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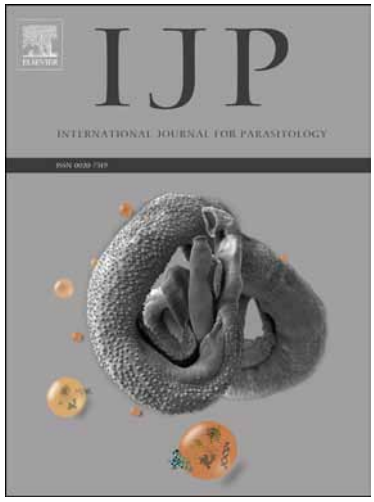

Mating strategies and multiple paternity, assessed by microsatellites, of the dispersal-limited, ectoparasitic tree-hole tick *Ixodes arboricola*

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Mating strategies and multiple paternity, assessed by microsatellites, of the dispersal-limited, ectoparasitic tree-hole tick *Ixodes arboricola*

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Abstract

Multiple mating is common among ticks, a large group of haematophagous ectoparasites, but multiple paternity has rarely been investigated. Multiple paternity may be common because the resultant increased genetic diversity allows ticks to rapidly evolve in relation to host responses and increases colonisation potential in novel habitats. Knowledge concerning mating systems is important because ticks may have profound effects on their hosts and are the principal transmitters of many pathogenic agents. In the current study, we investigated the mating system of the nidicolous tick *Ixodes arboricola*. These ticks attach to their bird hosts in the nest, which restricts gene flow but facilitates finding a partner off-host. Having genetically variable offspring may be beneficial for ticks which may encounter very different conditions when dispersed to the nest of another host type. We conducted an experiment in which female ticks fed on great tit nestlings and mated with two males in three treatments of the females: mating with both males before feeding, mating with one male before and the other male after feeding, or mating with both males after feeding. We investigated paternity with microsatellites. In a complementary experiment we investigated male preference for unfed or engorged females, and measured mating duration. We predicted (i) there would be multiple mating by *I. arboricola* males and females, leading to multiple paternity, and (ii) males would prefer to mate with engorged females and those matings would last longer because engorged females present a higher probability of successful reproduction. We found multiple paternity within clutches but no indications of sperm precedence. Males preferred to mate with engorged females and those matings lasted significantly longer, even including attachment beyond egg deposition. We suggest such mate guarding and male preference for mating after feeding is adaptive because there is no first male precedence. Male preference for mating after feeding may also be adaptive because dispersal is low and females are available after the blood meal.

Keywords: Mating strategies, Microsatellites, Multiple paternity, *Ixodes arboricola*, Ectoparasites

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1. Introduction

Multiple mating is common throughout the animal kingdom and in many species both sexes exhibit polygamous behaviour. For males, the benefits of polygamy are obvious. Their gametes are small and numerous, such that males can fertilise many partners. Multiple mating increases fecundity and therefore increases fitness (Yasui, 1998). Males therefore often compete for fertilisations (Parker and Pizzari, 2010). For females, on the other hand, promiscuous mating does not increase fecundity because all the eggs she can produce can be fertilised by one male alone (Bateman, 1948). However, females may obtain other benefits from multiple mating. Multiple mating may be a strategy of females to choose a high quality male in situations where it is either not possible to distinguish between males of different qualities or where encounters with males are unpredictable. Multiple paternity may also increase fitness if environmental conditions are unpredictable or rapidly changing, because it leads to offspring with higher genetic diversity (Watson, 1991; Yasui, 1998). In parasites, increased genetic diversity due to multiple paternity may aid in the arms race with the parasite's hosts and allow greater colonisation potential (Poulin, 2007).

Hard ticks (family Ixodidae, hereafter: ticks) are haematophagous ectoparasites of the superfamily Ixodoidea that infest a a wide range of vertebrate hosts (Hoogstraal and Aeschlimann, 1982; McCoy et al., 2013). It has been shown experimentally that *Borrelia* (Alekseev et al., 1999) and Rickettsiae (Hayes et al., 1980) may transmit sexually between ticks. Therefore, knowledge concerning tick mating systems may be useful for tick-borne disease prevention. It has been found that multiple mating occurs in many genera of hard ticks (Oliver, 1974), and multiple paternity has been confirmed in a limited number of tick species (McCoy and Tirard, 2002; Hasle et al., 2008; Cutullé et al., 2010; Ruiz-López et al., 2012). Yet, there is a great deal of diversity among tick species in several areas, e.g. host and habitat use, life history and mating strategies. It is currently largely unclear which factors have been important in the evolution of mating systems in ticks.

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Ticks reproduce following a coordinated series of stylised behaviours and physiological responses to discrete stimuli and complex developmental changes, largely regulated by pheromones (Kiszewski et al., 2001; for details, see Kaufman, 2008). After mounting the female, the male inserts his mouthparts into the female gonopore. Although this position may be maintained for 1-2 h, insemination generally follows quickly (Kiszewski et al., 2001; Kaufman, 2008). The male forms an external spermatophore in less than a minute, which he transfers to the female's genital pore with his mouthparts in less than a minute (Kiszewski et al., 2001). Mating pairs may remain in sexual contact for days after sperm transfer (Feldman-Muhsam and Borut, 1978; Kiszewski et al., 2001). Inside the female, sperm is stored in oviducts, where it survives for only a short period (Gladney and Drummond, 1971). As early as 1-2 weeks after the female begins to feed, eggs are released into the oviducts, where they are fertilised. Oviposition can occur several weeks after feeding, but tends to be seasonal and may be delayed by many months (Kiszewski et al., 2001). Females lay hundreds to thousands of eggs, depending on the species and size of the bloodmeal, and die soon after oviposition (Sonenshine, 1991).

Ticks are ideal study organisms to investigate mating strategies due to the potential differences in the life history constraints imposed on ticks by their hosts (McCoy and Tirard, 2002). There are two major factors that differ considerably among tick species. First, the Ixodidae are divided in two phyletic lines with contrasting life histories and mating strategies: the Prostriata and Metastriata. Currently, the former comprises a single genus (*Ixodes*), and the latter comprises 11 genera in four sub-families (Guglielmone et al., 2014). All hard ticks take a single blood meal in each instar (larva, nymph, adult) before moulting to the next instar or laying eggs (Sonenshine, 1991). In metastriate ticks, adults of both sexes require a blood meal for gonadal maturation, and mating can occur only during or after feeding (Oliver, 1974; Sonenshine, 1991). Metastriate ticks invariably mate while attached to their hosts (Kiszewski et al., 2001). In prostriate ticks gonadal maturation is completed in the late nymphal phase and therefore mating can occur before, during and after a blood meal. Adult males of most prostriate tick

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species do not take a blood meal and never seek hosts, but remain in the off-host habitat, where they mate with adult female ticks (Oliver, 1974; Sonenshine, 1991; Kiszewski et al., 2001).

Second, there is a marked source of variation in the ecology of ticks due to their host-seeking behaviour. Non-nidicolous ticks actively seek hosts when the seasons are suitable (Hillyard, 1996). They often feed on far-ranging hosts that are widely dispersed in the environment. In contrast, nidicolous ticks attach to their host inside the nest or burrow, where they also detach. The focal occurrence inside nests may be disadvantageous to the parasite, as dispersal events via their returning host are rare, and therefore gene flow is restricted (McCoy et al., 2003; Van Oosten et al., 2014a). On the other hand, nidicolous ticks are likely to find a partner more easily than non-nidicolous ticks (McCoy and Tirard, 2002). Attachment to a host as well as the nidicolous lifestyle come with certain risks, which may be reflected in the ticks' mating system. First, there is a risk imposed with feeding and being on the host because hosts may kill their parasites, e.g. by grooming or immunological defence (Wikel, 1996). If mating off-host reduces the time spent on a host, it is adaptive because partners are available in the off-host habitat. In species where gonadal maturation is completed in the nymphal phase, male ticks may avoid hosts altogether. Off-host mating is indeed common among nidicolous ticks (McCoy and Tirard, 2002; Gray et al., 2014). For male nidicolous ticks, the feeding risk for female ticks also implies that the best strategy would be to select only those females that have already taken a blood meal. However, in species where there is first male sperm precedence (i.e. a selective advantage for males that mate first), selection for postprandial (i.e. after feeding) mating may represent a trade-off with competition among males (Kiszewski et al., 2001; Kaufman, 2008). Second, there may be high mating competition because tick populations in nests occasionally reach high local densities (Haarløv, 1962). Such local competition could lead to multiple mating and sperm competition (Parker, 1970; Kiszewski and Spielman, 2002). Finally, multiple paternity and the resultant genetic diversity may be a selective advantage for female ticks because dispersal to nests of different host species can be associated with different environmental conditions (McCoy and Tirard, 2002; Van Oosten et al., 2014b).

