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Parthenogenesis as an alternative mode of reproduction in the Metastriate ticks *Rhipicephalus appendiculatus* and *Rhipicephalus zambeziensis* (Acari: Ixodidae).

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Abstract
Apart from sexual reproduction, a limited degree of parthenogenesis and artificial parthenogenesis was revealed in the ixodid tick *Rhipicephalus appendiculatus* but not in *Rhipicephalus zambeziensis*. In *R. appendiculatus* all offspring were females; no males were produced, indicating a thelytokous mode of parthenogenetic reproduction.Copulation of female ticks with ^{60}Co-irradiated males triggers engorgement of the female ticks and increases the number of parthenogenetic offspring, illustrating the importance of copulation on the completion of engorgement of female ticks. To test the possibility that symbiotic microorganisms are involved in the thelytokous reproduction of *R. appendiculatus*, specific primers for the 16S rDNA gene were used in a PCR analysis. Neither *Wolbachia*, nor the Cytophaga-like organism were detected using that method.

Keywords: *Rhipicephalus appendiculatus*, *Rhipicephalus zambeziensis*, parthenogenesis, thelytoky, reproduction, *Wolbachia*

Introduction
We tend to consider sexual reproduction as being the only "normal" way of producing offspring, but a surprising number of species reproduce differently, at least part of the time. Apart from asexual reproduction (i.e. fission and budding), parthenogenesis can be seen as an intermediate between sexual and asexual reproduction in which an individual develops from a single-celled egg, generally produced by an ovary or ovarian tissue (Hoffman, 1998). It is known to occur in a few kinds of lizards, snakes, salamanders, bony fishes, and in a variety of arthropods. Parthonogenesis can be obligate in some species (in which females are produced exclusively), or facultative, meaning reproduction is sexual, sometimes by parthenogenesis. Three types of parthenogenesis occur: arrhenotoky (haploid males produced from unfertilized, haploid eggs), pseudoarrhenotoky (males from fertilized eggs, but father's genome is silenced or eliminated), and thelytoky (only diploid females are produced, males are rare or absent).

Parthenogenic species are favoured in colonising new habitats (only one individual needs to arrive to start a new population) and often tolerate marginal physical conditions.

The symbiotic bacterium *Wolbachia pipientis* is a cytoplasmaticly inherited Proteobacterium, which manipulates host reproduction, including cytoplasmatic incompatibility (CI), male killing, thelytokous parthenogenesis induction (PI), and feminization (Stouthamer *et al*., 1999 for review). It has been detected in between 16% and 76% of arthropod samples (Werren *et al*., 1995; Jeyaprakash and Hoy, 2000). Recently, a cytophaga-like organism (CLO), related to Cytophaga-Flexibacter-Bacteroid (CFB) group, was found to be associated with thelytokous parthenogenesis in parasitoid wasps of the genus *Encarsia* (Zchori-Fein *et al*., 2001). The presence of *Wolbachia* in ticks has previously been speculated based on electron-microscopy figures. However, a molecular analysis has determined that these symbionts belong to other bacterial groups (Noda *et al*., 1997), and presently no *Wolbachia* was found in ticks. In 1984, however, Homsher *et al*. reported some degree of thelytokous parthenogenesis in the tick *Dermacentor variabilis*.

The African brown ear tick, *Rhipicephalus appendiculatus* (Neumann, 1901)(Acari:Ixodidae), the main vector of *Theileria parva* (Theiler, 1904), the causative agent of East Coast fever (ECF), is confined to parts of eastern, central and south-eastern Africa but its distribution is far from continuous (Norval *et al*., 1992, Walker *et al*., 2000). It is restricted to savannah and woodland savannah with a substantial annual rainfall pattern and suitable hosts (Norval *et al*., 1992, Norval and Lightfoot, 1982). *R. zambeziensis*, also vector of ECF, is more restricted to warmer and drier areas from Tanzania southwards to South Africa. It is often found in great river valleys and adjacent low-lying areas (< 1000 m)(Walker *et al*., 2000, Speybroeck *et al*., 2002). No previous records were made of parthenogenetic reproduction within the species or even the genus, except for sporadic parthenogenesis in the normally bisexual tick species *R. bursa* (Oliver, 1989). Few ticks such as *Amblyomma rotundatum* (Oliver *et al*., 1993) in the tropical and subtropical areas of the New World and some populations of *Haemaphysalis longicornis* in Oceania and East Asia reproduce successfully by parthenogeneis (Takenouchi *et al*., 1970).

