Factors influencing the outcome of male-male encounters in the entomopathogenic nematode *Steinernema* spp.

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Declaration of authorship

I certify that the work presented here is, to the best of my knowledge,

original and the result of my investigations,

except where explicitly acknowledged otherwise.

This work has not been submitted, either in whole or in part,

for a degree at this or any other university.

Annemie N. R. L. Zenner October 22nd 2011

Abstract

Steinernema infective juveniles (IJs) carry cells of symbiotic bacteria in their intestine and release these bacteria upon entry into insect-haemolymph. The bacteria kill the insect, providing ideal conditions for development and reproduction of the nematodes. About three *Steinernema* generations can develop within one insect cadaver leading to the production of thousands of IJs.

Steinernema longicaudum is the first nematode for which intraspecific male-male fighting behaviour was observed (O'Callaghan, 2006). Placing 2 males in a drop of haemolymph resulted in injurious or paralysing fighting within the hour in 20% of the drops. Not lethally injured males were less successful at siring offspring.

S. longicaudum males only produce sperm after several hours with a female (Ebssa *et al*, 2008). Such matured males fought, paralysed and killed at a higher speed than males that had not produced sperm. Previous victory also resulted in earlier fighting and paralysis and in longer fights with new partners. Prior residency, reproductive value of or presence of a female didn't have measurable effects on the occurrence of paralysis and death. A well-established culture of the symbiotic bacteria also enhanced fighting outcome.

IJs experience a different developmental pathway than juveniles that develop straight into adults, the pathway followed is determined by environmental conditions of the parental generation. The level of aggression and the influence of relatedness depended on the developmental pathway followed: IJ-males were more aggressive than non-IJ males.

Evidence of fighting avoidance mechanisms (e.g. assessment) could not be established for *S. longicaudum* males.

Other *Steinernema* species, from different clades, were also studied, but none was more aggressive than *S. longicaudum*. Types of fighting resulting in injury and possible mechanisms leading to paralysis and death were also studied.

1. Introduction

1.1. Introduction

Fatal fighting is an extreme behaviour and is rare in the animal kingdom. Most animal conflicts are resolved using ritualised displays and assessments of each other's capabilities. In case the contested resource has a major impact on the individual's lifetime reproductive success, current theory predicts higher possibility of fatal fighting to occur.

While studying the reproductive traits of the two closely related species of entomopathogenic nematodes, *Steinernema hermaphroditum* and *Steinernema longicaudum,* Dr Kathryn O' Callaghan observed fatal fighting behaviour. In her undergraduate project (O'Callaghan, 2000), she discovered male *S. longicaudum* nematodes could kill *S. hermaphroditum* hermaphrodites when direct contact was possible. Expanding on these experiments in her PhD thesis (O'Callaghan, 2006), she noted that interspecific killing behaviour towards females was also performed by males of other *Steinernema* species. However, female *S. longicaudum* nematodes would not kill any *Steinernema* female. O'Callaghan (2006) also reported that *Steinernema* males attacked and killed hetero-specific and even conspecific males. The proportion of encounters resulting in dead nematodes varied between species in both the intra- and interspecific encounters. In interspecific encounters some species were more prone to be the victim than other species or would even not attack females of a particular different species.

This thesis aims to further explore the intraspecific killing behaviour of different *Steinernema* species, specifically, to shed light on the specific mechanisms of paralysis and kill, the evolution of this fighting behaviour in the *Steinernema* genus and the circumstances that lead to the development of such an extreme behaviour that is fatal fighting, or to less extreme alternatives.

1.2. The Family Steinernematidae

Steinernematidae and Heterorhabditidae are the 2 families that are termed entomopathogenic nematodes (EPNs). These are obligate, lethal parasites of insects and are soil inhabitants only in their infective stage. Steinernematidae are ubiquitous (recovered from all continents except Antarctica (Griffin *et al*, 1991) and can infect a broad range of insect species. These characteristics make them effective biological control agents of insect pests in a variety of crops. The species comprising these families differ in infectivity, host ranges, suitability for commercial culturing and environmental limitations.

1.2.1. Life Cycle

Heterorhabditidae and Steinernematidae are characterised by a similar life cycle (see Figure 1.1) that only differs in the type of sexual reproduction. With the exception of *S. hermaphroditum*, *Steinernema* species are dicœcious, produce sexually dimorphic adults and reproduce through cross-fertilisation or amphimixis. *Heterorhabditis* species reproduce initially by self-fertilisation (automixis) of the hermaphrodites of the first generation. The following generation's hermaphrodites are joined by males and females and reproduction is both amphimictical and automictical.

The free-living, soil dwelling, invasive stage of *Steinernema* is the infective or dauer juvenile stage (IJ or DJ) (Figure 1.1-a) (Nguyen & Smart, 1992). This is a developmentally arrested form of the 3rd juvenile stage that carries symbiotic bacteria in an intestinal vesicle (Goodrich-Blair, 2007) (see below). Upon entry in the insect host, mainly through natural openings (Renn, 1998), the nematode releases these bacterial cells into the haemocoel (Figure 1.1-b,c) (Snyder *et al*, 2007). The bacteria rapidly multiply and within 24-48 h post-infection kill the insect by septicaemia or toxaemia. The infective juveniles recover and start feeding on the bacteria and degrading insect tissue. They develop through the 4th juvenile stage to adults of the 1st generation (Figure 1.1-d,e) (Nguyen & Smart, 1992). The females lay most of their fertilised eggs after mating, but some are retained in the female and develop into the first juvenile stages while still in the parental body, a process known as *endotokia matricida* (Baliadi *et al*, 2001;Baliadi *et al*, 2004;Nguyen & Smart, 1992). Dependent on the available resources in the host cadaver, one or more generations may occur. A low nutrient status and/or overcrowding of the cadaver prompt(s) the production of infective juveniles (Burnell *et al*, 2005). These emerge from the cadaver into the moist soil (Figure 1.1-f) and are

able to survive without feeding for several months until a suitable host is found or passes by (Griffin *et al*, 2005).

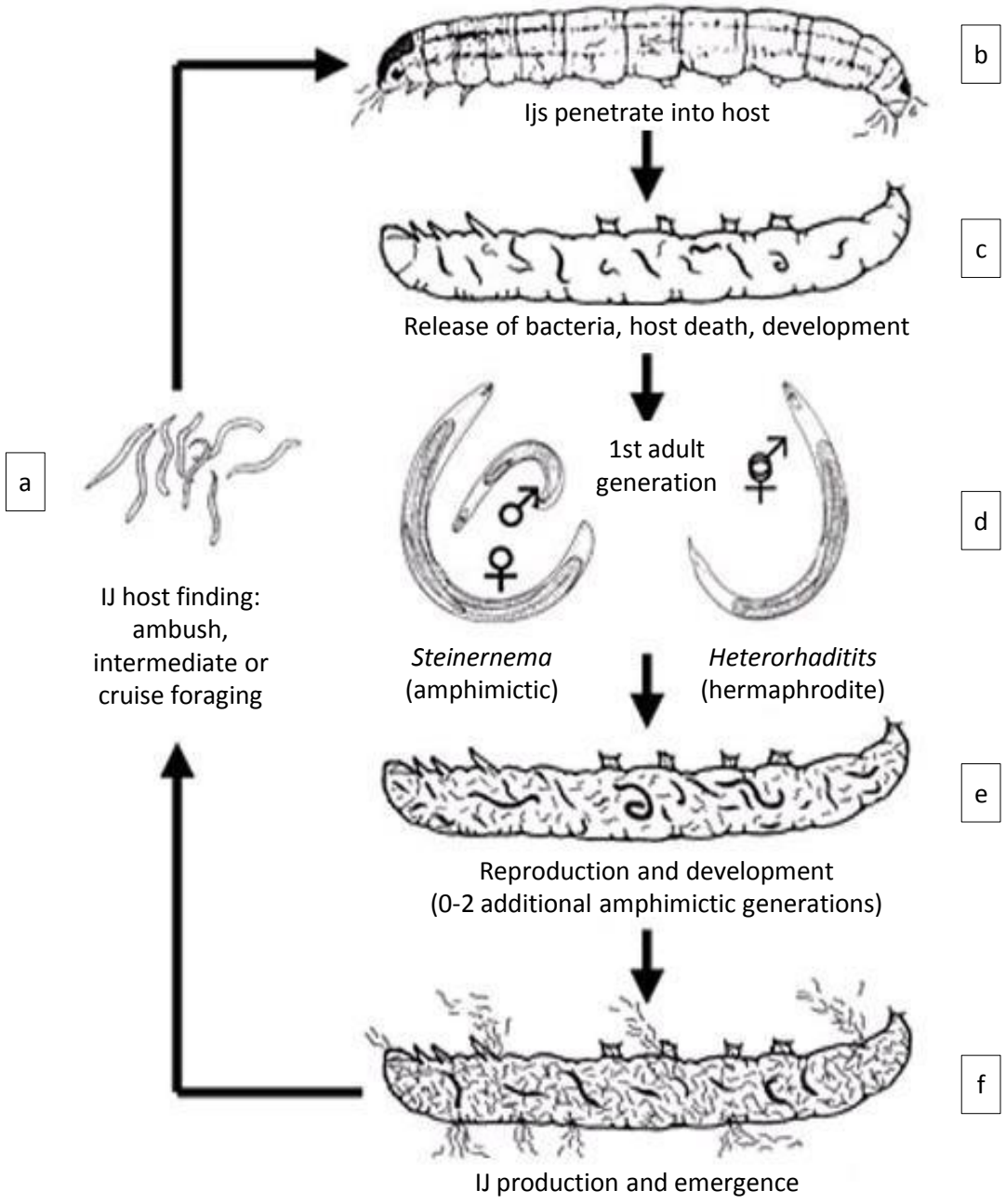

Figure 1.1 Simplified life cycle of entomopathogenic nematodes (Source: http://www.sipweb.org/nematodes).

In order to infect a suitable insect host, infective juveniles use a foraging strategy along a continuum between ambush and cruise foraging (Lewis *et al*, 2006). Ambushers raise most of their body off the substrate and attach to passing insects whereas cruisers actively move through the soil in search of a suitable host. *S. glaseri* is an example of a cruise foraging species, *S. carpocapsae* is an ambusher species and *S. feltiae* uses an intermediate strategy, the latter lift part of their bodies from the substrate but only for periods a few seconds long (Lewis, 2002).

1.2.2. Association with *Xenorhabdus*

The success of entomopathogenic nematodes as a biocontrol agent owes a lot to the unique association of a host-seeking nematode (Steinernematidae and Heterorhabiditae) and a lethal insect-pathogenic bacterium (*Xenorhabdus* spp. and *Photorhabdus* spp., respectively) (Forst *et al*, 1997).

The nematodes transport the bacteria between a depleted insect cadaver and a new host, protecting the bacterium against the external environment and impeding insect immune responses. The bacteria ensure the rapid death of the host insect, provide suitable nutritive conditions for the nematode and curtail competing organisms through the production of antibiotics and toxins. There are several species of *Xenorhabdus* known (Tailliez *et al*, 2006). Although the association is not obligate, both partners thrive better in terms of development and reproduction when occurring together with their natural symbiont (Sicard *et al*, 2003). The special intestinal vesicle of a *Steinernema* IJ can only be colonized by its natural symbiont. Even closely related *Xenorhabdus* species are unable to colonize the vesicle and thus can't be retained in the IJs of non-natural symbionts (Sicard *et al*, 2004). But different nematode species can have the same species of bacterium as natural symbiont (Fischer-Le Saux *et al*, 1999;Lee & Stock, 2010a;Tailliez, 2006). The *Steinernema* species used in this study are presented with their respective natural bacterial symbiont in Table 1.1.

