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RESEARCH ARTICLE

No barrier to fertilisation when different sexual populations of the mānuka stick insect are crossed

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ABSTRACT

The mānuka stick insect *Clitarchus hookeri* (White) is facultatively parthenogenetic, but females from sexual populations that have mated with males from their own population do not produce any offspring via asexual reproduction. In contrast, females from parthenogenetic populations of *C. hookeri* mate with males (in captivity) but show a partial barrier to fertilisation with more than 90% of their offspring resulting from asexual reproduction post mating. Captive crossing experiments with parthenogenetic females require the mating of individuals from different populations (sexual and parthenogenetic), thus potential intraspecific differences bring a confounding element to these experiments. Experiments mating sexual females with males from different sexual populations were undertaken to determine whether offspring resulting from such a cross would be the result of sexual or parthenogenetic reproduction. Virgin females and males were collected from two sexual populations known to represent distinct genetic lineages (Waikato and Whanganui). Eleven adult females were caged with non-local males and eggs collected post-mating. Approximately equal numbers of sons and daughters hatched (168 female; 210 male) suggesting all offspring were the result of sexual reproduction. In these intraspecific crosses no barriers to fertilisation were detected, suggesting that in the absence of males the decay of some sexual trait in Phasmids can occur in fewer than 100 generations.

KEYWORDS

Clitarchus hookeri; parthenogenesis; Phasmatidae; sexual reproduction

Introduction

The mānuka stick insect *Clitarchus hookeri* (White) is endemic to Aotearoa New Zealand and common over much of the country (Salmon 1991; Buckley et al. 2010). This nocturnal species is facultatively parthenogenetic as unmated females from any population can produce daughters via asexual reproduction. In the northern part of its range, populations have equal numbers of males and females and reproduction is sexual. In southern and eastern North Island and in South Island, populations of *C. hookeri* comprise only females who reproduce via parthenogenesis (geographic parthenogenesis; Morgan-Richards et al. 2010). After mating, females from sexual populations produce eggs that hatch equal numbers of sons and daughters (Figure 1). Females from sexual populations raised in captivity without males produce eggs that all hatch as daughters (Salmon 1955; Morgan-Richards 2010). In contrast, females from all-female populations and mated in captivity produce fewer than 5% sons, revealing a partial barrier to fertilisation (Figure 1) (Morgan-Richards et al. 2010, 2019). It is not known what limits sexual reproduction, but we can infer that this barrier to fertilisation has arisen twice within *C. hookeri* (Morgan-Richards

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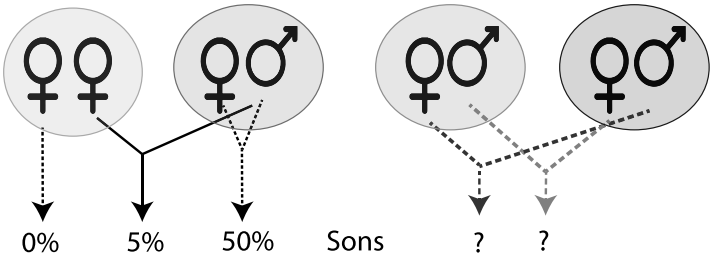

Figure 1. Summary of the known and unknown outcomes of sexual and parthenogenetic reproduction in the mānuka stick insect *Clitarchus hookeri* when females mate with local or non-local males.

et al. 2019). In contrast, we have not detected evidence of decay of traits involved in sexual signalling as when given a choice *C. hookeri* males prefer to mate with parthenogenetic females than with sexual females (Nakano et al. 2019).

Captive crossing experiments with parthenogenetic females require mating individuals from different populations as parthenogenetic populations do not have males, and thus intraspecific differences produce a confounding element to these studies. In previous crossing experiments, males came from populations that differed from the females by between 5.9% and 4.3% (mtDNA sequence divergence) and between 0.5 and 0.12 pairwise F_{ST} (14 microsatellite loci; Morgan-Richards et al. 2010, 2019). Captive experiments mating sexual females with males from different populations have not reported whether offspring resulting from such a cross would be the result of sexual or parthenogenetic reproduction. Thus, I undertook to cross virgin females from sexual populations with males from different populations and to use the sex ratio of offspring to infer whether nymphs were produced sexually or asexually.

