International Journal for Parasitology 39 (2009) 725–734

Contents lists available at ScienceDirect

International Journal for Parasitology

journal homepage: www.elsevier.com/locate/ijpara

Sex ratios and sex-biased infection behaviour in the entomopathogenic nematode genus *Steinernema*

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ARTICLE INFO

Article history:
Received 28 July 2008
Received in revised form 7 November 2008
Accepted 10 November 2008

Keywords:
Sex ratio
Male colonisation
Female-biased
Entomopathogenic nematode
Steinernema species
Nematoda

ABSTRACT

In experimentally infected insects, the sex ratio of first generation nematodes of five species of *Steinernema* was female-biased (male proportion 0.35–0.47). There was a similar female bias when the worms developed in vitro (0.37–0.44), indicating that the bias in these species is not due to a lower rate of infection by male infective juveniles (IJs). Experimental conditions influenced the proportion of males establishing in insects, indicating that male and female IJs differ in their behaviour. However, there was no evidence that males are the colonising sex in any species, contrary to what has previously been proposed. Time of emergence from the host in which the nematodes had developed influenced sex ratios in experimental infections. In three species (*Steinernema longicaudum, Steinernema glaseri* and *Steinernema kraussei*), early emerged nematodes had a higher proportion of males than those that emerged later, with the reverse trend for *Steinernema carpocapsae* and *Steinernema feltiae*. In a more detailed in vitro study of *S. longicaudum*, the proportion of males was similar whether or not the nematodes passed through the developmentally arrested IJ stage, indicating that the female bias is not due to failure of males to exit this stage. The sex ratio in vitro was independent of survival rate from juvenile to adult, and was female-biased even when all juveniles developed, indicating that the bias is not explained by failure of males to develop to adults. The female-biased sex ratio characteristic of *Steinernema* populations appears to be present from at least the early juvenile stage. We hypothesise that the observed female bias is the population optimal sex ratio, a response to cycles of local mate competition experienced by nematodes reproducing within insect hosts interspersed with periods of outbreeding with less closely related worms following dispersal.

1. Introduction

The sex ratio of acanthocephalan and nematode parasites is frequently biased towards females in both natural and experimental infections (Poulin, 1997), while that of schistosomes is typically male-biased (Mone and Boissier, 2004). The primary sex ratio of animals is expected to be balanced (Fisher, 1930) but later events such as differential survival and infection rates may result in biased sex ratios of parasites within hosts. Early mortality of males contributes to the female-biased sex ratio seen in many helminth parasites of vertebrates (Roche and Patrzek, 1966; Stien et al., 2005) while differential invasion rates of cercariae destined to be male or female contributes to the male-biased sex ratio of schistosomes (Boissier and Mone, 2000). Different behaviour of male and female infective stages has received little attention in parasitic nematodes, apart from the entomopathogenic nematodes *Steinernema* spp. Several studies have explicitly addressed the possibility that *Steinernema* infective juveniles (IJs) that are destined to be male and female differ with respect to dispersal and/or infection behaviour, affecting the within host sex ratio (Grewal et al., 1993; Lewis and Gaugler, 1994; Bohan and Hominick, 1997; Stuart et al., 1998; Fujimoto et al., 2007).

Steinernema spp. (Rhabditida: Steinernematidae) are parasites of a broad range of insects. Unlike most parasites they kill their host, with the aid of their associated symbiotic bacteria, *Xenorhabdus* spp. (Kaya and Gaugler, 1993). The life cycle is direct: IJs actively invade living insects and release their bacteria in the haemocoel, resulting in death of the host, often within 48 h. Sex is chromosomally determined (Poinar, 1967). IJs develop to amphimictic adults which reproduce within the host cadaver. There can be up to three generations within a host, depending on resources (Wang and Bedding, 1996). The *Steinernema* life cycle includes two developmental pathways: juveniles develop directly to adults within a host cadaver as long as conditions are favourable, but in less favourable conditions (generally believed to be due to crowding and resource depletion (san-Blas et al., 2008)), developmentally arrested IJs are formed which leave the cadaver to search for another host. IJs may develop from eggs laid by generations under poor conditions such as a crowded cadaver, but may also develop

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0020-7519/$36.00
doi:10.1016/j.ijpara.2008.11.003

in each generation from eggs which hatch within the mother i.e., in response to locally crowded conditions (Baliadi et al., 2004). The short life cycle, ready availability of naïve hosts and diversity of species make *Steinernema* an attractive model for addressing many biological questions (Stock, 2005).