As motile, Gram-negative, facultatively anaerobic rods, *Xenorhabdus* spp. are currently assigned to the Enterobacteriaceae. *Xenorhabdus* are however negative for nitrate reductase and catalase which are 2 major positive characters of the Enterobacteriaceae family (Akhurst, 1986;Forst *et al*, 1997). *Xenorhabdus* show phenotypic or phase variation. Only the Phase I variant has been isolated from the wild nematode and is characterized by specific dye absorption and the production of antibiotics. In *in vitro* subcultures of the bacteria, a proportion of Phase II cells occur that are different for a range of phenotypic characters

amongst which the 2 mentioned before. So far, there is no consistent ecological explanation for the role of Phase II variants (Griffin *et al*, 2005). In the stationary phase of phenotypic Phase I cells, metabolites are secreted into the insect haemolymph. These include exoenzymes, like lipase and protease, insecticidal and nematicidal toxins and several broad spectrum antibacterial and antifungal antibiotics (Brown *et al*, 2004;Brown *et al*, 2006a;Crawford *et al*, 2010;Mahar *et al*, 2008;Maxwell *et al*, 1994;Ribeiro *et al*, 2003;Webster *et al*, 2002). These antibiotics and toxins help to deter scavengers, bacteria and other species of nematodes, leading to a virtual monopolisation of the insect cadaver by the *Steinernema* nematode and its associated bacterium.

Table 1.1 *Steinernema* species used in this study and their corresponding symbiotic bacteria

***Steinernema* species**	***Xenorhabdus* species**
S. longicaudum Shen and Wang, 1992	*X. ehlersii* Tailliez, Pages, Ginibre and Boemare 2006
S. bicornutum Tallosi, Peters and Ehlers, 1995	*X. budapestensis* Lengyel et al., 2005
S. feltiae Filipjev, 1934	*X. bovienii* Akhurst, 1983
S. glaseri Filipjev, 1934	*X. poinarii* Akhurst, 1983
S. kraussei Steiner, 1923	*X. bovienii* Akhurst, 1983

1.2.3. Taxonomy & phylogeny

Despite the many similarities between the Heterorhabiditae and Steinernematidae, it is more likely that their shared unique characteristics have developed through convergent evolution. The Steinernematidae (Chitwood & Chitwood, 1937) family currently contains 2 genera: *Neosteinernema* (Nguyen & Smart, 1994) and *Steinernema* (Travassos, 1927).

Currently about 61 species of *Steinernema* have been described (Nguyen *et al*, 2010), however, many isolates remain to be fully characterised (Stock, 2005). Many morphological characters of species in the *Steinernema* genus are similar in structure and shape, but not origin (homoplasy). The use of combined phylogenetic analysis of 3 genes over 25 described species (with both a Chinese and an American isolate for *S. longicaudum*) put forward well-resolved and highly similar phylogenetic trees of the genus *Steinernema* shown in Figure 1.2

(Nadler *et al*, 2006). Although with reservations, Nadler et al. (2006) believe the best working phylogenetic hypothesis is yielded by a combined analysis (several molecular approaches plus morphology). On the basis of morphological features and different ITS alignments, Spiridinov et al. (2004) identified 5 major clades that are in general mostly recognised (Lee & Stock, 2010a;Lee & Stock, 2010b;Nadler *et al*, 2006;Nguyen, 2007). There are however different opinions on morphological characteristics that form the basis for these clades e.g. colour of the spicules and the gubernaculum (Nadler *et al*, 2006;Spiridonov *et al*, 2004;Stock *et al*, 2001a). Of great interest are future studies regarding co-evolution of *Xenorhabdus* and *Steinernema*. Speciation of 1 partner in a symbiotic relationship can lead to speciation in its symbiotic partner. Such cospeciation(s) can lead to mirrored phylogenetic relationships of the partners. Lee and Stock (2010) have proposed cophylogenetic hypotheses for Xenorhabdidae and Steinernematidae. Cophylogenetic methods can also resolve incompatibilities of unresolved phylogenies in one of the partners (Lee & Stock, 2010a;Lee & Stock, 2010b;Maneesakorn *et al*, 2011).

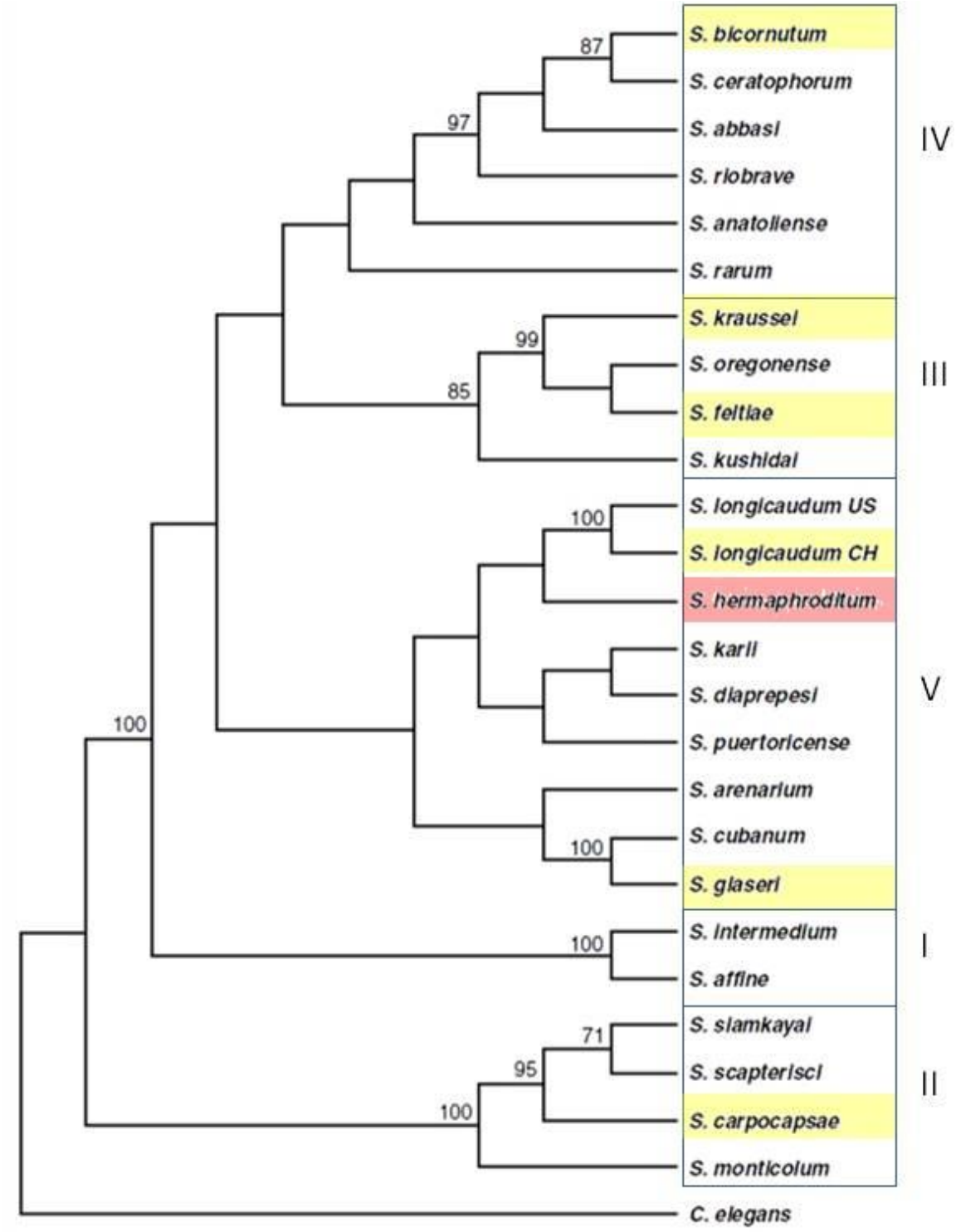

Figure 1.2 Phylogenetic tree of *Steinernema*: Most parsimonious tree inferred from combined molecular data (three genes, 1,639 characters, 565 parsimony informative) . MP boot strap clade frequency values ≥70% mapped above internal nodes. This tree is taken from Nadler *et al.* (2006). Roman numerals represent clades enumerated by Spiridonov *et al*. (2004). The species studied in relation to this thesis are highlighted in yellow. Highlighted in red is a species used in O'Callaghan (2006) but not in this work.

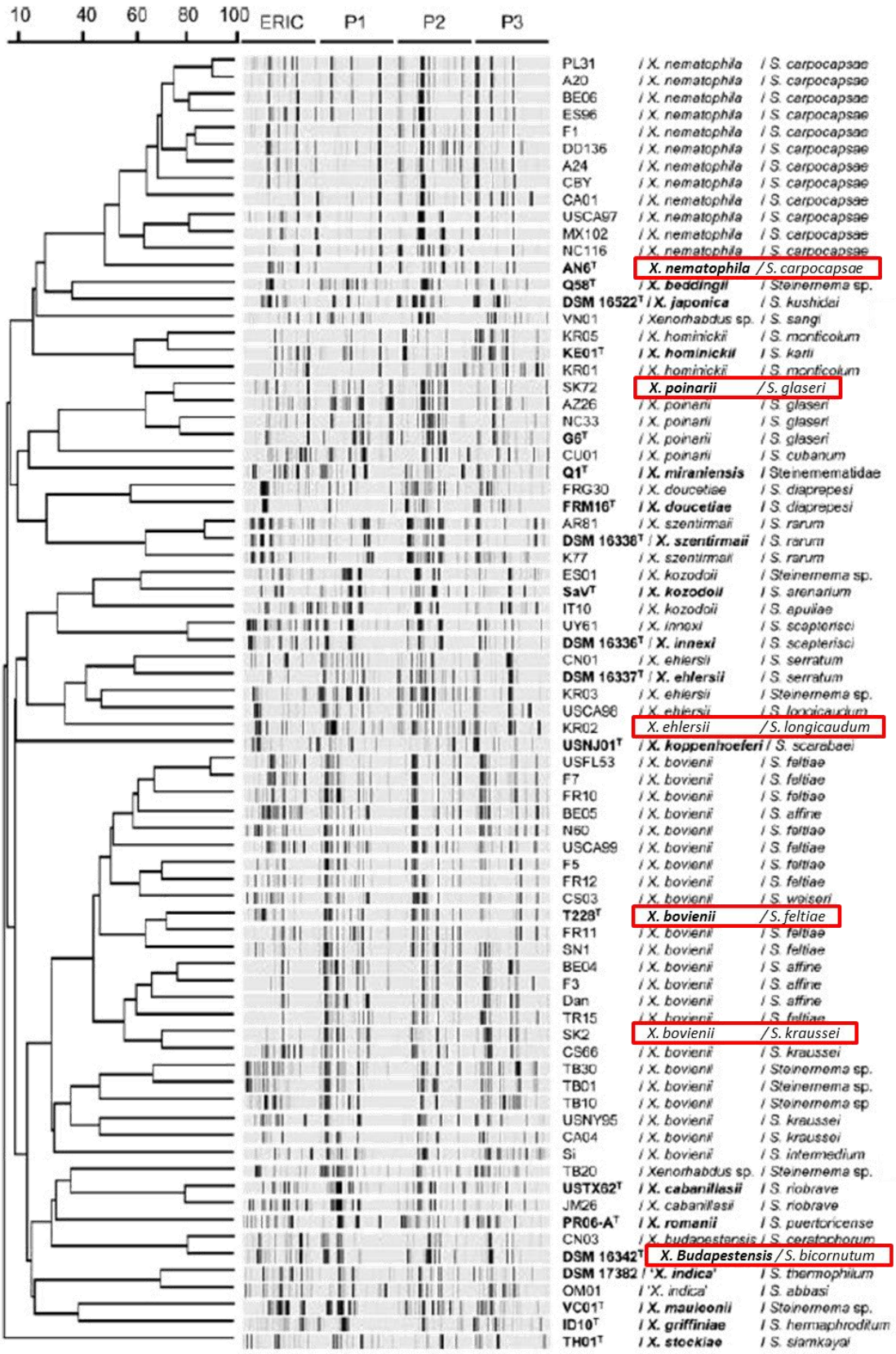

Figure 1.3 Comparison of the molecular typing profiles of 76 *Xenorhabdus* strains and isolates studied in Tailliez *et al*. (2006). One ERIC profile and three RAPD profiles were obtained (primers P1, P2 and P3, used in independent reactions), were combined for each strain. The combined molecular typing profiles were compared using Pearson's similarity coefficient. The corresponding similarity matrix was used to generate a dendrogram using

the UPGMA module of the GelCompar software (Applied Maths). Type strains are highlighted in bold. When known, the Steinernema species of the corresponding nematode host is indicated. This figure is taken from Tailliez *et al.* (2006). The species studied in relation to this thesis are highlighted in red boxes.