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In the current study, we investigated the mating system of the nidicolous, prostriate tick *Ixodes arboricola* Schulze and Schlottke 1929. This tick chiefly infests cavity-nesting birds, with great and blue tits (*Parus major*, *Cyanistes caeruleus*) as its principal hosts (Walter et al., 1979; Hudde and Walter, 1988; Petney et al., 2011). Although great and blue tits are very mobile, opportunities for *I. arboricola* to disperse among cavities are limited, leading to inbreeding within some cavities (Van Oosten et al., 2014a). Dispersal of adult female ticks may be even more restricted than the earlier instars, as adult females feed almost exclusively on nestling birds (Heylen et al., 2014). Very little is known about the mating system of this tick, but such knowledge is important because *I. arboricola* is the carrier and/or transmitter of several pathogens that are important for humans and livestock, including tick-borne encephalitis (TBE) virus, Rickettsiae and several *Borrelia* genospecies (Hillyard, 1996; Špitalská et al., 2011; Heylen et al., 2012a). Previous studies have shown that, although *I. arboricola* is rare in nest boxes throughout Belgium, adults of both sexes are often found together in nest boxes (Heylen et al., 2014; Van Oosten et al., 2014a). Moreover, as we have previously suggested, tick densities may be much higher in natural tree holes as these may be more suitable habitats than nest boxes (Van Oosten et al., 2014a). Multiple mating may therefore have important implications for the ecology and evolution of this tick.

In this paper we report on an experimental study in which we investigated multiple mating and paternity of *I. arboricola* as well as the role of female engorgement status. It is unknown what the effect of mating first or last is on paternity, nor is it known what the effect of the blood meal is on paternity. Therefore, female *I. arboricola* fed on great tit nestlings and mated with two males in three different treatments: both matings before the blood meal ("preprandial" treatment), one before and one after the blood meal ("transprandial" treatment), or both after the blood meal ("postprandial" treatment). We used microsatellite markers to investigate the contribution of both males to the offspring. We predicted that multiple paternity would be common within clutches as this promotes genetic diversity, but there were no a priori predictions of which male would sire most larvae because in some *Ixodes* ticks there is first male precedence (e.g. *Ixodes uriae*, McCoy and Tirard, 2002; *Ixodes ricinus*, Hasle et al., 2008) and in others

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last male precedence (e.g. *Ixodes scapularis*, Yuval and Spielman, 1990). In an additional experiment, we allowed males to choose between an unfed and an engorged female. We predicted that males have a preference for engorged females because these present a higher probability of successful reproduction.

2. Materials and methods

The current study took place during the breeding seasons of 2013 (May and June) and 2014 (May). All mating experiments were conducted in the laboratory with previously unmated ticks. All females (n = 58 in 2013; n = 91 in 2014) and the majority of males (n = 16 in 2013; n = 20 in 2014) were obtained from a laboratory stock, and they moulted from the previous instar in individual Eppendorf tubes. In 2013, we used five additional males that were collected as nymphs from nest boxes in Peerdsbos, Brasschaat, Belgium (PB; 51°16'29"N, 4°29'03"W), the Boshoek area, Lier, Belgium (BH; 51°07'43"N, 4°31'52"W) and Park de Warande, Oostmalle, Belgium (WA; 51°17'53"N, 4°43'46"W) during the winter of 2013, and moulted in the laboratory in individual Eppendorf tubes.

The laboratory stock was established in 2008 with ticks (303 larvae, 111 nymphs and 19 adult females) from nest boxes in the study areas PB and BH (Heylen and Matthysen, 2010). This stock has been maintained by allowing ticks to infest great tits, and occasionally other birds, in several studies (e.g., Heylen and Matthysen, 2011; Van Oosten et al., 2015), and we occasionally added ticks from PB, BH and two other nearby study areas: Wortel Kolonie, Wortel, Belgium (WK; 51°24'10"N, 4°49'29"W) and WA. Nest boxes have been present in these areas for many years as part of long-term population studies on great and blue tits (Matthysen et al., 2001, 2011). Engorged ticks in the laboratory stock were always kept at 25 °C and unfed ticks at 12 °C, both at 85% relative humidity. Before each breeding season, all adult ticks were put together in a vial (diameter 1.5 cm, height 12 cm) for several days to allow uncontrolled mating between ticks from all study areas.

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2.1. Mating

Artificial mating experiments were conducted in the laboratory by placing a single unmated female, either unfed or engorged, and a single male in an autoclaved 1.5 ml Eppendorf tube. The tubes had pierced lids for aeration. Males and females were paired randomly, but we ensured that different males were used for the first and second mating of each female.

Observations were carried out by registering mating status every 10 min for 4 h, and we recorded the time until mating commenced as well as the duration of each mating. Individuals that remained in copula after the observation period were kept together and investigated again after 9-10 h. If males were still attached, mating was interrupted by gently pushing the male with tweezers. Matings were interrupted because males were required for subsequent matings, and we assumed that insemination had been completed by that time, as males require less than 2 h to inseminate (Graf, 1978; Fourie et al., 1988; Sonenshine, 1991; Kiszewski et al., 2001; Kaufman, 2008). After mating, the male was moved to a separate 1.5 ml Eppendorf tube.

In 2014, a subset of the mating pairs was used to investigate whether males mate more frequently with either engorged or unfed females. To do this, 1.5 ml Eppendorf tubes were set up containing one male, one unfed female and one engorged female. We recorded the male's initial mate choice and whether or not mating took place with both females, in addition to the time until mating commenced and mating duration.

To investigate whether there was multiple mating and which male had the largest contribution to the offspring (see Section 2.5), all females were mated with two males. Due to a limited number of males, males mated with 4.6 ± 0.48 females (range 1 – 11). We set up three treatments with respect to female feeding status: either both matings took place before feeding (preprandial treatment, n = 16 females initiated of which six females were recovered from the nest boxes after feeding), both after feeding (postprandial treatment, n = 70 females initiated of which 55 were recovered), or one before and one after feeding (transprandial treatment, n = 23 females of which 13 were recovered). Preprandial mating took place on average 4.3 days before feeding commenced (range 0 - 13 days), and postprandial mating on

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average 2.7 days after the female was recovered from the nest boxes after feeding (range 0 to 14 days). The average time between both matings, irrespective of when they took place, was 3.4 days (range 1 - 13 days).

2.2. Feeding

Females ticks fed on great tit nestlings in nestboxes in BH or PB. In 2013, females were placed in a nest with nestlings (one tick per nest) approximately 8 days old (n = 58 ticks, of which 13 ticks were recovered from the nest boxes after feeding). In 2014, females were directly placed on a nestling approximately 12 days old, by inserting the tick on the occipital side of the head with a small brush (n = 91, of which 86 ticks were recovered). In case females had mated preprandially, only a single tick was placed in a nest box, allowing identification after the blood meal. In other cases, up to five ticks were placed on a single nestling in a nest box. Each nest box was inspected for the presence of ticks before infestation, and nest boxes where ticks were present were avoided. Nest boxes were inspected regularly starting 4 days after attachment because adult *I. arboricola* ticks typically take a little over 5 days to complete a blood meal (Heylen and Matthysen, 2011). After being recovered from the nestbox, females were weighed to the nearest 0.1 mg.