The aim of this study was to determine whether intraspecific differences could explain previously reported barriers to fertilisation exhibited by parthenogenetic lineages of *Clitarchus hookeri*. If individuals from genetically differentiated populations mate but do not produce sons, this would suggest that barriers to fertilisation are not linked to the switch to parthenogenetic reproduction. However, if crosses between individuals from distinct *C. hookeri* sexual populations produce equal numbers of sons and daughters, this would suggest reproductive strategy rather than genetic divergence per se is responsible for creating barriers to fertilisation in this species. One *C. hookeri* lineage switched from sexual reproduction to parthenogenetic reproduction in fewer than 100 generations (based on its translocation history from Taranaki to the Isles of Scilly, UK), so this has relevance for inferring the speed at which barriers to fertilisation can evolve in the absence of males.

Methods

Clitarchus hookeri were collected from two sexual populations in early summer 2020 (10 males and 10 females from each site). Adult and juvenile stick insects were collected from kānuka (*Kunzea ericoides*) during the day from the shore of Lake Karapiro, Waikato. Juvenile stick insects were collected during the night from mānuka (*Leptospermum scoparium*) in Bushy Park Tarapuruhi Forest Sanctuary, Whanganui. These two populations were represented in previous genetic studies, so are known to differ for both mitochondrial (3.1–5.3% sequence difference) and nuclear markers ($F_{ST} = 0.533$; Morgan-Richards et al. 2019).

In captivity, individuals from the two locations and two sexes were caged separately. The stick insects were provided with mānuka and pōhutukawa (*Metrosideros excelsa*) leaves to eat and subjected to natural daily light and temperature cycles. Two females collected as adults from Karapiro (assumed to have mated with local males) were held on their own for 4 weeks (from 5 December 2020 until 2 January 2021). Eggs from these two female stick insects were collected at three time points: end of week one, end of week three and end of the fourth week. The eggs from these two

females provide controls to ensure storage conditions were suitable for egg development and hatching.

When collected, the instar of juvenile specimens was not known. Sexual maturity was inferred from a combination of body length (females > 80 mm; male > 60 mm), operculum of females reached almost to tip of abdomen (Stringer 1970) and behaviour (females dropped eggs, males clasped females). Eleven female stick insects moulted to become adults while in captivity and thus were virgins until males were provided. When females moulted to maturity males from different populations were added to their cages. When mating was observed, females were transferred to separate boxes so eggs were collected from single females. Six females from Karapiro were each mated with one or two males from Bushy Park Tarapuruhi and five females from Bushy Park Tarapuruhi were mated with males from Karapiro. These 11 females were held until they died or were killed in April 2021 (between 3 and 11 weeks as adults). Eggs from each female were separated from the leaf litter and frass in their box, counted and transferred to damp paper in plastic boxes. Eggs from each female were held in the same room at temperatures between 10 and 16 °C for 12 months.

Eggs produced via sexual reproduction will hatch sons and daughters in equal proportions (Figure 1). The number of sons and daughters can be used as an estimate of how many nymphs are the result of sexual reproduction even when sons are uncommon. In previous work, genetic data from a non-random sample of offspring ($n = 30$) confirmed that 30% of nymphs were the result of sexual reproduction when 20% were sons (Morgan-Richards et al. 2019). Without genetic information one can infer all offspring are the result of sexual reproduction if 50% or more of nymphs are male. Parthenogenetic nymphs hatch on average later than sexual nymphs but within 11 months of laying (Stringer 1967; Morgan-Richards et al. 2010), and females can store sperm for many months (pers. obs.). Males can also be produced from parthenogenetic reproduction via rare mutation during egg production involving the loss of one X-chromosome, but this has been observed only once from hundreds of parthenogenetic eggs (Morgan-Richards et al. 2010).

When nymphs hatched, they were sexed (Stinger 1970) before being either transferred to cages for raising or preserved in ethanol. Nineteen nymphs were recorded as hatching but not sexed due to their death before removal from egg boxes. The null hypothesis of an equal sex ratio was tested for nymphs that hatched from each female using a two-tailed exact binomial test in Excel.