1.2.4. Reproductive biology

In Steinernematidae, sex is most likely determined by the X-O type chromosomal mechanism (Poinar, 1967) where females are of the homogametic XX-type (3-5 bivalent chromosomes) and males are of the heterogametic XO-type with 2-4 bivalent chromosomes and a single univalent chromosome (Curran, 1989). The main non-reproductive related morphological difference between the sexes is the difference in size. This size difference depends on the generation, but healthy females are always the bigger sex (Nguyen, 2007;Stock *et al*, 2001b).

Males have a single reflexed testis opening into a seminal vesicle, a vas deferens (glandular and ejaculatory part) and finally into the cloaca. In pouches in the cloaca is a pair of copulatory spicules. The spicules are made up of hardened cuticle with a cytoplasmic core through which a nerve runs ending in sensilla at the tip of the spicule (Lee & Atkinson, 1976;Liu & Sternberg, 1995;Sood & Kaur, 1983). The spicules are used for sperm transfer during copulation. The gubernaculum is another sclerotized structure which guides the spicules during copulation so that they do not pierce the cloacal wall. Spicules and gubernaculum are 2 of the characteristics that differ between males from the first generation and males from the subsequent generations. Morphological variations of the spicules and the gubernaculum are also used as important characteristics in the taxonomy of nematodes (Nguyen, 2007).

Females have 2 ovaries each opening into a seminal receptacle, then an oviduct and uterus; the uteri join and open via the vulva. Males of some species (particularly *S. longicaudum*) need the presence of a conspecific female to produce sperm (Ebssa *et al*, 2008). Since the males don't need to be in direct contact with the female, this is likely mediated through a pheromone produced by the females. Spermatozoa are only activated within the female's gonoduct (Spiridonov *et al*, 1999); activation involves bipolarisation, a reorganisation of organelles and the formation of pseudopods (Yushin *et al*, 2007). Male *S. carpocapsae* are attracted to virgin and not to mated females through female produced sex attractants (Lewis *et al*, 2002;Neves *et al*, 1998). The male attractant produced by hermaphrodite *C. elegans* are ascarosides (ascr), whereas hermaphrodite *C. elegans* are attrackted by indole ascarosides (icas) (Macosko *et al*, 2009;Sokolowski, 2010;Srinivasan *et al*, 2012). *P. redivivus* males produce

an ascaroside, identified as dhas#18, a dihydroxy derivative of ascr#18, which strongly attracks female *P. redivivus* (Choe *et al*, 2012a). Male *C. elegans* also produce ascarosides, but with a profile markedly different from the hermaphrodite produced ascarosides (Izrayelit *et al*, 2012). This. Ascarosides are also produced by both juvenile and adult Steinernematidae and also have an attractive or repelling action (Choe *et al*, 2012b).So far however, no pheromone production by male *Steinernema* has been reported.

When mating, the male coils its tail around the middle of the female's body and while moving up and down along the female, it searches for the vulva with its spicules. Having found the vulva, the spicules are inserted and sperm is transferred (Lewis *et al*, 2002). In other Nematoda, males might leave a copulatory plug in the vulva in attempt to prohibit subsequent mating with another male (Barker, 1994). So far, this has not been reported for Steinernematidae (Lewis *et al*, 2002). Male *Steinernema longicaudum* CB2B can start mating successfully from as early as 2 days old (Ebssa *et al*, 2008). The ages of 4-6 days are the optimal ages for reproduction for this species (Ebssa *et al*, 2008).

Sperm morphology varies between species of *Steinernema* with a group of species even showing sperm dimorphism (Spiridonov *et al*, 1999;Yushin *et al*, 2007). *Steinernema tami* and *S. longicaudum* are two such species having megaspermatozoa (diameter: 25-30 up to 110 µm) that carry microspermatozoa (1.5-3 µm diameter) (Spiridonov *et al*, 1999;Yushin *et al*, 2007). The immature microspermatozoa are attached to the surface of the immature megaspermatozoon by gap junctions. Mature microspermatozoa are immotile and can be found free in the uterus lumen or attached to the main cell body of the amoeboid megaspermatozoa. Species like *S. feltiae* have only 1 type of spermatozoa, amoeboid cells about 6-12 µm in diameter forming chains (Spiridonov *et al*, 1999;Yushin *et al*, 2007); typical Rhabditida spermatozoa.

Sperm competition has been observed in other Nematoda including *C. elegans* (Lamunyon & Ward, 1999;Lamunyon & Ward, 2002) and might be present in *Steinernema* as is suggested by the occurrence of sperm dimorphism in some species, but has not yet been proven (Yushin *et al*, 2007).

1.2.5. Fighting in *Steinernema*

In 2006, O'Callaghan reported the novel phenomenon of intraspecific male killing in Steinernematidae. Killing was observed both *in vitro* and *in vivo* and its frequency varied between species with *Steinernema longicaudum* being the most frequent killer, followed by *S.*

carpocapsae and *S. feltiae* brought up the rear with little or no killing reported (1 pair out of 19 had a dead male after 24-48 h) (O'Callaghan, 2006).

The observed fighting showed a male approaching another male and curling its tail around the victim upon which the latter immediately curled in on itself but soon appeared paralysed (O'Callaghan, 2006). Victims of fights also often looked injured: they were shrunken in size, had a damaged cuticle or showed a kink in their body (O'Callaghan, 2006). The rapid paralysis might indicate the involvement of a toxin, either produced by the nematodes or present in the medium in which case it might be produced by the symbiotic bacteria.

1.3. Fatal fighting

When a resource is limited, competition for this resource is likely to arise. Competition between individuals of the same species is called intraspecific competition. One direct way of competing over a resource is fighting over it. The essence of fighting is to harm, or threaten to harm, the other contestant so that it will leave the resource to the winner of the fight. In most animals behaviours and/or physiological adaptations have evolved that diminish the chance for actual harm to occur. The reason for this is that each individual is not only the potential winner of the fight, but also the potential loser. In case the loser possesses a technique to recognize itself as the loser and to retreat before it gets hurt, the loser has not lost all it could have if it had proceeded with the fight. The antagonists may test each other's fighting capabilities prior to a fight through assessment. Animals can size each other up on the basis of external traits like the height of the casque (head ornament) of male Cape dwarf chameleons (Stuart-Fox *et al*, 2006) or in harmless contests like the bellowing contests of red deer stags (Cluttonbrock & Albon, 1979). Assessment and display relate to the contestant's own resource holding power (RHP, the ability to win the fight)(Jennings *et al*, 2004), his perception of the opponent's RHP (Arnott & Elwood, 2009;Briffa & Elwood, 2009;Humphries *et al*, 2006;Hurd, 2006) and the value of the contested resource for each contestant (Subjective Resource Value) (Elias *et al*, 2010;Humphries *et al*, 2006). Escalation will occur when the opponents are matched in their RHP or when their perception of the value of the resource is very high (Brown *et al*, 2006b;Keil & Watson, 2010). The perceived value of the resource is likely to be different for the owner of a resource and the intruder wanting the former's resource (Leimar *et al*, 1991). A resident might have a higher motivation because he knows value of the contested resource and the intruder does not (Buena & Walker, 2008;Takeuchi & Honda, 2009), or the

owner might have put a lot of effort into preparing the territory for a certain use e.g. solving boundary disputes or building nests (Arnott & Elwood, 2008;Parker, 1974).

Even though fatal fighting is an extreme behaviour and is fairly rare in the animal kingdom, it still occurs (Cook *et al*, 1999;Enquist & Leimar, 1990;Piper *et al*, 2008;Stevens, 1993;van Wilgenburg *et al*, 2005). The existence of fatal fighting and the circumstances leading to its evolution are described by Enquist and Leimar (1990). The most important determinant of the occurrence of fatal fighting is the balance between the value of the future for each contestant and the subjective value of the resource for each contestant (Enquist & Leimar, 1990). When the subjective value of the resource is equal to or greater than the value of the future, assessment will be of no use and the contestant in question will be prepared to fight until serious injury or death occurs (Cook, 2005). In case the value of the future is close to nothing, the contestant will never give up and the fight will always end in the death of one of the combatants (Enquist & Leimar, 1990). This happens most when the restricted resources contested are mating opportunities since these have a very high impact on lifetime reproductive success (Fromhage & Schneider, 2005;West *et al*, 2001).

The phylum Arthropoda contains the two groups that have been most reported in the literature with highly lethal fighting: the Insecta and the Arachnida (Enquist & Leimar, 1990). Examples of species with a high occurrence of fatal fighting are fig wasps ((Bean & Cook, 2001;Cook *et al*, 1999;Cook, 2005;Greeff & Ferguson, 1999;Pereira & Do Prado, 2005;Reinholdt, 2003)), parasitoid wasps (Innocent *et al*, 2007;Reece *et al*, 2007), ants (Anderson *et al*, 2003;Batchelor & Briffa, 2010;van Wilgenburg *et al*, 2005) and spiders (deCarvalho *et al*, 2004;Leimar *et al*, 1991). Several species of fig wasps have been well studied and given their confinement to a fig mirroring the confinement of entomopathogenic nematodes to an insect cadaver, they make a good reference group for this study. In most of the taxa cited above, fighting is a sexually dimorphic character that is only displayed by one of the sexes. Fig wasps are sexually dimorphic and the males of fighting species use their huge mandibles as weapons in intraspecific fighting (Frank, 1987;Hamilton, 1979). Hamilton (1979) even notes the possibility of the use of venom in the fighting of some *Idarnes* species having regularly observed quick paralysis after a bite leaving only a mere puncture in the body of the victim.

Male fig wasps can be very closely related to each other within a fig (Moore *et al*, 2006;Nelson & Greeff, 2009;Reinholdt, 2003;West *et al*, 2001). Local mate competition is a term given by Hamilton to the process where the competition between highly related males for females leads to the selection of a female biased sex ratio (Abe *et al*, 2005;Bailey & Zuk,

2009). Fig wasps show both the highly female-biased sex ratios (Nelson & Greeff, 2009) and a high likelihood of competition between brothers (Hamilton, 1979;Moore *et al*, 2006;Nelson & Greeff, 2009;Reinholdt, 2003;West *et al*, 2001).

1.4. Homosexual encounters & cost of mating on lifespan

In quite a lot of arthropod species (mainly insects) but also in lizards, mating can reduce the lifespan of females (Chapman *et al*, 1995;Fowler & Partridge, 1989;Maklakov & Bonduriansky, 2009;Rankin *et al*, 2011;South & Lewis, 2011). In *Drosophila melanogaster,* it is a toxin in the seminal fluid that shortens the lifespan of mated females (Chapman *et al*, 1995;Wigby & Chapman, 2005). Mating also reduces the lifespan of hermaphrodite *C. elegans*, as do homosexual interactions between male *C. elegans* (Gems & Riddle, 1996;Gems & Riddle, 2000). Homosexual mating in the AB2 wild isolate of *C. elegans* consists of the deposition of a mating plug over another male's excretory pore occurring most probably after attempted copulation.