2.3. Egg and larva recovery

After mating and feeding, females (n = 13 in 2013; n = 86 in 2014) were individually placed in vials (diameter 1.5 cm, height 12 cm with a fine mesh under the cap to prevent escape of larvae) under controlled climatic conditions (25 °C, 85% relative humidity, 12 h light:12 h dark). To record the moment the first egg was deposited each vial was inspected every 3 days. In 2014, clutch size was recorded after egg laying had finished, whereas in 2013 clutch size was not recorded. In both years the female was stored in 70% alcohol for DNA analysis after egg laying had finished. The eggs were inspected every 3 days and we recorded the moment the first larva hatched. Once the eggs hatched the number of larvae was

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determined and approximately 10-15 larvae were stored in alcohol for DNA analysis. Male ticks were stored in alcohol as soon as all matings had been conducted.

2.4. Genotypic analysis

Using NucleoSpin Tissue kits (Macherey-Nagel, Düren, Germany), DNA was extracted from whole individual larvae and from three legs of adults. The first pair of legs was never used, as these are important for species identification for future reference. Ticks were genotyped using seven polymorphic microsatellite loci (Ixaf3, Ixaf6, Ixaf8, Ixaf11, Ixaf15, Ixaf16 and Ixaf19) designed for *I. arboricola* (Van Houtte et al., 2013). Amplifications were carried out in 10 µl containing 1 µl of template DNA, 5 µl of 1X Qiagen (Belgium) Multiplex PCR Master Mix (including Hot Star Taq DNA Polymerase, Multiplex PCR Buffer and dNTP Mix) and 0.4 µl of each forward and reverse primer (5 µM). Amplification conditions were 95 °C for 15 min followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 56 °C for 90 s, extension at 72 °C for 90 s, and a final elongation step at 72 °C for 10 min. Fragment length analyses were performed on a 3130XL Genetic Analyser (Life Technologies, Carlsbad, CA, USA), and alleles were scored with GeneMapper Version 3.7 (Life Technologies). If paternity assessment was not possible due to unsuccessful scoring, we opted to reanalyse individuals, particularly adults, but this was not done for all ticks. In this way, genotyping was possible for all 60 adult females (27 partially, i.e. 2 – 6 loci), all 36 adult males (12 partially) and 580 out of 584 larvae (421 partially).

For adult ticks, GenePop 4.0 (Rousset, 2008) was used to estimate observed (H_O) and expected (H_E) heterozygosity, the number of alleles and the frequency of null alleles, test for linkage disequilibrium between pairs of loci and test for deviations of genotype frequencies from Hardy-Weinberg equilibrium (HWE) expectations using an exact probability test. Ticks from different years were treated as separate populations. The sequential Bonferroni's procedure was applied to the tests of linkage disequilibrium and HWE to correct for multiple testing.

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2.5. Paternity assignment

Paternity was assigned by manually comparing the genotypes of individual larvae across all loci with those of both potential fathers, whilst taking the mother's genotype into account. Among the seven microsatellite loci used, assignment to a potential father was permitted if it was possible to match alleles at two or more loci, but no father was assigned if there were conflicting parentage assignments among loci (i.e. different loci pointing to different fathers). One mismatch, i.e. an allele in a larva not found in any of the parents, was accepted to allow for the possibility of novel mutations arising during the production of a large offspring cohort and to correct for potential genotyping errors (Vandeputte et al., 2006). A mismatch was only allowed if there were no conflicts among loci.

We calculated the probability of detecting multiple paternity per clutch using the software PrDM (Neff and Pitcher, 2002). PrDM accounts for the effects of population allele frequencies, number of loci, number of alleles, clutch size, number of putative fathers and their respective fertilisation proportions. The software was used to consider two sires with equal fertilisation proportions.

2.6. Statistical analyses

All data analyses were done in R version 3.2.2 (R Foundation for Statistical Computing, Vienna, Austria). Generalised linear models were fitted to test the following associations: (i) mating duration and female feeding status (unfed or engorged), ii) mating duration and previous mating experience of males, (iii) mating duration and female mating status when feeding (preprandially mated or unmated), (iv) mate selection by males and feeding state of females, (v) female engorgement weight and female mating status when feeding, (vi) engorgement weight and previous mating experience of males, (vii) clutch size and engorgement weight, (viii) clutch size and female mating status when feeding, (ix) hatching success and clutch size, (x) hatching success and female mating status when feeding, (xi) paternity and treatment (preprandial, transprandial or postprandial mating), (xii) paternity and which male was interrupted due to prolonged attachment (none, first, second or both), (xiii) paternity and the relative previous mating experience of both males and (xiv) paternity and mating duration.

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Male ID was fitted as a random effect, with the package LME4 version 1.1-10 (Bates et al., 2015), to the models for choice of female by males and paternity in relation to mating interruption, to correct for the non-independence in the response variable. Mating duration was analysed with survival analysis, with right censoring from package parfm version 2.5.3 (Rotolo and Munda, 2013), with male ID as a random effect. We used positive stable Frailty models with a Weibull baseline hazard distribution. In all models a stepwise selection procedure was used in which the model was iteratively refitted after exclusion of the least significant fixed effect. Terms were not removed if they were part of a higher order significant interaction. In cases of significant interactions, data were subset along all levels of the main effects and tested separately with Bonferroni correction. All estimates are reported as mean ± S.E., unless otherwise mentioned. P values < 0.05 were considered significant.

Previous mating experience of males was analysed with both the number of previous matings (ordinal) and the total time of previous matings (continuous). Because these covariates are highly correlated ($r_{212} = 0.93$, $P < 0.001$), separate analyses were conducted. For all response variables, we report both effects and only the most significant interaction.

The model for mating duration (identity-link, right-censored) included as fixed effects female feeding state (unfed or engorged), previous mating experience of males, female mating state (mated or unmated) and all interactions. All couples that remained in copula once the observations ended (> 4 h) were handled as right-censored data.

The model for choice of female by males in favour of engorged or unfed females (logit-link, binomially distributed residuals) included as fixed effect previous mating experience of males. Cases where no mating occurred were excluded from the analysis.

The model for female engorgement weight (identity-link, normally distributed residuals) included as fixed effects female mating status (mated or unmated) and previous mating experience of preprandially mated males.

The model for clutch size (identity-link, normally distributed residuals) included as fixed effects female engorgement weight, female mating status and their two-way interaction (engorgement weight :

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mating status). Only females from 2014 were used, and females that failed to lay eggs were excluded from the analysis.

The model for hatching success (identity-link, normally distributed residuals) included as fixed effects female mating status, clutch size and their interaction (mating status : clutch size), while correcting for female engorgement weight.

Paternity (logit-link, binomially distributed residuals, weighted by the number of assignable larvae) was evaluated in three models with differing fixed effects. The first model included as fixed effects treatment (preprandial, transprandial or postprandial mating), which mating was interrupted due to prolonged attachment (none, first, second or both) and their two-way interaction (treatment : interruption). Post-hoc testing was conducted for each treatment-interruption combination for which larvae from at least four clutches were available, with Bonferroni correction for multiple testing. The second model for paternity included the following fixed effects: the relative previous mating experience of the first male, treatment (preprandial, transprandial, postprandial), and their two-way interaction (male mating : treatment). The third model for paternity was conducted only with males which were not manually interrupted, and included the relative mating duration as a fixed effect.