Preliminary studies in our laboratory on interspecies reproduction indicated the existence fertile offspring. This study was initiated with the principal aim of elucidating alternative mechanisms of reproduction, i.e. parthenogenesis and/or artificial parthenogenesis, of the two species and investigating the presence of known parthenogenesis inducing bacteria.

Materials and methods

Tick stocks

Two *R. appendiculatus* stocks and one *R. zambeziensis* sto ck, recently isolated from Zambia, were used in this study. They originated from Wafa (13°35’S, 32°30’E, ±980m) in Eastern and from Nkonkola (16°15’S, 27°54’E, ±1200m) in Southern Province. The *R. zambeziensis* stock was isolated from Keemba (16°03’S, 27°22’E, ±1100m), Southern Province. These ticks were collected as engorged females (± 20 per tick stock) from the ears of local cattle in the respective villages. They were transferred to The Institute of Tropical Medicine Antwerp and allowed to oviposit in individual plastic specimen bottles. All stages were maintained in a controlled environment room at 22±0.5°C (STAEFA Temperature control), 14h:10h (Light:Dark, (L:D)) regimen and 87±2%RH (STULZ Ultrasound humidifier). The larvae were allowed to feed on tick naive rabbits. They are referred to as KBA for the Keemba stock, NKA for Nkonkola and WAF for the ticks collected in Wafa village.

Experimental design

Three groups were designed: (i) a control group containing 30 female and 30 male ticks of each stock, all fed on an individual rabbit (WAF, KBA and NKA); (ii) a second group containing 60 female and 60 gamma irradiated males ticks of each stock (^{60}Co – 6.15krad, 24 hours prior to feeding) fed on rabbits (WAF irr, KBA irr and NKA irr); and (iii) a group of two times 30 female ticks fed on a rabbit (KBA rab, NKA rab) and a calf each (KBA bov, NKA bov), except for WAF which was only fed on a rabbit (WAF rab). All the tick groups were applied on the ears and engorged ticks were collected daily. Engorgement time, weight, pre-oviposition, and eclosion were recorded and percentage of eclosion estimated.

The irradiated ticks were dissected for inspection of their reproductive system.

The offspring of each group were allowed to feed on tick naïve rabbits until the adult stage. The sex of the adult ticks was recorded.

Screening for known parthenogenesis-inducing bacteria

DNA was extracted from the three tick stocks as described by de Kok *et al.* (1993). Two ticks of the same gender were used per extraction. PCR primers specific for the 16S rDNA gene of *Wolbachia* (O'Neill *et al*., 1992) were used to determine whether the different tick stocks were infected with that bacterium. In order to detect the CLO (EB of Zchori-Fein *et al.*2001), two sets of sequence-specific primers were used in PCR. The first was the EPS-f/EPS-r primer set that was designed to specifically amplify the bacterium found in *Encarsia* (Zchori-Fein *et al*., 2001). The other primer set was designed to be broader and detect other CFB bacteria (ixod-f 5'-tactgtaagaataagcaccggc and ixod-r 5'-gtggatcacttaacgctttcg). Annealing temperature for the second primer set was 57ºC. *Encarsia pergandiella* served as a positive control for the CLO and *E. formosa* for the *Wolbachia* presence.

Statistical analyses

The data collected in the parthenogenesis trial were analyzed by means of a non-parametric Kruskal-Walis test, BIAS for Windows, version 7.0.

RESULTS

The results of the engorged ticks are presented in Table 1.

Within the different tick populations, a statistical difference was found for the engorgement time among all treatments of the WAF groups, among KBA and all other treatments of the KBA group and among NKA, NKA irr and NKA rab or NKA bov (χ^2=132, df=10, p<0.0001).

Engorgement weights of the different WAF and NKA groups were statistically different, except forNKA rab and NKA bov, which had similar weights. For KBA groups, only KBA was statistically heavier than other KBA groups (χ^2=103, df=10, p<0.0001).

The pre-oviposition period was only statistically shorter between NKA and other NKA treatments, pre-oviposition periods for KBA and WAF groups were cognate (χ^2=16, df=10, p<0.098).