Homosexual behaviour is also widespread in the animal kingdom (Abele & Gilchrist, 1977;Bailey & Zuk, 2009;Gems & Riddle, 2000;Levan *et al*, 2009;Switzer *et al*, 2004). Explanations for the occurrence and persistence of same-sex sexual behaviour are social bonding (Connor & Mann, 2006), intrasexual competition (Abele & Gilchrist, 1977;Preston-Mafham, 2006) and practice for intersexual mating (Dukas, 2010;Vervaecke & Roden, 2006). Non-adaptive explanations have also been explored, including weak sex recognition (perception error hypothesis) (Ryne, 2009) and deprivation of intersexual mating behaviour due to the absence of the opposite sex (Field & Waite, 2004). Homosexual behaviour can also be associated with a reduction of male life span as has been recorded *inter alia* in *Musca domestica* (Ragland & Sohal, 1973), *Tribolium* (Spratt, 1980), Cimicidae (Ryne, 2009) and *C. elegans* (Gems & Riddle, 2000).

1.5. Objectives

The objectives of this thesis were:

1. to describe fighting behaviour and related agonistic behaviours in a focus species (*Steinernema longicaudum*) that has fairly high rates of fighting and subsequent paralysis and death. Related agonistic behaviours include assessment and defensive behaviour. (Chapter 3). Additional objectives were pursued using the focus species *S. longicaudum* and included in Chapter 3;
2. to determine factors influencing the resource holding potential of a male, such as age, sexual maturity and prior residency in a haemolymph drop (Chapter 3);
3. to explore the essential features that elicit fighting behaviour, using normal, incapacitated and dead males, and inert male-sized objects (Chapter 3)
4. to vary the value of the contested resource (e.g. presence of virgin or mated female) and study the effect of its value on male-male competition (Chapter 3);
5. to study the distribution of fighting behaviour and its evolution in the *Steinernema* genus, using species from different clades (Chapter 4);
6. to describe the effects of fighting and investigate the mechanisms behind paralysing and killing (Chapter 5).

All experiments were carried out in vitro, in drops of insect blood.

2. General Materials and Methods

2.1. Origin of nematodes

All strains that were used for this thesis and its related studies came from the NUIM culture collection and had been maintained in culture since they were isolated or obtained from other sources.

Steinernema longicaudum strain CB2B was originally isolated from the Shandong province in China (Shen and Wang, 1992) and obtained from CABI Biosciences, UK. This was the only tropical species used in this study.

Steinernema bicornutum strain IRA7 was isolated in Iran and obtained from Naser Eivazian Kary, University of Azerbaijan.

Steinernema feltiae isolate 4CFMO was isolated from County Mayo, Ireland by Aoife Dillon, NUI Maynooth, Ireland.

*Steinernema glaseri (*NC1 strain) was isolated from North Carolina and obtained from Albrecht Koppenhofer, Rutgers University, USA.

Steinernema kraussei was isolated from the UK and obtained from Becker Underwood UK.

2.2. Cultivation and storage of nematodes

The cultivation of *Steinernema* nematodes comprehends the infection of an insect host for which late instar larvae of *Galleria mellonella* (greater wax moth) were used. These larvae were obtained from the Mealworm Company, Sheffield, UK. Prior to use, the wax moths were stored at 15 °C in sawdust filled plastic containers with air holes.

2.2.1. Infection

A sheet of 9 cm filter paper was placed on the bottom and on the lid of a 9 cm Petri dish. The filter paper was moistened with a suspension of infective juveniles (approx. 1000 IJs/ml). Five to eight *Galleria mellonella* larvae were placed on the filter paper in the Petri dish and covered with the lid and its moist filter paper. These Petri dishes were inverted and placed in an incubator (temperature and period depending on the species, see Table 2.1). This incubation period allowed the nematodes to develop and multiply in the insects.

Table 2.1 The conditions for incubation, harvest and storage of the different *Steinernema* species used.

Steinernema species	Incubation temperature (°C)	Incubation period for the production of adults (days)	Storage temperature (°C)	Trap used for harvest
S. bicornutum	20-23	3-4	9	White trap
S. carpocapsae	20	3-4	9	White trap
S. feltiae	20	4-6	9	White trap
S. glaseri	20	4-6	9	Modified White trap
S. kraussei	15	4-6 (adult: 3-4)	9	White trap
S. longicaudum strain CB2B	27	2-3	20	Modified White trap

2.2.2. Harvest

After a nematode species specific period, the successfully infected larvae were placed on White traps (Kaya & Stock, 1997;White, 1927) or on modified White traps based on the model of Woodring & Kaya (1988). These traps allowed the infective juveniles to leave the host and migrate into water. This water was periodically harvested and the trap was then replaced in fresh water. The infective juveniles were cleaned before storage at a nematode specific temperature.

Modified White traps were used to harvest infective juveniles from *S. longicaudum and S. glaseri,* as the nematodes emerge as pre-IJs and entry directly into water may impede development to IJ. About 5-7 days after infection, the infected larvae were placed on moist filter paper in the lid of a 9 cm Petri dish. The lid was then placed in the base of a 15 cm Petri dish. The base of the big Petri dish was covered with tap water (up to 50% of the height of the base). Small pieces of Blu Tack attached to the side of the lid kept it away from the side of the dish and hence prevented wicking of water into the lid. The 15 cm Petri dish was covered with its lid and placed into the appropriate incubator.

White traps were used to harvest infective juveniles from all other species. An inverted lid of a 9 cm Petri dish was placed in the base of a 15 cm Petri dish and covered with a piece of filter paper (approx. 12 cm diameter). This filter paper was then moistened with a couple of drops of tap water. The infected wax moth larvae (about 5-7 days after infection) were placed on this filter paper. The base of the big Petri dish was covered with tap water (up to 50% of the height of the base) while making sure the 9 cm Petri dish lid did not start to float. The 15 cm Petri dish was covered with its lid and placed at 15 – 27 °C depending on the species (Table 2.1).

The traps were checked every other day until infective juveniles appeared in the water. They were left to aggregate (without overcrowding) for about 2 days after which the infective juvenile suspension was poured into a clean jar. The nematodes were left to settle and washed 4 times with tap water. White traps were harvested from more than once, so the dish was covered again with water and put back at the appropriate temperature. Different harvests were kept separate.

2.2.3. Storage

The infective juveniles were stored at a concentration of approx. 1000 IJs/ml in 40 ml aliquots in re-sealable plastic tubs (9 cm diam.) at 9 or 20 °C depending on species (Table 2.1).

2.3. Cultivation of nematodes in hanging drops

Nematodes were cultured *in vitro* using the hanging blood drop method (Figure 2.1; Poinar, 1967) as follows:

2.3.1. Surface sterilisation of infective juveniles

Infective juveniles were surface sterilised in 0.1% (0.4 mM in H_2O) Hyamine® 1622 solution (51126, Aldrich). One ml of 1% Hyamine® and 9 ml of nematode suspension were added to a 50 ml graduated cylinder. The cylinder was sealed with Parafilm and inverted 3 times. When the nematodes had settled (no more than 15 min), the cylinder was opened in a sterile bench environment (by use of a Bunsen burner) and the excess liquid was poured off. The nematodes were then washed by sedimentation with sterile tap water at least 4 times and then used. Alternatively, these surface sterilised juveniles were stored in sterile plastic tubs at the appropriate temperature (Table 2.1) for 2-3 days.

2.3.2. Extraction of *Galleria mellonella* haemolymph

Galleria mellonella late instar larvae were surface sterilised with 70% (v/v) alcohol before they were used for the collection of haemolymph. While holding the larva taut between the thumb and index finger, it was pierced with a 25 g needle (25 Gauge, 0.5 mm diameter, 16 mm length) on the ventral side just below the last 2 prolegs. Slight pressure was applied so that a haemolymph drop formed but no other contents (intestine or fat body) burst out. The haemolymph from 8-10 larvae was collected in a 1.5 ml sterilised eppendorf and was used straight away before melanisation occurred. The used larvae were submerged in 70% (v/v) alcohol to ensure quick death.

2.3.3. Construction of hanging haemolymph drops

On the lid of a 3.5 cm Petri dish, 3 drops of about 25 µl haemolymph each were pipetted using a P200 Gilson pipette with a cut off tip. The lid was then inverted and placed on top of the base that was filled with ± 0.5 ml distilled water.

2.3.4. Inserting a sterile IJ into a drop

A drop of about 40 µl surface sterilised IJ-suspension was used to pick individual IJs. Using a mounted platinum wire (0.10 or 0.20 mm diameter) with a hook, and observing with a dissecting microscope, 1 infective juvenile was lifted out of the drop and inserted individually in a drop of haemolymph. Instead of using a mounted platinum wire, a microcapillary tube (pulled in a flame until the desired width was achieved) attached to an aspirator was also sometimes used to pick up a single infective juvenile in a minimum of water (≤5 µl) and transfer it to a haemolymph drop. The lid with the infected haemolymph drops was then inverted and placed back on the water containing dish. This 3.5 cm Petri dish was placed in a larger Petri dish that was sealed with Parafilm and incubated at the species appropriate temperature (Table 2.1).

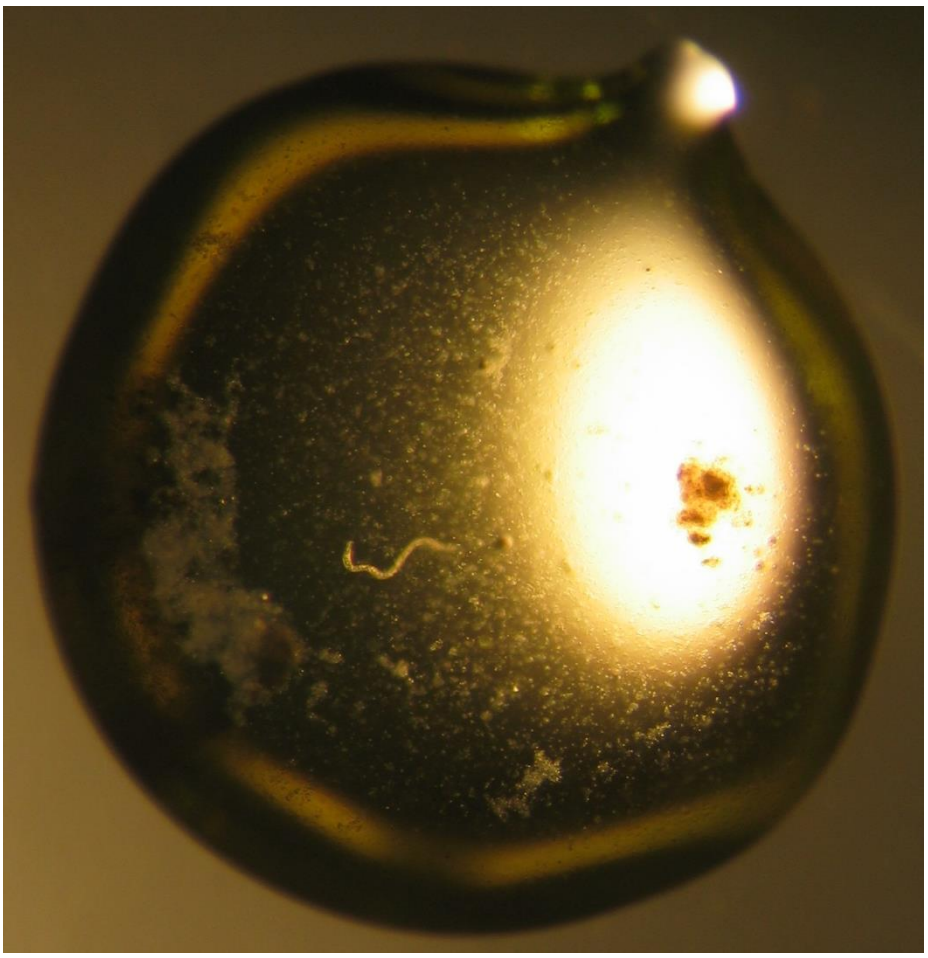

Figure 2.1 Infective juvenile of *Steinernema longicaudum* in a hanging haemolymph drop.