3. Results

3.1. Mating duration

Mating duration was recorded 214 times (n = 130 female ticks). There was a significant interaction between mating status and feeding status of the females ($\chi^2_{1,166} = 6.92$, $P = 0.009$; Fig. 1, Table 1). Specifically, the average mating time of unfed, unmated females was 50 ± 3.6 min (n = 51) and of unfed mated females was 48 ± 6.6 min (n = 16) and all finished within the observation time. In contrast, 67 out of 79 engorged unmated females (84.8%) and 63 out of 68 engorged mated females (92.6%) did not finish mating within 4 h of observation, of which respectively 58 (73.4%) and 48 (70.6%) were separated manually another 9-10 h later. Mating duration was not influenced by the number ($\chi^2_{1,166} = 0.73$, $P = 0.393$; Table 1) or the total duration ($\chi^2_{1,166} = 1.20$, $P = 0.273$) of previous matings by males. Two

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males that remained attached to an engorged female were still attached when the female started depositing eggs, more than 2 weeks after mating onset.

3.2. Choice of female by males

In the mate choice experiment (n = 70 trials; 16 males were used four times and two males were used three times), engorged females were preferred 46 times (75.4%) and unfed females were preferred 15 times (24.6%), which is a significant difference ($z_{1,61}$ = -3.77, $P < 0.01$; Fig. 2). Male choice was not influenced by the number ($z_{1,62}$ = -1.11, P = 0.27) or the total duration ($z_{1,62}$ = -0.56, P = 0.58) of their own previous matings. Mating did not occur in nine trials. Of the males that initially mated with an unfed female, 12 (80%) mated with the engorged female afterwards, whereas this never occurred for males that initially mated with an engorged female.

3.3. Engorgement weight, egg laying and hatching success

Engorgement weight (n = 99 female ticks) of preprandially unmated females was 39.45 ± 1.04 mg, whereas preprandially mated females were significantly lighter at 34.53 ± 1.97 mg ($\chi^2_{1,73}$ = 6.22, P = 0.013). There were no differences between females that mated once or twice before feeding ($\chi^2_{2,73}$ = 5.16, P = 0.076). Female engorgement weight was not influenced by the number ($\chi^2_{1,37}$ = 0.29, P = 0.590) or the total duration ($\chi^2_{1,37}$ = 0.16, P = 0.693) of previous matings of males.

Eggs were laid by 79 out of 99 females (79.8%) and the average clutch size was 319.4 ± 12.8. Heavier females laid significantly more eggs ($\chi^2_{1,61}$ = 30.48, $P < 0.001$; Fig. 3), but whether they mated before or after the blood meal had no influence ($\chi^2_{1,60}$ = 0.05, P = 0.816), nor was there a significant interaction between both terms ($\chi^2_{1,59}$ = 1.50, P = 0.22). There was a statistically significant positive correlation between clutch size and hatching success (r_{64} = 0.46, $P < 0.001$; Fig. 3).

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3.4. Microsatellites

Allele size, the number of alleles, H_O and H_E, the frequency of null alleles and amplification success are given in Table 2. Amplification success across loci was 0.905 ± 0.022 for adults and 0.709 ± 0.022 for larvae. After Bonferroni's correction, five out of the 14 year–locus combinations deviated significantly from HWE expectations (Table 2). These deviations were observed in both years for Ixaf6 and Ixaf15, and only in 2013 for Ixaf8. There was significant linkage disequilibrium for only one year-locus combination (Ixaf6 – Ixaf8 in 2014).

3.5. Paternity

Paternity assignment was possible for 257 out of 584 larvae (44%) from 52 clutches. Mismatches, i.e. larvae showing novel alleles not found in any potential parent, were found in 248 larvae (30.2%), for a total of 365 loci (average 1.47 loci per larva). Genotypes and paternity assignment are provided in Supplementary Table S1, and paternity ratios for each clutch in relation to previous experience of males in Supplementary Table S2.

The probability of detecting multiple paternity was 0.958 ± 0.009 across clutches. Multiple paternity, i.e. larvae in a clutch sired by different males, was present in 22 clutches (42.3%; Fig. 4). Overall, there was no precedence of a particular male (ratio 0.47), whereas there was overdispersion of larvae assignment ($\chi^2_{3,51}$ = 172.50, ratio = 3.83, $P < 0.001$). Paternity was lower for males whose mating was manually interrupted ($\chi^2_{3,51}$ = 45.68, $P < 0.001$; Table 3). As such, when both males mated postprandially more larvae were sired by the first male when the second male was interrupted ($P < 0.01$, ratio 0.88), and more larvae were sired by the second male when the first male was interrupted ($P < 0.01$, ratio 0.19). No such effect was observed when neither or both males were removed, irrespective of when mating occurred in relation to feeding ($P > 0.07$; Table 3). Paternity was not affected by treatment ($\chi^2_{2,48}$ = 4.99, $P = 0.233$), nor was there a significant interaction between treatment and interruption ($\chi^2_{1,46}$ = 2.99, $P = 0.083$).

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Paternity was lower for males that had more previous matings ($\chi^2_{1,49} = 7.34$, $P = 0.007$), but there was no effect of the total duration of previous matings ($\chi^2_{1,49} = 0.90$, $P = 0.342$). There was no effect of treatment ($\chi^2_{2,48} = 1.51$, $P = 0.470$), nor was there a significant interaction between treatment and male previous experience ($\chi^2_{2,47} = 0.28$, $P = 0.868$). In cases where neither male was manually interrupted ($n = 11$), there was no effect of mating duration on paternity ($\chi^2_{1,8} = 0.57$, $P = 0.449$).

4. Discussion

Multiple mating is commonly observed in different tick species (Oliver, 1974), and multiple paternity has been confirmed in tick species with varying life histories (McCoy and Tirard, 2002; Hasle et al., 2008; Cutullé et al., 2010; Ruiz-López et al., 2012). Our results indicate that multiple mating is also common in *I. arboricola* and may indeed lead to multiple paternity, at least under laboratory conditions. In addition, mating between males and engorged females took place more often and lasted considerably longer than between males and unfed females. Finally, heavier females had larger clutches, from which more larvae hatched. Our work therefore suggests that engorgement weight is a reliable fitness proxy, corroborating previous studies (Gladney and Drummond, 1970a; Chen et al., 2009; Ma et al., 2013).

It is known that *Ixodes* ticks sometimes remain in genital contact for several weeks, even though fertilisation is completed within 2 h – usually within minutes (Graf, 1978; Fourie et al., 1988; Sonenshine, 1991; Kiszewski et al., 2001; Kaufman, 2008). However, our study is, to the best of our knowledge, the first to report a marked difference in mating duration between unfed and engorged females. On an ultimate, or evolutionary, level, prolonged attachment (i.e. mate guarding) prevents other males from mating with the same female and is therefore adaptive if there is no first male sperm precedence (Falk-Vairant et al., 1994; Kiszewski et al., 2001; Kaufman, 2008). Contrarily, in species with first male precedence, such as *I. uriae* and *I. ricinus* (McCoy and Tirard, 2002; Hasle et al., 2008), mating preprandially should be adaptive and there would not be strong selection on mate guarding. Indeed, in an experimental study with *I. ricinus*, where there is first male precedence, Zemek et al. (2002) found fewer matings with engorged females than with unfed or semi-engorged ones. This contrasts with our findings

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of *I. arboricola*, for which we found no indications of sperm precedence (see discussion below). The current data do not inform about the proximate mechanisms. One possibility is that the diversion of female metabolic processes to sperm digestion and absorption, and vitellogenesis, reduce the rate at which females are able to accept and store sperm. Similarly, there might be mechanical or chemical processes that cause physical entrapment of males by engorged females. It may also be possible that the process of insemination is different in engorged and unfed females, for instance due to morphological differences. Further investigation of these possibilities will be worthwhile as it is currently unknown what processes dictate mating duration in *Ixodes* ticks (Kaufman, 2008).

We found multiple paternity in less than half of the clutches investigated. At the same time, paternity ratios were overdispersed, i.e. there was less mixed paternity than expected based on the overall paternity ratio. Despite using laboratory stock, the markers used contained considerable allelic diversity, and the probability of detecting multiple paternity in each clutch with the given loci was high. This suggests that the observed overdispersion is not a methodological artefact, such as the presence of null alleles or the low number of genotyped larvae per clutch (although the detection probability may have been overestimated because the analysis does not take into account the degree of amplification success in the larvae). Rather, the observed overdispersion may have been caused by manual interruption of mating males, which drastically lowered their share of the progeny and, hence, led to less mixed paternity than expected by chance. An untested hypothesis is that qualitative differences between males, which have not been accounted for in the current study, may have led to increased paternity of a particular male. Because males and females were paired randomly, qualitative differences would lead to an overall paternity ratio of approximately 0.5, but strong overdispersion among clutches.