Out of the 11 groups that produced eggs, in only two groups (WAF rab and KBA rab) no larvae emerged. In the other groups, a statistical difference in pre-eclosion period was demonstrated between NKA irr and all other NKA groups (χ^2=28, df=8, p<0.0001) with NKA irr eclosing earlier than the other groups. For the KBA groups, KBA bov eclosed significantly earlier than the other KBA groups.

Looking at the percentages of eclosion, the three control groups (WAF, NKA and KBA) gave similar eclosion results which were significantly higher than those observed in all other groups (χ^2=40, df=8, p<0.0001).

From all the groups that produced larvae (control groups WAF, KBA and NKA not taken into account), only larvae of WAF irr and NKA irr engorged successfully and moulted to the nymphal stage. Nymphs of these groups also successfully developed into the adult stage. All of the individuals that reached maturity were females.

The dissection of all irradiated fed male ticks revealed very small, atrophied testes in comparison with testes of normal fed males.

Neither *Wolbacia* nor the CLO were detected in the different tick extracts.

Discussion

A general trend was observed, for both engorgement time and weight. Mated and fertilized females of the control, showed shorter engorgement time than groups with gamma irradiated males, which in turn engorged faster than groups without males (except for KBA). Engorgement weights of the former were always significantly heavier than that of the two latter groups. These differences probably resulted from the absence of copulation and/or the absence of fertilization. In Metastriate ticks, the absence of males during female engorgement lengthens the slow feeding phase, withholding the rapid feeding period as in copulated female feeding (Sonenshine, 1991). A cumulative effect of copulation and fertilization could be hypothesized: copulation without fertilization (with gamma irradiated males) resulted in intermediate engorgement time and weight. Although eggs and larvae were produced in both the gamma irradiated and the group without males (apart from the control groups), only larvae of the groups with gamma-irradiated males could be reared until the adult stage (only females produced = thelytoky). Larvae of the pure parthenogenetic groups were not viable. Whether or not this is a result of copulation without fertilization is still unclear. Undoubtedly engorgement weight, influenced by the physical stimulus of copulation, determines the number of eggs produced (with significant within-species population variation), but probably not the viability of the larvae. The only possible influence of copulation with gamma-irradiated males is an effect brought about by the sterile spermatophore. In certain species of moths, salamanders and fishes, interspecies copulation of closely related species triggers egg development without fertilization of the egg (=gynogenesis). The sperm received by the female contributes no genetic material to the offspring. It could be hypothesized that interspecies copulation between the two closely related species *R. appendiculatus* and *R. zambeziensis*, which have the same predilection sites and are in some parts of their distribution sympatric (Walker *et al*., 2000), takes place, favouring the former species. Under experimental conditions interspecies breeding has been reported (Zivcovic *et al*., 1986, Wouters, 1989, Madder unpublished results). Copulation with gamma-irradiated males can in this context be similar with interspecies copulation without fertilization. The advantage for *R. zambeziensis* is unclear, maybe both species produce similar attractant and mounting sex pheromenes attracting each other during feeding, and thus acting as allomones, interspecific messenger chemicals benefiting the emitter, *R. appendiculatus*.

The importance of this special combined type of parthenogenesis (thelytoky but with the necessity of copulation without fertilization =gynogenesis) is most probably limited to the marginal distribution range where *R. appendiculatus* and *R. zambeziensis* occur sympatrically. Females of *R. appendiculatus* are able to initiate a new population in the absence of, or in attendance for, the arrival of males, brought in by ranging or migrating hosts (naturally or forced by cattle owners frequently observed in epidemic East Coast fever areas).

Our analysis shows that *Wolbachia* and CLO are not responsible for the partial thelytokous reproduction exhibited by *R. appendiculatus.* These results are not surprising for several reasons: 1) Despite some effort, no *Wolbachia* was found in ticks yet; 2) To date no known case of occasional symbiont-induced thelytoky is known, and 3) The sex determination mechanism of the ticks involved in the present research is not haplodiploid and so they are less vulnerable to *Wolbachia* influence. However, ixodid ticks are known to harbor a number of symbiotic microorganisms (Noda *et al*., 1997), and the possibility that one of those will interfere with the normal sex determination of its host can not be ruled out.

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