2.3.5. Incubation and identification of the sex of adult worms

Depending on the species, *Steinernema* nematodes incubated at the species appropriate temperature, had developed enough in 2-5 days to identify the sex of the worm. Males were identified by the presence of spicules in the tail tip. Females were identified by the lack of spicules, their larger size and the presence of a vulva in the middle of the body.

Unless the nature of the experiment did not allow it (e.g. previous fighting experience), *Steinernema longicaudum* males were put together in a drop for fighting when both had developed in a haemolymph drop for 3-5 days. Experiments by Lemma Ebssa (unpublished results) had shown that drops with 3-5 day old, same-aged males displayed more mortality 24 and 48 h after the males had been put together than older males (ages 6-9 days).

2.4. Procedures used in experiments

2.4.1. Handling nematodes in a drop

Both juveniles and adult nematodes in a drop were manipulated with a platinum wire of 0.10 mm or of 0.20 mm diameter mounted on a glass Pasteur pipette. The platinum wire was sterilised by passing it through a high temperature flame.

When males or objects were placed together in a drop, 1 of the males was always the resident, unless it is specified otherwise. The resident male was lifted up and placed back in its drop at the beginning of the experiment, after which the intruding male or object was added to this drop.

2.4.2. Observation of nematode behaviour in a hanging haemolymph drop

After addition of the treatment (nematode or object), the timer was put to zero and observation of the first 15 min started when the drop was then placed under a dissecting microscope. When possible, observations were recorded on VHS video tape. For each attack, the following specifics were recorded when possible: start time of the attack in relation to the start of the specific observation period, identity of the attacker and of the victim, position of the attack on the body of victim, duration of the attack, intensity of the attack (did the attacking male perform squeezing behaviour, how much of the attackers body was used in the wrap around the victim), immediately visible reactions to the attack. To prevent dehydration of the drop during the observations the lid with the drop that was being observed was placed in a 9 cm Petri dish along with two 3.5 cm Petri dishes that were half filled with distilled water. When necessary, a small drop of sterile Ringer's saline (full strength, made from tablets; Oxoid) was added to rehydrate the drop.

Verification of paralysis or death consisted of prodding an immobile nematode gently with the tip of the platinum wire. When no reaction to prodding was observed, the nematode was lifted up and immediately placed back into the drop. When this still yielded no reaction the nematode was considered not capable of moving or completely paralysed. In case the nematode was moving, but certain parts were not moving normally, the nematode was scored as suffering impeded movement. A nematode was considered dead when absolute immobility

was accompanied by tissue disintegration, probably the result of naturally occurring bacterial activity.

2.4.3. Construction of an agarose pad

Using a Pasteur pipette, a drop of melted 2% (w/v) agarose mix was put on a microscope slide. A 3.2 cm round cover slip was gently placed onto the drop which flattened under the weight of the cover slip. The agarose was left to set for a couple of minutes taking care not to let it dry. When set, the cover slip was slid off making sure the agarose stuck to the cover slip and not to the microscope slide. The cover slip with agarose pad was left on the bench overnight to dry and then stored in the original cover slip container.

2.4.4. Artificial culture medium

Some experiments (e.g. injection of fighting medium) needed a medium other than haemolymph. Based on Aguillera and Smart (1993) and Stoll (1953) a liquid medium with Brain Heart Infusion Broth as the main component was used.

Brain heart infusion broth (16.65 g), liver concentrate (1.85 g), peptone (0.185 g) and dextrose (1.25 g) were added to 500 ml distilled water and autoclaved for 20 min at 121 °C. After allowing the broth to cool to about 50 °C in the laminar flow, filter sterilised cholesterol (0.01 g) was added under sterile conditions.

2.4.5. Cultivation of axenic nematodes

Xenorhabdus-free *S. longicaudum* nematodes were cultivated based on a protocol for aXenic *S. carpocapsae* (Mulroy-Hehir, 2008).

Gravid 1st generation females were harvested from *in vivo* culturing in *Galleria mellonella* larvae, washed and chopped up in sterile M9 buffer (see below). The resulting egg-M9 mixture was filtered through a 70 µm cell filter (Falcon) and centrifuged (2 min at 6000xg). The supernatant was removed using a Pasteur pipette and replaced with sterile M9. The eggs were resuspended using a vortex mixer, the eggs were washed by repeating this process several times until the supernatant was clear. The eggs were then sterilised with NaOCl sterilisation solution (1.5 ml 4 M NaOH, 500 µl NaOCl and 10 ml distilled water, strictly 4 min). Working in the laminar flow from then onwards, the eggs were washed once more with M9. The eggs

were transferred to 24 well plates in glucose-M9 solution (M9 supplemented with 1% (w/v) glucose) for hatching. The addition of a drop of nutrient broth to 2-6 wells allowed checking for contamination. After 2 days in the *27* °C incubator, the plates were checked daily for signs of hatching. Only plates that showed no bacterial growth in the nutrient broth enriched wells were used to collect the 1st stage juveniles which were washed by centrifuging at 1.2 rpm (this was the lowest possible) for 2 min. The juveniles were then added to autoclave-sterilised chopped up mice liver on modified YPC agar plates (see below) and incubated at 27 °C. These nematodes were left to develop and reproduce and eventually gave rise to infective juveniles.

Xenorhabdus free infective juveniles were also always surface sterilised before use as described in section 2.3.1.

Mice livers were obtained from the Epithelial Immunobiology and Cellular Immunology Laboratory at the National University of Ireland, Maynooth.

Growth, media and Buffers

Modified YPC agar

Yeast extract (1 g), soy peptone (5 g, enzymatic digest), liver digest (3 g) and agar (12.5 g) were added to 500 ml distilled water and autoclaved for 20 min at 121 °C. After allowing the agar to cool to about 50 °C in the laminar flow, filter sterilised cholesterol (0.1 g) was added under sterile conditions. Also under sterile conditions, about 30 ml of this agar was then poured into 9 cm Petri dishes. The plates were allowed to set and then stored at 4 °C.

M9 buffer (Kaya & Stock, 1997)

KH_2PO_4 (3 g), Na_2HPO_4 (6 g), NaCl (5 g), $MgSO_4.7H_2O$ (1 M) (1 ml) were added to 1000 ml distilled H2O and autoclaved for 20 min at 121 °C. The solution was allowed to cool and was stored at room temperature.

2.5. Microscopy & photography

2.5.1. Routine microscopy and photography

Images were captured using a Canon PowerShot S45 mounted on a Nikon Optiphot 2 SMZ-U compound microscope (objectives x4, x10, x40 and x100 with oil immersion; eyepiece 10x) or on a LEICA MZ9.5 dissecting microscope with cold (fibre optic) light source. Images were taken through the ocular tube, but without the ocular lens. The sizes of blowups comparable to the main image were noted. For images that needed some retouching like modifications to the brightness or cropping, Canon Utilities ZoomBrowser EX 6.3 version 6.3.0.7 was used.

Specifications of the images acquired with the Canon PowerShot S45: Dimensions: 2272 x 1704 pixels; Horizontal and vertical resolution: 180dpi; Bit depth: 24; Resolution unit: 2; Colour representation: sRGB; Compressed bits/pixel: 3; Metering mode: Pattern; Digital zoom 1; EXIF version 0220.

2.5.2. Micro-injection microscope setup

As shown in Figure 2.2, a Wild Heerbrugg M5 dissecting microscope was used for the general manipulations that did not need the use of the micro-injector. A Zeiss Axiovert 135 microscope (Figure 2.2) with gliding stage had two micromanipulators attached to it: a 3 axis coarse Narashige positioner/micromanipulator was mounted on the microscope stage and a Narashige MO-202 joystick hydraulic micromanipulator for fine positioning was beside the microscope. The pressure regulator was attached to a nitrogen gas tank regulated by a solenoid valve timer on the bench. The microinjection needle (Eppendorf Femtotip II: catalogue No 5242957000= sterile glass injection capillary, 0.5 µm inner and 0.7 µm outer diameter) was backfilled using a freshly pulled micro-capillary pipette. The needle was screwed into the capillary holder and its tip was broken off by moving it against debris on the agarose. No injection oils were used and when buffer was added, M9 was used. Injections and stabs were aimed at the middle of body of the worm, more precisely at the uterus.

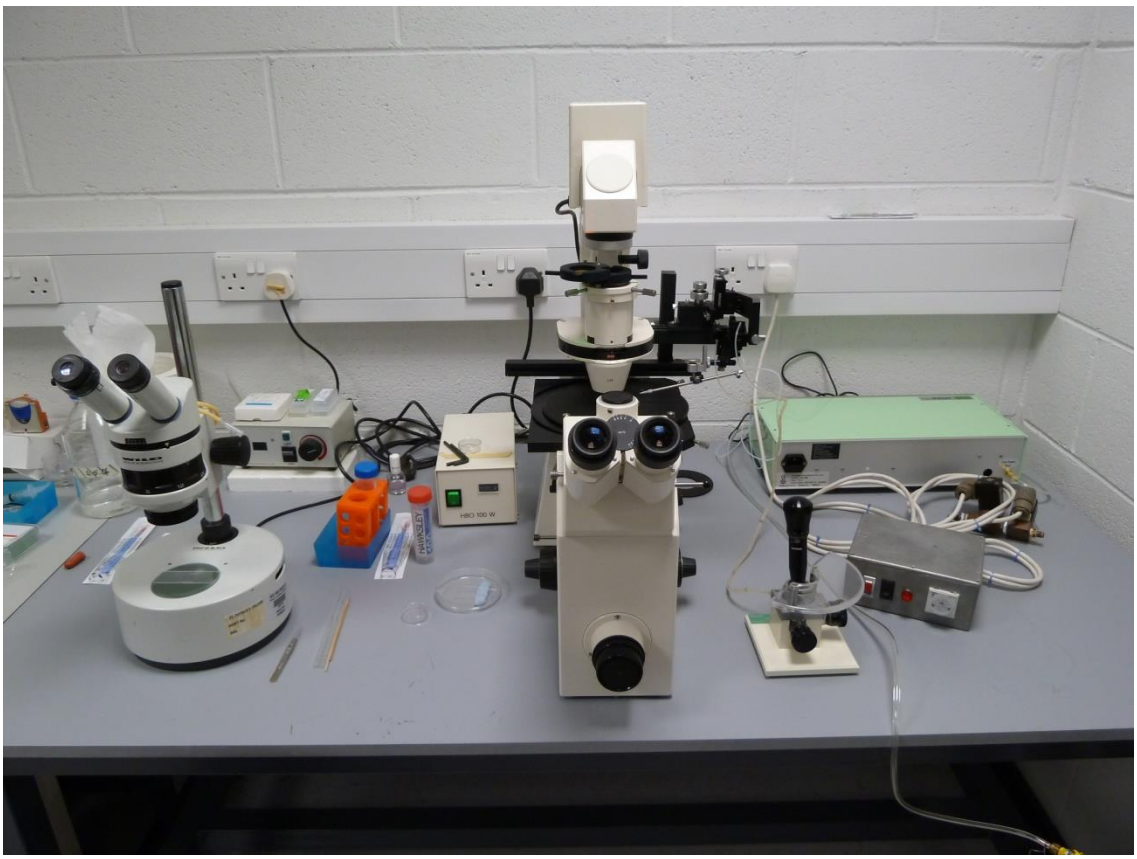

Figure 2.2 The micro-injection suite. On the left is a Wild Heerbrugg dissecting microscope and on the right a Zeiss Axiovert 135 inverted microscope with gliding stage. A Narashige micromanipulator is mounted on the microscope stage of the Zeiss Axiovert 135 inverted microscope for course manipulation and a Narashige MO-202 joystick hydraulic micromanipulator is also attached for the finer positioning of the needle.