Our data on paternity indicate that there was no sperm precedence in *I. arboricola* (i.e. higher paternity of either the first or last male). There is ambiguity concerning sperm competition in *Ixodes* ticks. In some species there is first male precedence (e.g. *I. uriae*, McCoy and Tirard, 2002; *I. ricinus*, Hasle et al., 2008), whereas in others there is last male precedence (e.g. *I. scapularis*, Yuval and Spielman, 1990). Although never identified, there could also be sperm loading, in which the male providing the largest

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number of spermatozoa sires most larvae, or sperm selection, in which there is non-random use of spermatozoa by the female (Kaufman, 2004). As such, differences in paternity could be associated with male quality or female choice. The current data do not allow us to investigate this, and to the best of our knowledge no studies are available that have done so.

Two factors that were measured in our experiment indicate that qualitative differences between sires may have some influence. First, the number of previous matings reduced the males' share of paternity. It is known for several Metastriata that males are able to inseminate tens of females (Gladney and Drummond, 1970b; Kaufman, 2004), whereas a limited number of studies suggest Prostriata males may achieve only about three inseminations (Graf, 1978; Yunker et al., 1991; Kiszewski et al., 2001). Our investigations suggest *I. arboricola* males are able to inseminate more than three females (see Supplementary Table S2), but, given the fact that males do not feed, sperm eventually will become limited. It is currently unknown how many successful fertilisations can occur, and the current data do not allow such an investigation. Second, we found that paternity was lower in the cases of very long matings with engorged females that were manually interrupted. Because we found no effect of mating duration on paternity, and because *Ixodes* ticks finish insemination within 2 h (Graf, 1978; Fourie et al., 1988; Sonenshine, 1991; Kiszewski et al., 2001; Kaufman, 2008), males in our study may not have prolonged attachment for insemination (although it may be possible that previous observations do not apply to all *Ixodes* ticks). An alternative, untested explanation is that males of low quality cannot deliver much sperm for sufficient sperm competition and thus prevent other males from mating with the female by staying attached. Conversely, males of high quality have a high share of the brood due to high sperm quantities, and can further increase their fecundity by finding and fertilising other females.

Multiple paternity is not only characteristic of nidicolous ticks, as it is common in ticks with varying life histories (McCoy and Tirard, 2002; Hasle et al., 2008; Cutullé et al., 2010; Ruiz-López et al., 2012). Multiple paternity can be adaptive because it may lead to offspring with variable genotypes, thus allowing ticks – and parasites in general – to rapidly evolve in relation to host responses (Wikel, 1996). In social insects, experimental studies have shown that multiple paternity may indeed result in higher fitness

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when individuals are faced with varying environmental conditions (Baer and Schmid-Hempel, 1999). For nidicolous ticks, maximising genetic diversity may be particularly important, because the number of different host individuals encountered is often much lower than in non-nidicolous ticks (Hoogstraal and Aeschlimann, 1982). Similarly, increased genetic diversity may counteract the negative effects of inbreeding due to low dispersal capability (McCoy et al., 2003; Van Oosten et al., 2014a). In addition, although their environments are temporally stable, nidicolous ticks may be faced with very different off-host conditions when dispersed to the nest of another host type (Van Oosten et al., 2014b).

There may be evolutionary feedback between the moment of mating and dispersal capability. Female *I. arboricola* ticks feed almost exclusively on nestling birds, which usually do not leave the nest before ticks detach (Heylen et al., 2012b, 2014). Therefore, female ticks rarely disperse and there is limited selective advantage for mating preprandially in terms of dispersal capability. This is different for ticks with greater dispersal potential such as *I. ricinus*, where pre- and perprandial (i.e. during feeding) mating, especially due to multiple paternity, may yield considerable colonisation opportunities (Hasle et al., 2008). Similarly, in the nidicolous *I. uriae*, a tick common to seabirds, dispersal capability is larger than in *I. arboricola* because besides nestlings, adult hosts are also frequently infested (McCoy et al., 2003). In *I. uriae,* multiple paternity has been confirmed and most fertilisations seem to occur preprandially (McCoy and Tirard, 2002). Preprandial mating has been suggested as an adaptive strategy, as the probability of a tick successfully colonising a new site would be greatly improved if all dispersed females were already fecund (McCoy and Tirard, 2002).

In our mate choice experiment, mating occurred more frequently between males and engorged females than between males and unfed females. Males may prefer to mate with engorged females because these present a higher probability of successful reproduction than unfed females, which have yet to find and feed on a host. We cannot exclude the possibility that mating occurred more frequently with engorged females due to female motivation rather than male preference. However, movement of engorged females was limited compared with males and unfed females (our personal observations). It therefore seems unlikely that engorged females were more motivated, but more explicit experiments may be required to

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identify the proximate causes of our findings. Similarly, we cannot exclude the possibility that male preference for engorged females was a mechanical consequence of higher contact rates due to their much larger body size. Yet, there are three observations that make it more likely that males prefer engorged females over unfed ones. First, a great number of males that initially chose the unfed female also mated with the engorged female afterwards, whereas the reverse was never observed even when mating was short. Second, prolonged mating was only observed with engorged females. Third, males in several trials made contact with both partners, sometimes several times, before mating (our personal observations).

It remains to be investigated what the consequences of male preference are for tick biology in nature. Because adult female ticks prefer feeding on nestlings, which are available for a limited time (Heylen et al., 2014), the majority of female ticks may engorge simultaneously. Thus, for male ticks, there may seldom be a choice between unfed and engorged females. Rather, males may fertilise females both preprandially and postprandially depending on their availability. To investigate the relative importance of unfed and engorged females for males under natural circumstances, it may be possible to investigate how many females are fertilised preprandially, e.g., by isolating females directly after the blood meal and observing whether egg laying occurs (which would suggest preprandial fertilisation has taken place) and by genotyping offspring (which could indicate the number of preprandial partners).

We found clear indications that the size of a female's blood meal is directly linked to her fecundity, corroborating previous studies (Gladney and Drummond, 1970a; Chen et al., 2009; Ma et al., 2013). Therefore females should always try to maximise the size of their blood meal, although remaining small may reduce detection by the host (Sonenshine, 1991; Heylen et al., 2012b), and there may be physical constraints in reaching a large engorgement weight (Slowik and Lane, 2009; Dietrich et al., 2014; Van Oosten et al., 2014b). We also found that mated females were lighter than unmated females, irrespective of the number of matings. Male fecundity may therefore be lower when mating preprandially rather than postprandially. Whether this is the case will depend on the frequency of host-induced female mortality. Future research is required to investigate to what extent preprandial mating and host-induced

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mortality affect the population dynamics of *I. arboricola* because, to the best of our knowledge, this is currently unknown.

Female fecundity may be reduced by male-male competition. It is known from many non-tick systems that female fecundity can decrease due to seminal peptides that increase the relative contribution of males to the offspring (Morrow et al., 2003). This effect may persist even in the absense of competitors. Indeed, in ticks that mate on-host a protein from the testis, originally named 'male factor', hastens the onset of salivary gland degeneration and ovarian development (Kaufman, 2004). Further investigations may demonstrate whether there are indeed competitive seminal peptides in *I. arboricola.* As an alternative explanation to the difference in engorgement weight between mated and unmated females, unmated females might invest more in their own survival by retaining more water from the blood meal, hence increasing the chance of postprandial mating. Perhaps this allows mating with males from the next generation that fed on the same nestlings as nymphs, because development of *I. arboricola* is relatively fast (Liebisch, 1996; Heylen et al., 2014). To our knowledge this has not yet been investigated.