Results

In captivity, adult females dropped between 11 and 96 eggs after mating. The two control females from Karapiro who had mated with local males laid 50 and 57 eggs each over 4 weeks. From 107 eggs, 68 nymphs had hatched by the end of 2021 (63.6% hatching rate after 12 months); 37 nymphs were daughters and 31 sons (female:male ratio ~ 1.19; Figure 2). Male offspring were produced from all three collection time points and over the full hatching window.

A total of 601 eggs were collected from 11 females after they had mated with non-local males (Table 1). After 12 months, 397 nymphs had hatched. Hatching rate per female ranged from 100% to 11% (average 66%; Table 1). All 11 females produced sons; in total 168 nymphs were female and 210 were male (female:male ratio ~ 0.8; Figure 2). None of the females produced fewer sons than expected from 1:1 ratio (lowest p-value = 0.125; Table 1).

Discussion

Hatching rate of *Clitarchus hookeri* eggs from control females (local × local; 64%) was similar to the hatching rate of eggs from my crossing experiment (66%). Sons were produced as often as daughters when stick insects from Waikato mated with stick insects from Whanganui. The equal sex ratio observed suggests all offspring were the result of sexual reproduction not parthenogenetic reproduction despite crosses between individuals from genetically distinct populations. Therefore,

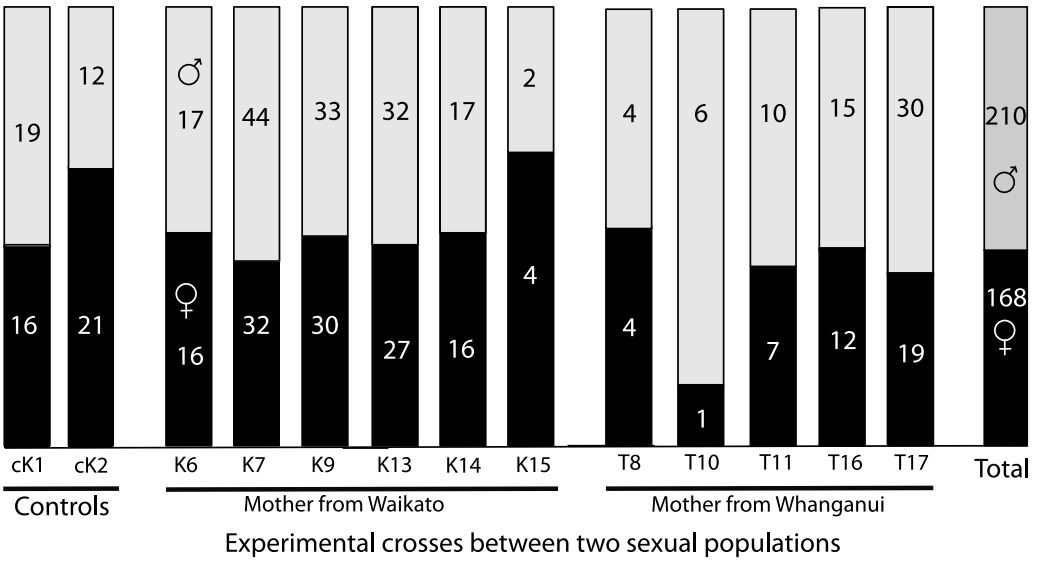

Figure 2. Sex ratio of *Clitarchus hookeri* nymphs produced by 13 females in captivity. Two control females mated with local males (cK1 and cK2). Eleven females mated with non-local males in captivity. Crosses consisted of parents from Karapiro, Waikato (K) and Bushy Park Tararuruhi, Whanganui (T) with origin of female indicated. Black bar = daughters, grey bar = sons. Total consists of non-local crosses only.

I found no evidence of a barrier to fertilisation between intraspecific pairings of *C. hookeri* involving two genetically distinct sexual populations.