2.6. Statistics

Statistical analysis was performed using Minitab Release 15 for Windows (Minitab Inc.). The normality of data was checked using the Kolmogorov-Smirnov or Ryan-Joiner test. In case the data were not normal, statistical analyses using ANOVA were done on the square root transformed data. In graphs and tables, the untransformed data are shown. If data could not be transformed, Kruskal-Wallis was used as non-parametric alternative.

A large amount of data was binomial and was thus analysed using Chi-square tests in Minitab. When multiple comparisons between treatments were necessary, α adjustment according to Bonferroni-Holm step down was used (Hochberg, 1988).

When the expected values were too low for reliable 2 by 2 Chi-square tests, a Fisher's exact test was used.

3. Fighting in *S. longicaudum*

3.1. Introduction

Steinernema longicaudum strain CB2B is the first nematode species in which intraspecific fighting behaviour was observed. Dr Kathryn O'Callaghan came upon this behaviour when studying the reproductive traits of 2 closely related species: *S. hermaphroditum* and *S. longicaudum (O'Callaghan, 2006)*. Since this discovery, several *Steinernema* species were examined for fighting behaviour (see Chapter 4). The speed in which fighting can be observed and in which the effects of fighting are visible combined with the relative ease to culture and rear *S. longicaudum* make this an ideal basic study species. To define the different aspects of fighting behaviour and to examine internal and external influences on aggression and fighting in Steinernematidae, the research was thus focussed on *S. longicaudum* strain CB2B.

3.2. Objectives

In this chapter, the fighting behaviour of *Steinernema longicaudum* is studied in detail. The objectives of each section are specified below and the specific hypotheses can be found at the end of the introduction of each section.

In 3.3, fighting between 2 male *Steinernema longicaudum* is described and analysed to find indications of assessment or defensive behaviours. Immediate and long term consequences of losing a fight (paralysis, death or loss of reproductive potential) are also considered in 3.3.

Residency influences both an individual's resource holding potential and its perception of the value of the contested resource (Buena & Walker, 2008;Enquist & Leimar, 1987;Haley, 1994;Kasumovic *et al*, 2011;Rillich *et al*, 2011;Takeuchi & Honda, 2009). In order to distinguish between the resident and the intruder and due to the lack of appropriate markers, one of the 2 males was matured, while the other was kept immature (Figure 3.2). Therefore, in 3.4 the effects of both residency and maturity on the fighting behaviour of 2 males are examined. First, effects of maturity on fighting behaviour are discussed (3.4.3.1 & 3.4.3.2), only then the influence of residency can be assessed. The effect of the presence and varying value of the contested resource (a conspecific female) are investigated in 3.5.

In 3.6, males are presented with different male-sized opponents so that the characteristics that elicit fighting behaviour can be studied.

It seemed in preliminary observations that males that were constantly forced to be in close proximity to each other, were more inclined to fight. This is quantified in 3.7.

Previous fighting experience influences the perception of a male's own RHP and might make it more capable of assessing its opponent's RHP (Elias *et al*, 2008;Hsu *et al*, 2006;Rutte *et al*, 2006). The effect on subsequent fighting of having already won a fight is examined in 3.8.

In 3.9 the influence of relatedness on *Steinernema* male-male fighting behaviour is explored in both the founder generation and in subsequent generations (all other studies were performed on first generation males), and differences between the generations in tendency to fight are quantified.

3.3. "Normal" fighting in *S. longicaudum*

3.3.1. Introduction

Fighting probably evolved on several occasions and independently in different animal groups (Eibl-Eibesfeldt, 1977). Fatal fighting is however fairly rare and most animal species tend to avoid very costly aggressive contests by assessing the fighting abilities of the opponent and/or itself. This often involves ritualised displays and might escalate when the contestants feel equally potent of winning a fight.

For example, the ant *Cataglyphis niger* settles intraspecific contests (Nowbahari *et al*, 1999) using the following agonistic behaviours: escape, defensive immobility (i.e., remaining motionless in the nymphal position), wide opening of the mandibles (threat), biting and venom spraying (the gaster is flexed forward and venom is sprayed through its acidopore). Size is an important determinant of the outcome of a fight (Nowbahari *et al*, 1999) and depending on its size, an ant will first assess its opponent's size and will then respond by escaping or escalating the aggressive encounter (Nowbahari *et al*, 1999).

Other examples are Varanid (monitor lizard) contests that are made up of 5 distinct phases; each characterised by rituals performed in a certain sequence and with new behaviours emerging as the combat escalates (Murphy and Mitchell, 1974; Carpenter et al., 1976; Vogel, 1979; Auffenberg, 1988, 1994; Thompson et al., 1992; Horn, 1994; Horn et al., 1994). The first phase is called "display" in which the rivals engage in series of head bobs/jerks and exhibit intense tongue flicking (Hurd, 2004). The second phase, "encompassing" is characterised by the side-by-side orientation of the combatants. Lateral display, intense head-jerking and arching-of the-back are often performed. "Clinch" is the third phase in which the contestants rise up and embrace each other, the bipedal stancebrachial embrace (Murphy & Lyndon, 1974). The "catch" phase sees the rivals twisting and tilting each other around in wrestling bouts. The subordinate male recognizes its defeat and is mounted (pseudocopulation) by the dominant, victorious male in the "suppressive" phase. Interspecific differences of fighting behaviour have been described for varanids: not all of these phases or behaviours have been recognised in the 19 observed varanid species/subspecies (Earley *et al*, 2002). Similar escalating phases in fighting have been described in invertebrates such as spiders (deCarvalho *et al*, 2004;Elias *et al*, 2008).

This first section aims to describe all behavioural aspects of intraspecific male-male fighting of *Steinernema longicaudum* CB2B and to identify any assessment behaviour, escalation and/or ritualised aggressive behaviours. The second and third sections quantify the outcomes of fights in terms of paralysis, death or reduced reproductive potential.

Hypotheses

- Male-male fighting in *Steinernema longicaudum* is composed of several distinguishable behaviours beginning with assessment of self and/or the opponent and gradual escalation of fighting.
- Paralysis and death increase over time.
- Male-male fighting reduces the fitness of the looser and yields the winner an advantage in attaining a higher fitness.

3.3.2. Materials and methods

3.3.2.1. Description of fighting

Over all the fighting observations in this study, the different components of fighting were described. This was thus not based on a single observation, but on many, of which the recorded fight on the attached CD is a prime example.

3.3.2.2. Paralysis over time

Three days after incubation of infective juveniles in haemolymph drops at 27 °C, a male was taken out of its drop and placed together with another male of the same age. Every hour for up to 8 h after the males were placed together in a drop, they were checked for paralysis and death. They were then checked again for paralysis about 24 and 48 h after the males were paired.

3.3.3. Results

3.3.3.1. Description of typical fighting

Some time after two males were put together in a drop, a fight would usually occur: one of the males wrapped its tail around the body of its victim. A “wrap” could be loose, where the attacker did not keep its victim in a hold and the victim could move out of the coil made by the body of the attacker, but a fight was defined as a “wrap” where the attacker took a strong hold of its victim and wrapped itself around the victim. Initially, the victim reacted by vigorously moving its whole body, but as the fight continued the movements of the victim diminished, until the victim did not move anymore. A male could be attacked at any place on its body. Depending on where the victim was grabbed by the attacker, the victim might be able to use its tail to counterattack the attacker (i.e. wrap around some part of its body) or to scrape its tail along the attacker’s body where it had wrapped the victim. Subjectively, this scraping looked like the victim was trying to pry off its attacker, although the victim almost never freed itself by doing this. When a male was grabbed close to its tail, it could no longer coil its tail and a counter-attack was then not possible. But when a male was grabbed in the head region, it was not uncommon for the victim to be able to counterattack the attacker. After a counterattack both males could end up with signs of paralysis, indicating that a counterattack could be efficient. From the time a fight had advanced a couple of seconds, the attacking male might lift up the anterior half of its body, and “rock” this back and forth, thus giving a subjective appearance of squeezing to the behaviour. Most of the time, the victim was already moving its body less vigorously when this behaviour occurred. A fight was over when the attacking male released its victim. The victim might be temporarily or permanently paralysed and was likely to die. Some fights resulted in visible physical damage to the victim, this is described in more detail in Chapter 5. For the victor, new fights were possible straight away.

The beginning of a fight was not preceded by any obvious changes in the behaviour of either worm that would indicate assessment or even recognition of the presence of another male. Also, there were no obvious differences in behaviour between single males and paired males except for the wrapping and fighting described above.

The attached CD contains a recording of a straightforward fight with immediate paralysis that occurred between 2 *S. longicaudum* males (*S. longicaudum* Fight.mpg).

3.3.3.2. Paralysis over time

When 2 males were reared singly in haemolymph drops and put together when they were 3-4 days old, paralysis as the outcome of a fight was highly likely to occur. The occurrence of paralysis and death in the drops with 2 males was significantly different to the 1-male controls at all time points (X^2, 1 d.f., $p < 0.05$). When given enough time (48 h), there was at least 1 male paralysed in 90 % of the drops with 2 males put together (Figure 3.1). Fighting was seen in the first hour, and resulted in paralysis of a male in nearly 20 % of the observed drops. The effects of a fight (paralysis and death) became clearer as time went by (Figure 3.1). In addition, a total of 57 single males were observed, but none of the single males showed paralysis or death within the 72 h observation period.

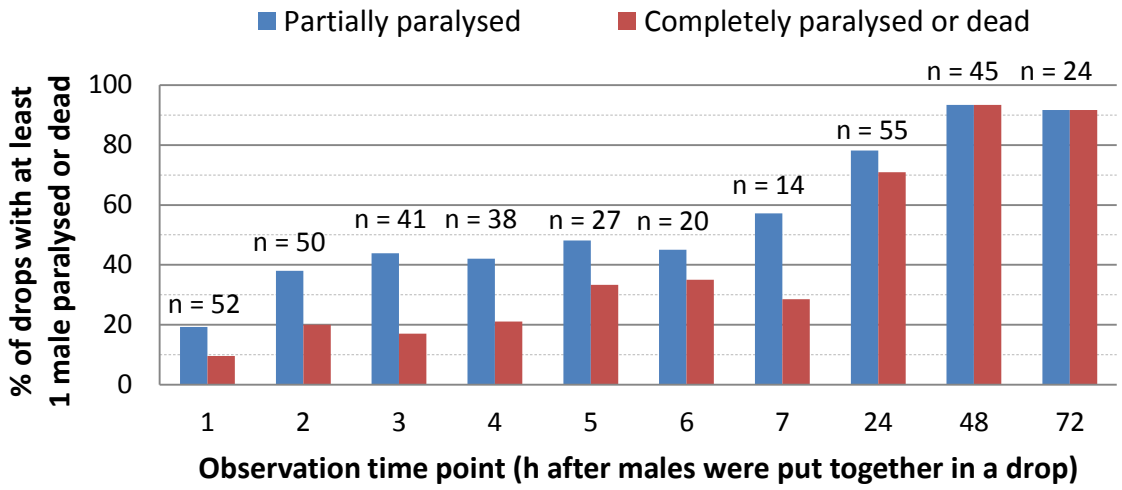

Figure 3.1 In a drop in which 2 adult males were put together at time point 0: paralysis and/or death of at least 1 male over time. The "Partially paralysed" refers to worms that were at least partially paralysed, and includes "completely paralysed and/or dead". Observations were made hourly, for up to 7 h, and at about 24, 48, 72 h after the males were put together in a drop. In none of the 57 drops where only 1 male was put for the same amount of time and for which observations were made at the same time points, paralysis or death was observed. The number above the bar is the number of drops observed at each time point.