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Legends to figures

Fig. 1. Kaplan-Meier survival plot of mating duration of *Ixodes arboricola* female ticks that were either unmated (virgin) and unfed (n = 52), mated and unfed (n = 16), unmated and engorged (n = 93) or mated and engorged (n = 96). The vertical line indicates the moment the observations ended.

Fig. 2. Preference of *Ixodes arboricola* males, in mate choice trials, between one engorged and one unfed female tick (n = 16 males over 70 choice trials). Male ticks that mated with both females in a trial are indicated in dark grey. Significantly more males chose engorged females ($P < 0.001$).

Fig. 3. Relationship between engorgement weight, clutch size and hatching success for female *Ixodes arboricola* ticks that were either unmated (virgin) or mated preprandially, i.e. before feeding (n = 66). Females that failed to lay eggs were excluded from the analysis. There was a significant positive relationship between engorgement weight and clutch size ($P < 0.001$, $r = 0.683$), as well as between clutch size and hatching success ($P < 0.001$, $r = 0.461$).

Fig. 4. Proportion of *Ixodes arboricola* larvae (n = 257 assigned larvae) per individual female tick (n = 38) sired by the first and second male. Treatments represent both matings after the female's blood meal ("postprandial"), mating with one male before and the other male after the blood meal ("transprandial") or both matings before the blood meal ("preprandial"). The number below each bar represents the number of larvae that could be assigned to each female. Only females for which >3 larvae could be assigned were used.

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Table 1. Results for survival analysis of differences in mating duration between unfed and engorged *Ixodes arboricola* females, considering mating status of females and the number and total duration of previous matings of males.

Fixed effect	χ^2	Degrees of freedom	*P*
$mating_{male}$	0.73	1	0.33
feeding status : $mating_{female}$	6.92	1	0.01
feeding status : $mating_{male}$	1.40	1	0.17
$mating_{female}$: $mating_{male}$	0.29	1	0.64
feeding status : $mating_{female}$: $mating_{male}$	3.53	1	0.05

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Table 2. Values of allele size, allelic diversity, expected (H_E) and observed heterozygosity (H_O), null alleles and amplification success for adult *Ixodes arboricola* (n = 96) for seven microsatellite loci, and amplification success for adults and larvae (n = 680). Hardy–Weinberg equilibrium (HWE) tests were conducted with adult ticks only.

Locus	Allele size	No. of alleles	H_E (2013/2014)	H_O (2013/2014)	Frequency of null alleles (2013/2014)	Amplification success (adults/larvae)
Ixaf3	118-130	8	0.612 / 0.700	0.793 / 0.810	0.000 / 0.003	0.958 / 0.685
Ixaf6[a]	156-184	12	0.866 / 0.860	0.500 / 0.655	0.269 / 0.120	0.896 / 0.623
Ixaf8[b]	157-189	13	0.677 / 0.671	0.464 / 0.667	0.205 / 0.000	0.885 / 0.649
Ixaf11	78-99	7	0.687 / 0.646	0.600 / 0.581	0.011 / 0.014	0.958 / 0.733
Ixaf15[a]	109-150	9	0.750 / 0.537	0.217 / 0.444	0.353 / 0.093	0.802 / 0.765
Ixaf16	188-199	5	0.166 / 0.329	0.103 / 0.286	0.107 / 0.058	0.958 / 0.777
Ixaf19	194-218	7	0.661 / 0.594	0.444 / 0.491	0.133 / 0.102	0.875 / 0.736

[a] Locus deviates from expected heterozygosity values under Hardy–Weinberg equilibrium in both years.
[b] Locus deviates from HWE in 2013.

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Table 3. Proportion of *Ixodes arboricola* larvae sired by the first male compared with the second male in relation to which mating was interrupted. Rows represent when males mated; in parentheses the number of larvae followed by the number of clutches.

	First interrupted	Second interrupted	Both interrupted	None interrupted
Postprandial	0.19 [a] (53; 10)	0.88 [a] (40; 8)	0.42 (74; 16)	-
Transprandial	-	0.66 (35; 7)	-	0.55 (20; 4)
Preprandial	-	-	-	0.50 (30; 6)

[a] Ratios that remained significant after Bonferroni correction.

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Figure 1 colour

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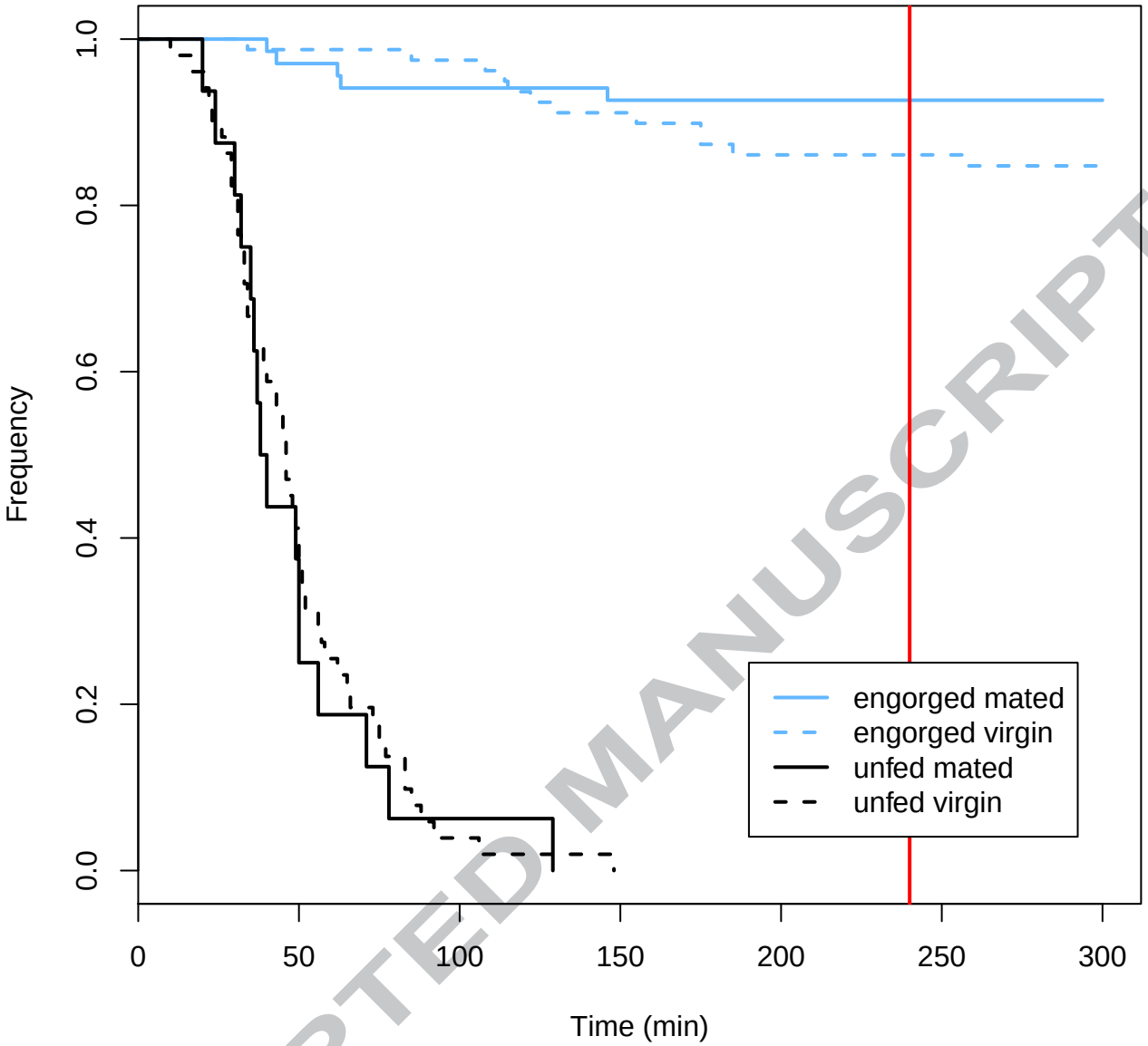

