the relative predominance of genetic or environmental sex determination varies among individuals, families, and populations (reviewed in Conover and Heins, 1987). Variance of this sort could exist for *K. marmoratus* as well, for there is no reason to believe that all Twin Cays genotypes have the same potential for maleness or are influenced by the environment to the same extent. If the variance is substantial, then our treatment of ‘maleness’ as a single trait is likely an oversimplification. In fact, the variation in male frequencies among the F_1 Twin Cays broods is consistent with variation in the strength of ‘maleness’. It is also tempting to speculate that the reduction in the frequency of males in the F_2 versus F_1 generation stems from the elimination of the most ‘male-prone’ genotypes, which, under our conditions, produced the all-male F_1 broods.

A relatively novel hypothesis that fits the data sees the development of males rather than hermaphrodites as a result of outcrossing *per se*, with the tendency to become male directly correlated with individual heterozygosities. In an androdioecious population, outcrossing increases heterozygosity, while self-fertilization decreases it. Thus, the progenies of androdioecious matings would contain a higher proportion of males than those of selfers. If the frequency of outcrossing is itself related to the frequency of males, then the frequencies of both could rise very rapidly in a population by an amplification process: a single outcross mating could produce several males, and these mating with hermaphrodites would produce more males, who would mate with more hermaphrodites, and so on. From the perspective of the developmental stability of the hermaphrodite phenotype, this hypothesis, in effect, treats 'maleness' as a form of outbreeding depression. The presumptive scenario is obviously speculative, but we note that it is testable: laboratory lines derived from Twin Cays and maintained by selfing should show correlated losses of heterozygosity and maleness and correlated variances of both traits. Progenies of laboratory crosses between genetically divergent non-Twin Cays stocks should be more prone to develop into males than either parental line maintained under the same conditions.

Regardless of whether the maleness of the Twin Cays population is a function of overall levels of heterozygosity or of the presence of specific male-promoting genetic factors, two lines of evidence argue that low levels of outcrossing characteristic of mainland populations was most likely the ancestral condition. First, there is no indication that the Twin Cays population is 'basal' in any sense. MtDNA haplotypes detected by restriction fragment comparisons (Weibel *et al.*, 1999) and D loop sequences (M.T. Fisher and B.J. Turner, unpublished) are shared with other populations in the region in which males are rare, including Dangriga and other mainland localities. Second, phylogeographic data strongly suggest that the species originated in the neotropics and migrated northward to the Caribbean region, rather than the reverse: *K. marmoratus* and its sister species, *K. caudomarginatus*, comprise a well-supported clade distinct from other '*Rivulus*' species (Murphy and Collier, 1996; Hrbek and Larson, 1999). The latter occurs in southeast Brazil, where its range overlaps completely with the southern range of *K. marmoratus* [apparently terminating about the latitude of Rio de Janeiro–Santos (Huber, 1992)]. The two other species in the recently recognized genus *Kryptolebias* [and the new subfamily, Kryptolebiatinae (Costa, 2004a, 2004b)] also have restricted neotropical distributions. Thus, 'ancestral' populations of *K. marmoratus* are more likely to be found in the southern part of its range and not, as in Belize, nearer to its northern limit.

If the argument above holds, then evolution of high levels of outcrossing in the Twin Cays population was essentially a transition from ancestral hermaphroditism to androdioecy, and it is arguably the first documented case of evolution in that direction. Androdioecy is a rare mating system, but in the two animal species in which it has been most studied, the ancestral form was apparently dioecious and not hermaphroditic [see Chasnov and Chow (2002) and Stewart and Phillips (2002) for *Caenorhabditis elegans*; see Sassaman (1995) and Hollenbeck *et al.* (2002 and references therein) for the conchostracan clam shrimp, *Eulimnadia texana*]. In plants, dioecy is generally held to be the likely ancestral condition [documented in *Datisca glomerata* by Rieseberg *et al.* (1992)]. Theoretically, putative transitions from hermaphroditism to androdioecy require that 'invading' males must have fertilities at least double the 'male fertility' of hermaphrodites. If partial selfing occurs, then males must have an even greater advantage, for fewer eggs would be available for fertilization (Charlesworth, 1984; Fritsch and Rieseberg, 1992; but see Pannell, 2002). If the ancestral state approached complete selfing, as it apparently did in *K. marmoratus*, then, by this logic, the

fitness differential of males and/or of their outcrossed progeny would need to be implausibly large. It is fair to conclude that the evolution of maleness, and consequently of high levels of outcrossing, in the Twin Cays population of *K. marmoratus* might well require some modification of current ideas about the factors that can promote or constrain transitions among different breeding systems.

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GLOSSARY OF TERMS

Androdioecious (Bot.): Having perfect and staminate flowers on different plants. More generally, a breeding system consisting only of hermaphrodites and males, usually as separate individuals, in which ova from hermaphrodites can be fertilized by sperm (or pollen) from males.

Chasmogamous (Bot.): Of, or relating to, a flower that opens to allow for pollination; in self-fertilizing species. Contrast with *cleistogamous*.

Cleistogamous (Bot.): Of, or relating to, a flower that does not open and is self-pollinated in the bud. More generally, anatomically obligate self-fertilization.

Gonochoristic: Of, or relating to, breeding systems in which males and females are separate individuals. [Noun form: *gonochorist(s)* (seldom used).] Contrasted with *hermaphroditic (-ite)* or ‘co-sex’.