3.3.3.3. Effect of paralysis/injury on production of offspring

Victors and surviving victims were each put together with 2 females to test production of progeny. Victors and victims that had not sustained any injury or paralysis from the fights were able to produce progeny (44 out of 44 males tested sired offspring). Surviving victims that had sustained injuries or had been paralysed from the fights were significantly less able to sire offspring: only 6 out of 14 (43 %) produced progeny (Fisher's exact test: $p < 0.001$). Differences in the number of offspring were not estimated.

3.3.4. Summary & conclusions

Under these experimental conditions more than 50 % of paired males engaged in fights resulting in paralyses within 7 h which lead to death in most of the cases. Over time, the number of drops with paralysis and death increased, indicating that the chance of a fight occurring and resulting in paralysis or worse, increased as the males were in each other's company for longer.

Even when a fight did not result in death, an injured or paralysed victim was about 57 % less likely to sire offspring than males that were not affected by a fight or did not fight. Paralysis or injury that does not lead to death thus still potentially bestows the winner of a fight with a reproductive advantage.

Observations of fights did not produce evidence of assessment or ritualised behaviour. Therefore I cannot say that any signalling of Resource Holding Potential (RHP, see section 1.3) or assessment before fighting took place. Defensive techniques, apart from trying to wrap and counterattack the opponent, have not been observed. The lack of observations of these behaviours in this thesis does not imply they don't exist. One reason why these behaviours might not have been observed could be due to the artificial environment of the haemolymph drop. An insect cadaver offers a lot more possibilities to interact and react and whereas a drop offers almost no possibilities for hiding, the insect cadaver offers a larger space to flee in and many more opportunities for hiding. The amount of coiling in on itself, tail-flicking and scratching itself with the spicule are behaviours that were also observed in single males. These behaviours might be used in assessment, signalling and/or defensive behaviours. An elaborate study, quantifying these behaviours in multiple or coupled male drops and in single male drops might show differences. Analysis of the sinusoidal movements of single and multiple or coupled males might also reveal behavioural differences, however, due to the nature of these movements differences are hard to perceive. The use of a system allowing automated recording and analysis of nematode movement (Cronin *et al*, 2005) may allow analysis of assessment and fighting behaviour on this level. Since *Steinernema* do not have eyes, any change in movement of an opponent would be sensed either through direct contact or through vibrations of the medium. Nematodes are however well equipped with chemosensory organs and use pheromones for communication (Chasnov *et al*, 2007;Golden & Riddle, 1982;Golden & Riddle, 1984;Neves *et al*, 1998;Reyes-Vidal & de la Torre, 2009). The possible involvement of pheromonal signalling in assessment and fighting was not investigated.

Assessment, signalling, displaying or ritualised behaviour don't need to be part of a fighting behaviour repertoire (Crespi, 1988;Enquist & Leimar, 1990). All of these evasive behaviours still confer costs on the contestants (Just & Morris, 2003). Displaying might make the rivals more vulnerable to predation (Brick, 1998). However, because adult nematodes are in the "protective" environment inside an insect cadaver, it doesn't seem likely that they are under a high predation pressure. Signalling and assessment are often seen as the evolutionary preferred strategies to reduce energy costs by avoiding energetically more costly fighting behaviour (Marden & Rollins, 1994). These evasive behaviours however still demand energy and thus, maybe, in fighting *Steinernema* species, the cost of fighting does not outweigh these behaviours (Crespi, 1988;Enquist & Leimar, 1990). Fight avoiding strategies like mutual assessment and aggressive rituals also bear time-costs: time spent on assessing or displaying is time not used for feeding, finding a mate or mating (e.g. sneaky males do not invest in displays/territorial behaviour and can steel mates from a territorial male (Arak, 1984;Byrne, 2004;Clutton-Brock & Albon, 1979;Taborsky, 1997)). As mathematically demonstrated by Enquist and Leimar (1990), the balance between the value of the future and the value of the contested resource will determine the occurrence of fatal fighting and will also influence the evolutionary development of fatal or costly fighting avoidance behaviours (Krebs, 1993). In situations where the value of the battle outweighs the value of the future without the resource, contestants will have little or nothing to lose and can be expected to ignore asymmetries and pursue ownership of the resource at all costs, this is also called the "Desperado effect" (Cronin & Monnin, 2010).

3.4. Maturity and residency of the male

3.4.1. Introduction

Steinernema longicaudum CB2B males only mature sexually when they are in the presence of a female (Ebssa et al., 2008). It takes 6 - 48 h for males to mature. In a mature male the reproductive tract is more developed and macrosperm can be observed in the seminal vesicle (Figure 3.2 and Figure 3.3). Preliminary data indicate that when mature males were no longer in the presence of a female for some days, the macrosperm disappeared (Shortall, 2009). Because of the possibility to observe macrosperm in live males and after dissection (Figure 3.3), maturity is a way to identify individual males. Other methods such as vital staining and feeding worms fluorescent latex microspheres proved unreliable (Ruth Brennan, NUIM, unpublished).

Fighting is a very costly behaviour (Briffa & Elwood, 2005): all the energy and time spent on fighting is not spent on reproductive behaviour (e.g. looking for mates or mating) or feeding; healing after injury needs lots of energy and when dead, reproduction is no longer possible. Therefore, the fought-over resource needs to be high in quality to make up for all those possible costs (Enquist & Leimar, 1987;Enquist & Leimar, 1990). A male residing in a territory, will know this territory and the values of the resources in it better than an intruder. It can then be expected that a resident male will be more driven to fight and win in order to preserve its rights over the territory and thus the contested resource(s) (Bentley *et al*, 2009;Haley, 1994;Krebs, 1993).

To be able to distinguish between the resident male and the intruder, mature and immature male nematodes were used because the differences in the gonads were easily observed (Figure 3.2). When these experiments were started, more fights seemed to be won by mature males. In order to research residency, the effect of maturity on fighting thus needed to be investigated first. Indeed, maturity can change an individual's willingness to fight (Fromhage & Schneider, 2005;Killian & Allen, 2008). Subordinate *Acheta domesticus* cricket males that were allowed to contact a female (via copulation or only through chemo-tactile cues) showed higher levels of aggression towards (dominant) males (Killian & Allen, 2008). Previous studies in which increased aggressiveness after mating was observed, explained this as post-copulatory mate guarding. However, the males in Killian & Allen's experiment also showed elevated aggressiveness after only chemo-tactile contact with a female. It is then

possible that the confirmation of the presence of a female elevated the value of the resource was what urged them to escalation of their fighting behaviour.

Thus, the objectives of this section are to explore the effects of both residency and maturity on fighting in *S. longicaudum*.

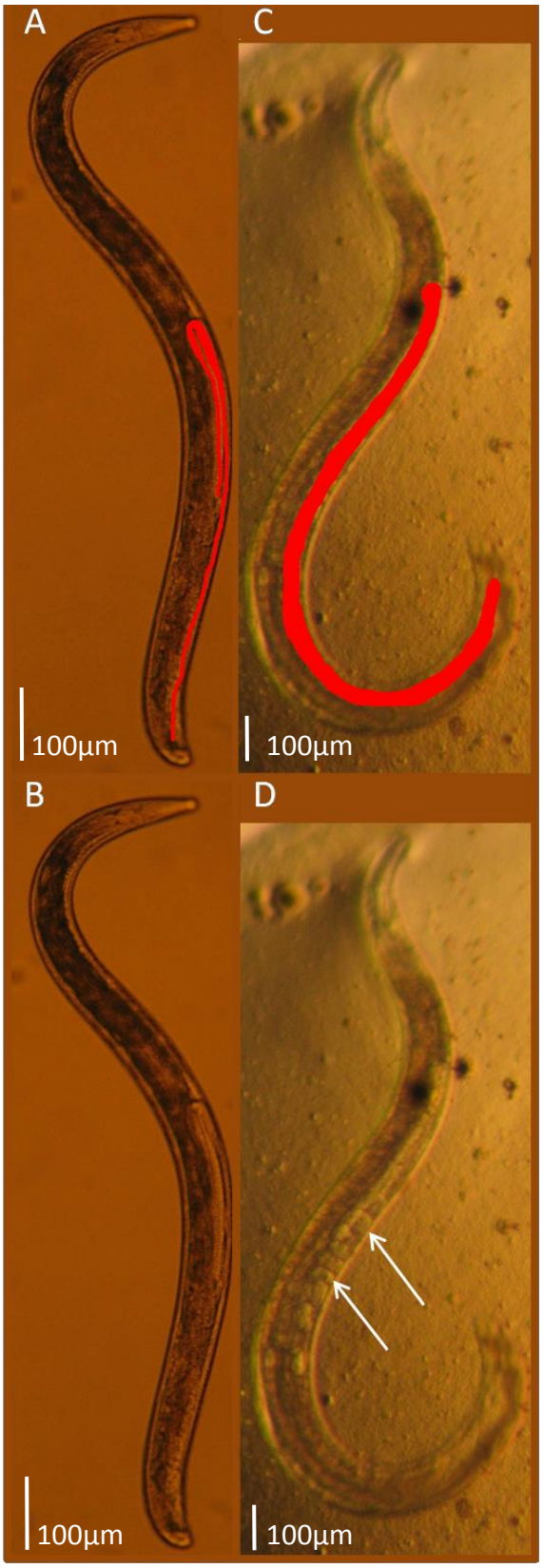

Figure 3.2 The reproductive tract of *Steinernema* males is a simple tubular structure. A & B: immature male, note the reflexed testis. C & D: mature male. A & C: shown in red is the location of the gonad. D: the white arrows indicate macrosperm. Identification of live mature and immature males is possible due to differences such as presence of macrosperm in a mature male, and the reproductive tract is a lot narrower in the immature male.

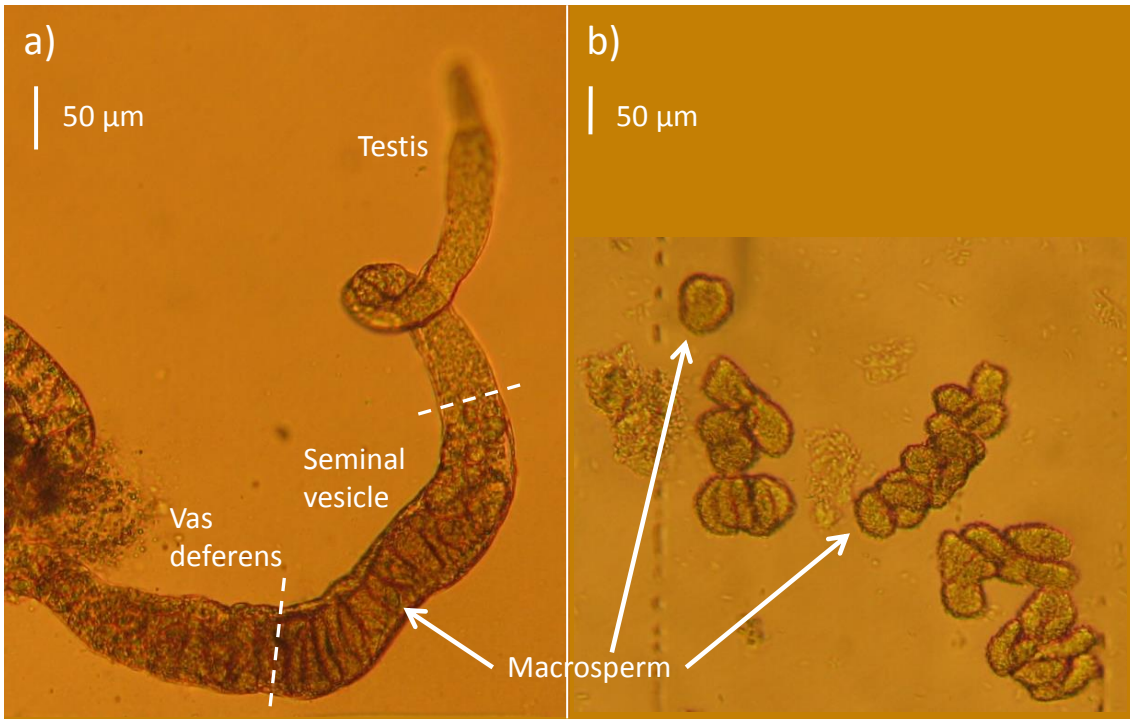

Figure 3.3 Reproductive tract from a dissected mature male. 1) Detail of the male gonad with macrosperm in the seminal vesicle (a dilated sperm storage region continuous with the testis). 2) Detail of macrosperm released after dissection. White arrows indicate macrosperm.