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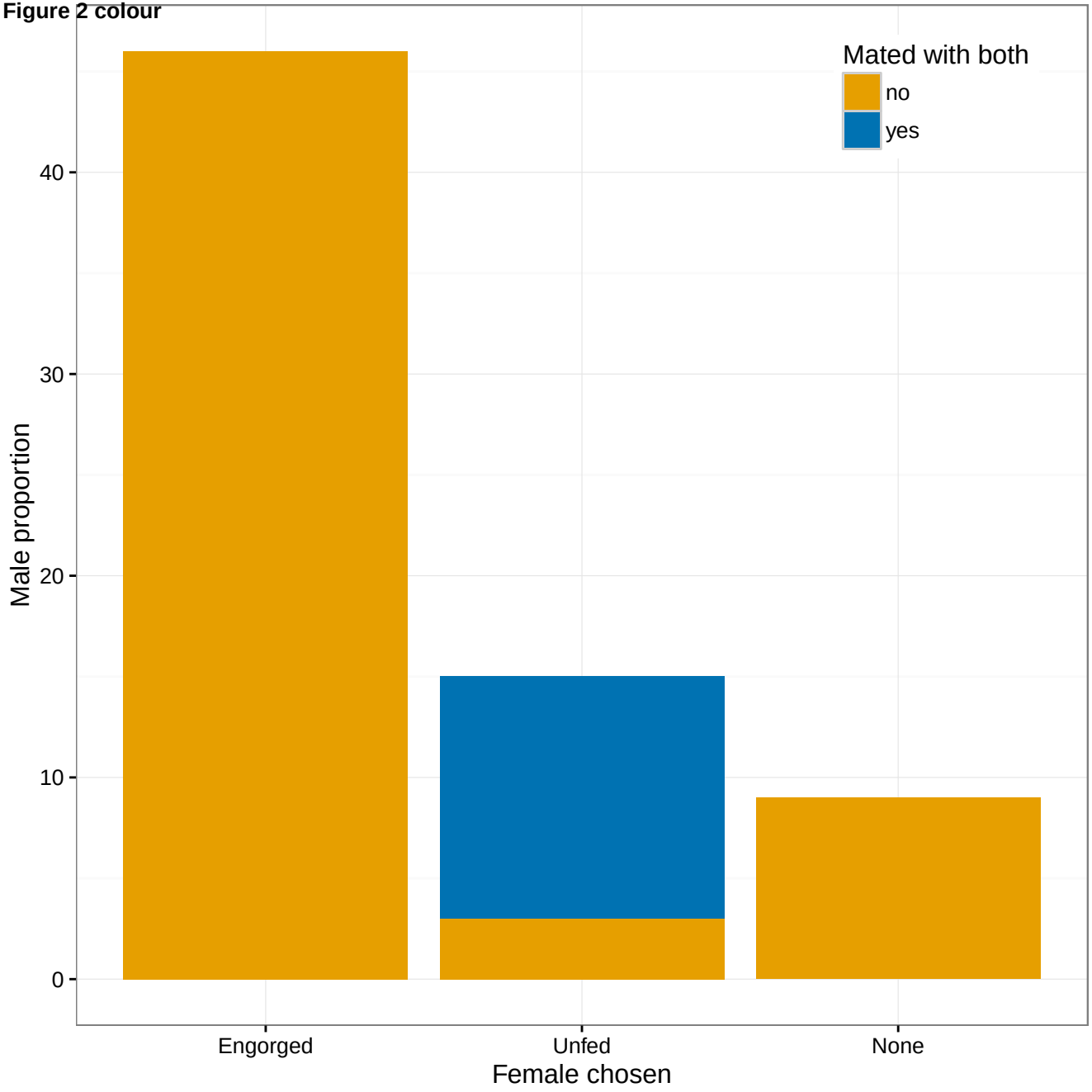
Figure 2 colour



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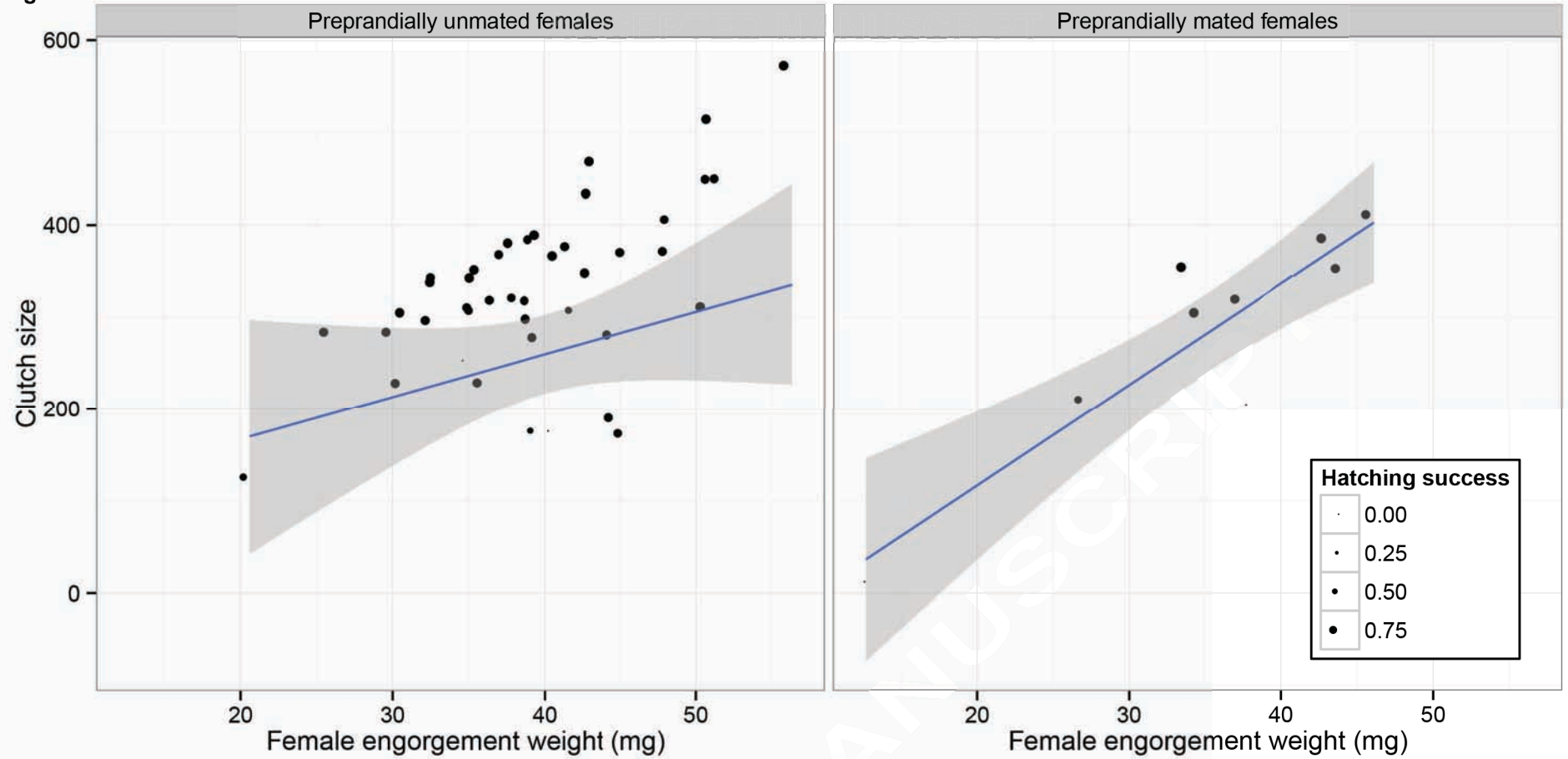
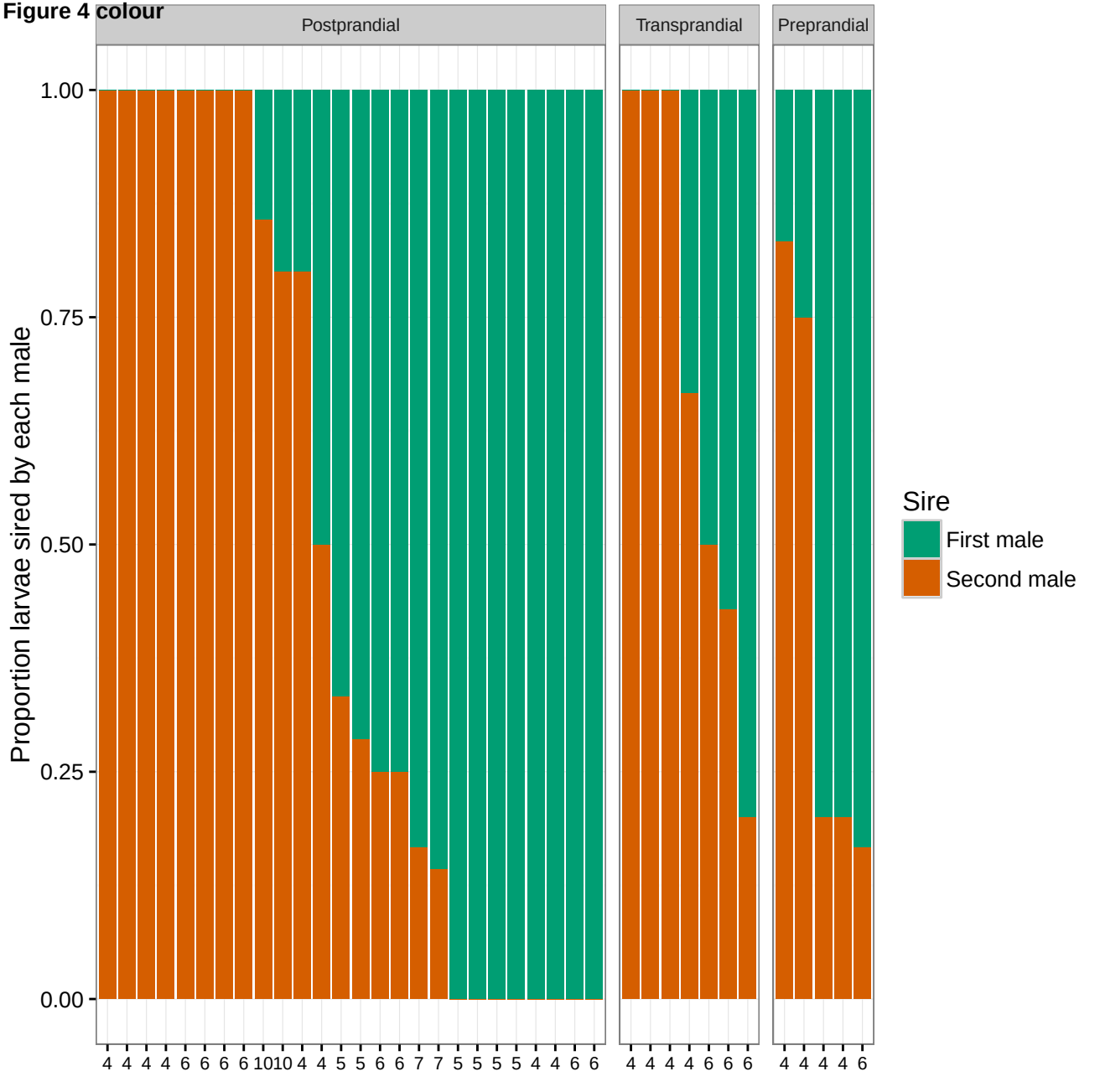