The partial barrier to fertilisation previously observed in parthenogenetic *C. hookeri* could be interpreted as evidence of evolution – an example of the decay in sexual traits as seen in other parthenogenetic lineages (Carson et al. 1982; Schwander et al. 2013). In the North American stick insect lineage *Timema*, the decay of reproduction traits in females (altered airborne and contact signals, modified sperm storage organs, barrier to fertilisation) was inferred to have been driven by selection (Schwander et al. 2013). However, the lack of sexual reproduction observed in *C. hookeri* from all-female populations might also be explained by genetic divergence as the sperm donor, by definition, must come from a different (sexual) population. In Wellington a parthenogenetic *C. hookeri* population has apparently regained sexual reproduction via *in situ* production of males – suggesting local males may be more likely to overcome barriers to fertilisation (Morgan-Richards et al. 2019). In contrast, there is little evidence of reproductive barriers between some distinct sexual taxa. For example, in northern New Zealand successful mating between *C. hookeri* and *Clitarchus tepaki* has resulted in populations dominated by hybrids (Myers et al. 2017) and *C. hookeri* is a parental taxon in the hybridisation that produced the *Acanthoxyla* lineage (Morgan-Richards & Trewick 2005; Trewick et al. 2008; Morgan-Richards et al. 2016), suggesting inter-species mating can be successful.

The experiment here tested whether sexual reproduction was limited between distinct sexual lineages of *C. hookeri* by crossing individuals from different populations. From the ratio of sons to daughters I infer that all nymphs were the result of sexual reproduction. Although I have tested just two of the many sexual populations of *C. hookeri*, it seems likely that previous observations of barriers to fertilisation exhibited by females from two distinct parthenogenetic lineages of *C. hookeri* are the result of changes linked to their new mode of reproduction. Evidence that reproductive strategy rather than divergence per se is responsible for creating barriers to fertilisation in this species is important for the interpretation of the observed decay of sexual traits in parthenogenetic lineages. For example, an all-female lineage of *C. hookeri* has established in the UK after an accidental translocation of individuals from a sexual population in Taranaki (Morgan-Richards et al. 2019). As the UK population established within the last 100 years, we can infer that in the

Table 1. Virgin female *Clitarchus hookeri* stick insects from two sexual populations were mated with non-local males and their offspring sexed to infer mode of reproduction.

Cross code	Mother's origin	Father's origin	Number of eggs laid	Number of nymphs hatched	Hatching rate (%)	Number of daughters	Number of sons	Number unsexed offspring	Ratio female: males	Exact binomial test (*p*-values)
cK1	Karapiro	Karapiro	57	35	61.4	16	19	0	0.84	0.736
cK2	Karapiro	Karapiro	50	33	66.0	21	12	0	1.75	0.163
K6	Karapiro	Tararuruhi	39	33	84.62	16	17	0	0.94	1
K7	Karapiro	Tararuruhi	77	77	100	32	44	1	0.73	0.207
K9	Karapiro	Tararuruhi	86	68	79.07	30	33	5	0.91	0.801
K13	Karapiro	Tararuruhi	96	70	72.92	27	32	11	0.84	0.603
K14	Karapiro	Tararuruhi	52	34	65.38	16	17	1	0.94	1
K15	Karapiro	Tararuruhi	54	6	11.11	4	2	0	2.00	0.688
T8	Tararuruhi	Karapiro	16	8	50.0	4	4	0	1.00	1
T10	Tararuruhi	Karapiro	11	7	63.64	1	6	0	0.17	0.125
T11	Tararuruhi	Karapiro	24	18	75.0	7	10	1	0.70	0.630
T16	Tararuruhi	Karapiro	80	27	32.50	12	15	0	0.80	0.701
T17	Tararuruhi	Karapiro	67	49	73.13	19	30	0	0.63	0.152
			601	*397*	*66.06*	*168*	*210*	*19*	*0.80*	

Two females mated with local males (controls; cK1, cK2), but totals are for intra-population crosses only. Exact binomial tests are two-tailed using null of an equal number of sons and daughters.

absence of males a barrier to fertilisation evolved in fewer than 100 generations. This is faster than the decay of sexual traits inferred in *Timema* species (less than 100,000 years; Schwander et al. 2013) but very similar to the 60 generations required for the fly *Drosophila mercatorum* to show reduction in sexual behaviour in one parthenogenetic lineage (laboratory strain maintained for 7 years, with ~8 generations per year; Carson et al. 1982). Thus, the translocated population of *C. hookeri* provides a useful microevolutionary system for studying the drivers of rapid trait decay.

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