Grewal et al. (1993) reported that male IJs of four species of *Steinernema* (*Steinernema glaseri*, *Steinernema carpocapsae*, *Steinernema scapterisci* and *Steinernema anomali*, but not *Steinernema feltiae*) disperse, locate and establish in distant live hosts before females, and proposed that males of these species are colonisers, invading the host before females and making the infected host more attractive to female IJs. This male colonisation hypothesis was not supported by a later study on *S. glaseri* (Stuart et al., 1998) and has remained controversial (Lewis et al., 2006). In contrast, Bohan and Hominick (1997) reported that female *S. feltiae* IJs invaded insect hosts before males, leading to a markedly female-biased sex ratio during the initial phase of the infection, but the sex ratio became balanced as the infection progressed. Earlier arrival at the breeding area by one sex or the other is common across animal taxa, and protandry, where males arrive at the breeding site earlier than females, is the more common form of sex-biased arrival timing (Morbey and Ydenberg, 2001). Although its adaptive significance is not well understood, protandry is common in arthropods, birds and other taxa (Morbey and Ydenberg, 2001). Protandry may result if males either develop to adults earlier or disperse to the breeding site earlier, on average, than females. Both forms of protandry have been reported for species of *Steinernema*; early dispersal of males from a given cohort of IJs (Grewal et al., 1993) and early emergence of male IJs from the host (Lewis and Gaugler, 1994; Fujimoto et al., 2007). Lewis and Gaugler (1994) reported that in *S. glaseri* (but not *S. carpocapsae)* IJs that will develop into adult males emerge from their natal cadavers before those that develop into females. They proposed that protandrous emergence is more likely to be adaptive in species such as *S. glaseri* which employ "cruising" as a strategy to seek their hosts, rather than "ambush" foragers as is the case with *S. carpocapsae*. Since ambush foragers rely on host movement it is unlikely that either sex would find a host before the other. However, Fujimoto et al. (2007) reported earlier emergence of male IJs in *S. carpocapsae* also. There are thus two behavioural traits that may lead to a biased sex ratio in experimental infections of *Steinernema*: a greater tendency of one or the other sex within a population of infective juveniles to infect (Grewal et al., 1993; Bohan and Hominick, 1997), and a skewed population made up of unequal numbers of males and females due to differential time of emergence from a host.

We report here a systematic study of *Steinernema* sex ratios in vivo in experimentally infected hosts and in vitro. The first objective was to look for evidence of differential dispersal or infection behaviour of male and female *Steinernema* juveniles as a contributory factor to skewed sex ratios of first generation adults. For this, we used the approach of Grewal et al. (1993), comparing the sex ratio of nematodes established in near and distant hosts (using filter paper and sand assays, respectively), based on the assumption that distant hosts should contain a higher proportion of the dispersing sex. We also include a sequence of exposure times in the sand assay, based on the assumption that if one sex invades before the other, the sex ratio should initially be skewed, but should then stabilise (Bohan and Hominick, 1997; Stuart et al., 1998). Experiments were repeated using two cohorts of IJs, early and later-emerging ones, as time of emergence from the natal cadaver is reported to influence sex-biased infection patterns (Lewis and Gaugler, 1994; Rolston et al., 2006; Fujimoto et al., 2007). For comparison, IJs were also reared in vitro to eliminate the effects of infection and dispersal behaviour on sex ratio. The second objective was to examine the role of factors other than IJ behaviour in influencing adult sex ratio, such as differential recovery rates of males and females from the developmentally arrested IJ stage, or their survival to adult. This more detailed study was done on *Steinernema longicaudum* reared in vitro. All determinations of sex were made on adult worms, since IJs cannot easily be sexed. We include five species of *Steinernema* representing varied phylogenetic clades and foraging modes (Table 1).

Table 1
Species and strains of *Steinernema* used in the experiments together with their foraging strategies and their phylogenetic relationships.

Species and strain	Foraging strategy[a]	Clade[b]
Steinernema carpocapsae All	Ambush	II
Steinernema glaseri NC1	Cruise	V
Steinernema longicaudum CB2B	Cruise	V
Steinernema kraussei L137	Cruise	III
Steinernema feltiae 4CFMO	Intermediate	III

[a] Campbell et al. (2003).
[b] Spiridonov et al. (2004) and Nadler et al. (2006).

2. Materials and methods

2.1. Source and cultivation of nematodes

Nematodes were cultured in larvae of *Galleria mellonella* (Lepidoptera: Pyralidae; the greater wax moth) using standard procedures (Woodring and Kaya, 1988). Culturing was at 20 °C with the exception of *Steinernema kraussei* which was cultured at 15 °C. Cadavers were placed in White traps (10 insects per trap) from which the emerging IJs were harvested. Modified White traps, in which juveniles must climb up the side of a Petri dish before reaching the surrounding water (Woodring and Kaya, 1988) were used for *S. glaseri* and *S. longicaudum*. Modified traps suit species in which the nematodes emerge as pre-IJs. IJs that were harvested on the 1st day of emergence ("early emergers") and on the 10th day after emergence began ("late emergers") were used in experiments. Infective juveniles that emerged in the intervening period were discarded. For both standard and modified White traps, the day on which IJs entered the water of the trap was counted as the day of emergence, and in both cases reflects the departure of newly formed IJs from the vicinity of the source cadavers. Harvested IJs were washed three times by sedimentation, the concentration was adjusted to 2000 IJs/ml, and the suspension was stored in tap water for 2 days at 9 °C except for the tropical species *S. longicaudum* which was stored at 20 °C.

2.2. Experimental infections

The experimental assay of Stuart et al. (1998) was adopted for this study. Nematodes were applied to the top of sand columns containing a wax moth larva at the bottom. For most species 80 ml vials, forming sand columns 4 cm high and 2.5 cm diameter, were used. Since *S. carpocapsae* did not migrate through the 4 cm sand column, cells of a 24 multi-well plate, forming sand columns of 8 mm height and 5 mm diameter, were used for this species. Fine and coarse sand particles were removed by sieving over 150 and 450 μm sieves, respectively. The sand was then sterilized at 120 °C for 24 h. The moisture content of the sand was adjusted to 8% w/w using tap water. Early or late emerging IJs of one of the five *Steinernema* spp. at a concentration of 100 IJs in 100 μl tap water were added to a depression in the top of the sand surface and the columns were capped. Columns were incubated at 20 °C except for *S. kraussei* which was incubated at 15 °C. The nematodes were allowed to migrate through the sand column for periods ranging from 4 to 48 h, after which the insects were removed from the column, washed to remove adhering IJs and incubated at the