Figure 4 colour



*Graphical Abstract (for review)

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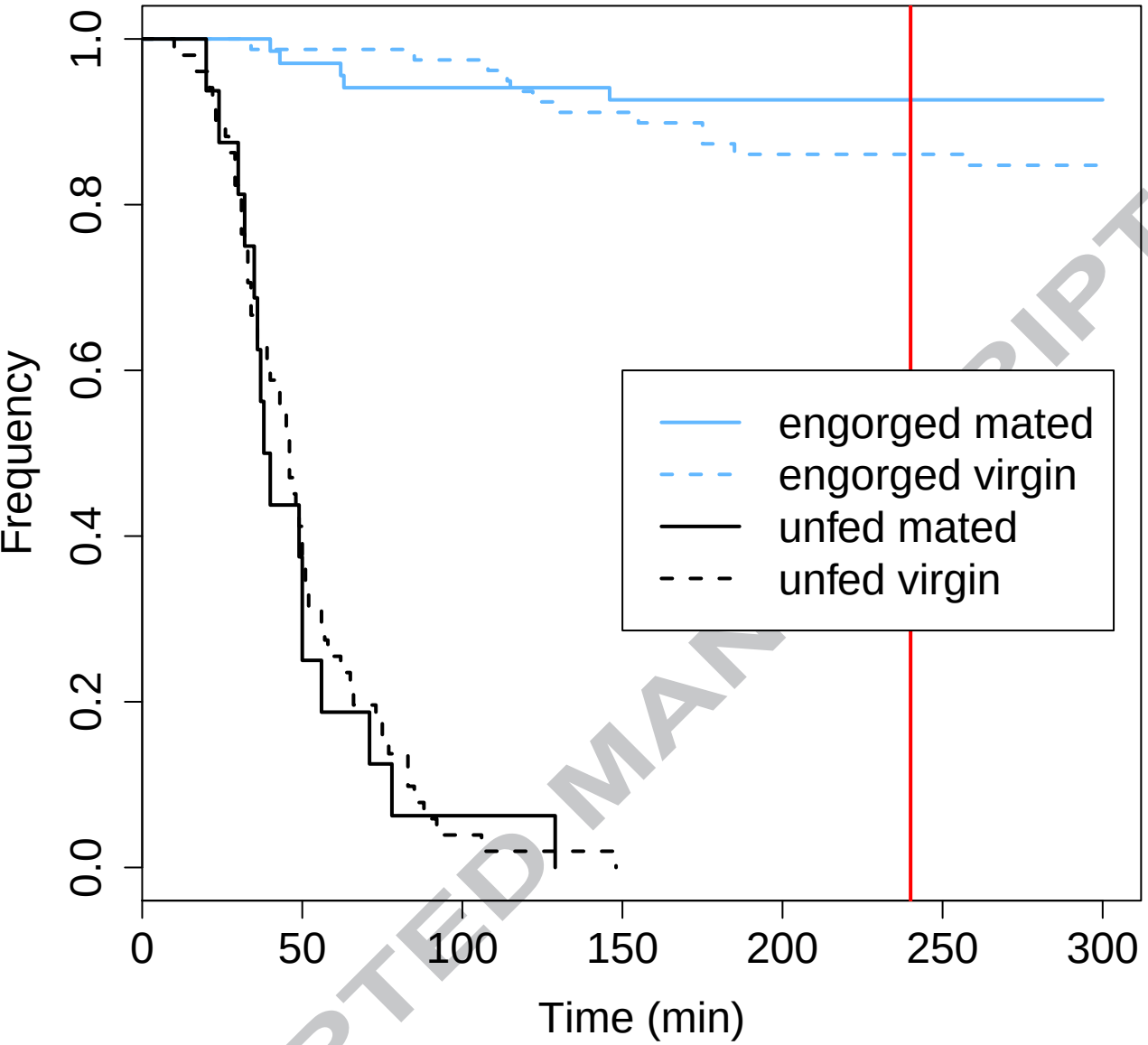

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Highlights

- Multiple mating is common in ticks, but multiple paternity is largely unexplored.
- We examined mating and paternity in the nidicolous tick *Ixodes arboricola*.
- Male ticks prefer mating with engorged females and exhibit mate guarding.
- There is multiple paternity but, overall, no precedence of a particular male.

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