Hypotheses

- Mature males also perform fighting and killing behaviour.
- Mature males do not show different fighting, paralysis or killing rates than immature males.
- Resident males show higher fighting, paralysis and/or killing rates than intruding males.
- There is no interaction between maturity and residency on fighting parameters.

3.4.2. Materials and methods

3.4.2.1. Do mature males kill?

Until now, all experiments on fighting were done using immature worms. In this preliminary experiment I paired mature males to see whether they would also fight.

After 2 days of development at 27 °C, some males were each paired with 2 females to induce sexual maturity, while other males were left alone in their drop. The drops were replaced at 27 °C for 16-20 h, after which the males were assigned to the following treatments:

- Mature male paired with a mature male (4 drops)
- Mature male paired with an immature male (4 drops)
- Single mature male (7 drops)
- Single immature male (12 drops)

Males were only considered mature when they had sired progeny. Any male that failed to sire progeny was excluded from the analysis.

In all these drops, neither male was resident, both were intruders. The males were put together in drops in which a male or a female had developed but had been removed.

About 24 h after the males were put together, the drops were checked for paralysis and death. This set was only used to assess the occurrence of fighting between 2 mature males and 1 mature versus 1 immature male. The identity of the victor in the mixed treatment (mature/immature) was not assessed.

3.4.2.2. Comparison of fighting in 2 mature and 2 immature males

Do mature males fight each other more often or more intensely than 2 immature males?

In this experiment, the behaviour of pairs of mature males was compared to the behaviour of pairs of immature males.

Some individual 2-day old males that developed at 27 °C were each paired with 2 females of the same age to induce sexual maturity, while other 2-day old males that developed at 27 °C were left alone in their drop. The drops were replaced at 27 °C for 16-20 h, after which the males were assigned to the following treatments:

After 24 h, males were paired in the following treatments:

- 2 mature males
- 2 immature males

Immediate observations were normally made for about 30 min during which fighting attempts were recorded. Observations were also made at the end of the observation day, and after 24 h and 48 h, at which point paralysis and death were recorded. Age of males at observations varied, but 2 males in a drop were always of the same age. Females used for the maturing of males were examined 3-6 days after mating and presence of progeny was noted. Males that had been with a female to induce maturity but did not produce any progeny - even when macrosperm may have been observed - were taken out of the dataset.

3.4.2.3. Effect of maturity and residency on fighting

Infective juveniles were reared to adults and were then assigned to mating or no mating as in 3.4.2.1 above. After 24 h the males were put together with another male to give the following treatments:

- mature intruder versus immature resident
- mature resident versus immature intruder
- 2 immature males

Where the resident was a mature male, it had developed and matured in the drop used for fighting: the 2 females were put in its drop for mating and were relocated to its original drops 24 h later. The drops containing females that had been used for mating were examined 3-6 days after mating for scoring the presence of progeny. When none of the females that were paired up with a certain male produced offspring 6 days after mating, this male was discarded from the dataset so that all the mature males in the dataset were males that had produced offspring.

The drops were observed for 30 min immediately after the 2 males were put together. About 4, 24 and 48 h after the males were put together, the drops were checked for paralysis and death. The drops were also checked at the end of the observation day which could range per drop from 5 to 8 h after the males were put together. At each examination, the identity (mature or immature) of the paralysed or dead male was ascertained by noting the presence or absence of macrosperm in the seminal vesicles. When the victim was dead or moribund, the males were put on a slide and examined for the presence of macrosperm under the high power (x40) of a Nikon Optiphot microscope. Pictures were taken using a Canon PowerShot S45 mounted on the microscope.

When possible Chi² tests with df=1 were used. In the cases where expected numbers were lower than 5, a Fisher's exact test was done.

3.4.3. Results

3.4.3.1. Do mature males fight?

This preliminary experiment showed that mature males fight each other (4 out of 4 drops had 1 male dead after 24 h), mature males and immature males fight each other (4 out of 4 drops had 1 male dead after 24 h) and mature single males showed no death after 24 h.

3.4.3.2. Comparison of fighting in 2 mature and 2 immature males

It appears that the **speed** with which 2 mature males paralysed and killed is higher than the speed with which 2 immature males paralysed and killed (Figure 3.4). At the end of the observation day (Figure 3.4-a), there were significantly more drops with a paralysed or killed male when 2 mature males were put together than when 2 immature males were paired ($p = 0.01$ resp. $p = 0.03$, Fisher's exact tests). Similarly, after 24 h (Figure 3.4-b), drops with a dead male were significantly higher in number for drops with 2 mature males (X^2 (1, n = 57) = 5.90, $p = 0.02$), but after 48 h (Figure 3.4-c) this difference was no longer significant ($p = 0.15$, Fisher's exact test).

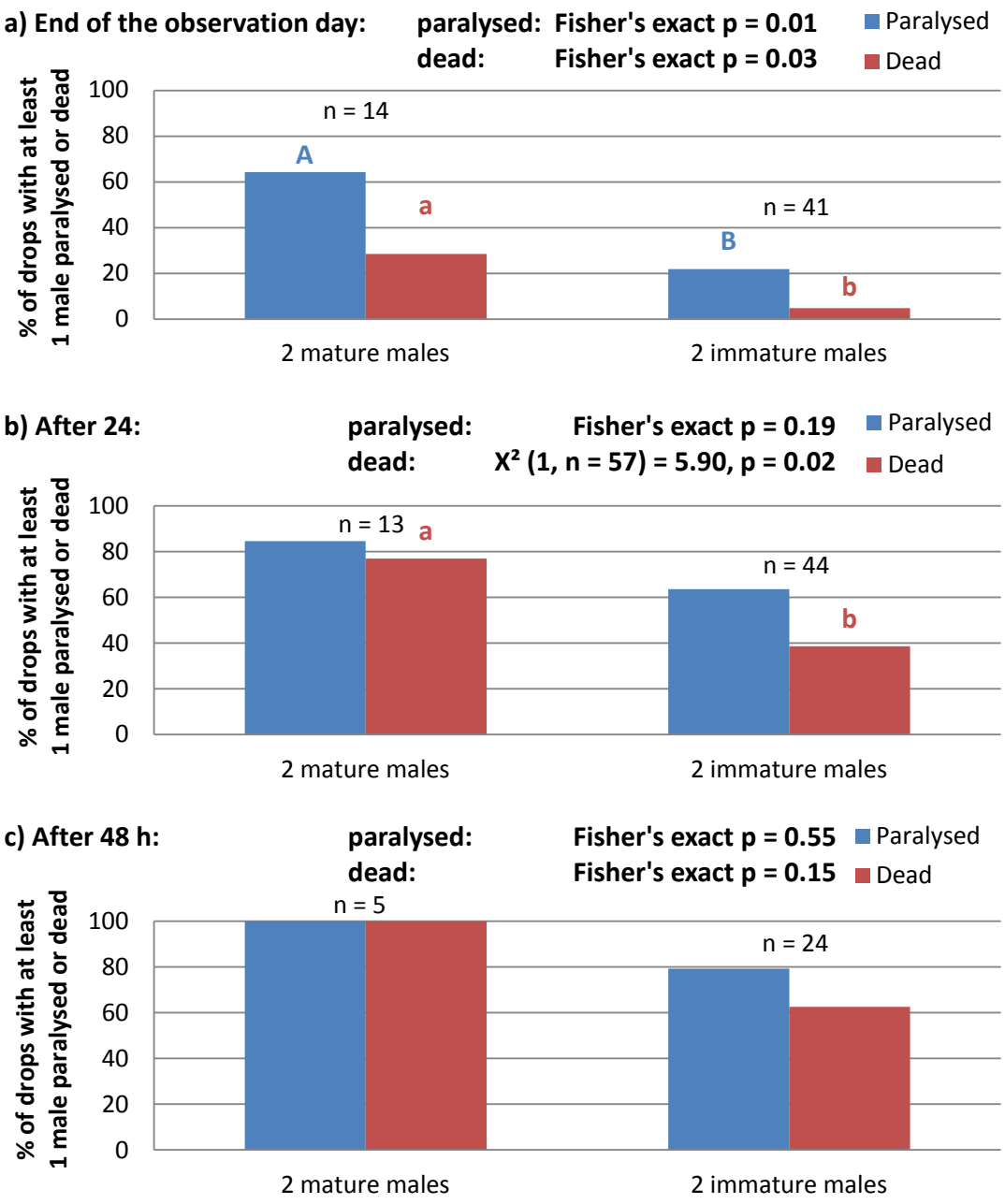

Figure 3.4 **Effect of the maturation status of the males on paralysis and death of 2 males. Observations were made at 3 time points: a) at the end of the observation day, b) about 24 h after the start of the experiment and c) 48 h after the start of the experiment. Within a graph, bars accompanied by no letters are not significantly different, n is the number of drops for that treatment.**

3.4.3.3. Effects of maturity and residency on fighting

I first examine the effect of maturity on fighting, thereby ignoring the residency status of the males (sections 3.4.3.3.1 and 3.4.3.3.1). Pairs of males where one was mature and one was immature are compared to pairs of males where both were immature in (3.4.3.3.1) and to two mature males in (0).

Finally, in 3.4.3.3.3, I include the residency status of the males in the analysis of the results from the present experiment.

3.4.3.3.1. A mature male versus an immature male compared to 2 immature males

We can see from Figure 3.5-a, that pairing an immature and a mature male resulted in 84.2 % of drops with at least 1 male paralysed in the first 4 h, whereas 2 immature males only had 50 % of drops with at least 1 paralysed male (Fisher's exact test: $p = 0.083$). At the end of the observation day, there were significantly more drops with at least 1 paralysed male (X^2 (1, n = 74) = 13.005, $p < 0.001$) when one male was mature (81.1 %) than when both males were immature (38.1 %) (Figure 3.5-b). So, when one of the males had been given the opportunity to mature and mate before being put together with an immature male, **paralysis** (number of drops with at least one male paralysed) tended to occur more (significant at the 10 % level) after 4 h and occurred significantly more (at the 5 % level) at the end of the observation day than when both males were immature. The difference in drops with paralysis was no longer significant after 24 and 48 h (Fisher's exact tests: $p = 0.115$ resp. $p = 0.234$; Figure 3.5-c & -d), more than 50 % of the drops had at least 1 male paralysed in both treatments.

No deaths had occurred 4 h after 2 immature males had been put together (Figure 3.5-a), whereas pairing an immature and a mature male resulted in 31.6 % of drops with at least 1 male dead after the first 4 h (Fisher's exact test: $p = 0.068$). At the end of the observation day, there were significantly more drops with at least 1 male dead (X^2 (1, n = 74) = 7.370, $p = 0.007$) when one male was mature (35.9 %) than when both males were immature (4.8 %) (Figure 3.5-b). And this difference was still significant after 24 and 48 h (X^2 (1, n = 70) = 9.434, $p = 0.002$ resp. $p = 0.026$ (Fisher's exact test)). When one of the males had been given the opportunity to mature and mate before being put together with an immature male, **mortality** (number of drops with at least one male dead) was higher than when both males were immature. This was significant at the 10 % level after 4 h, but was significant at the 5 % level for all 3 observation time points after that.

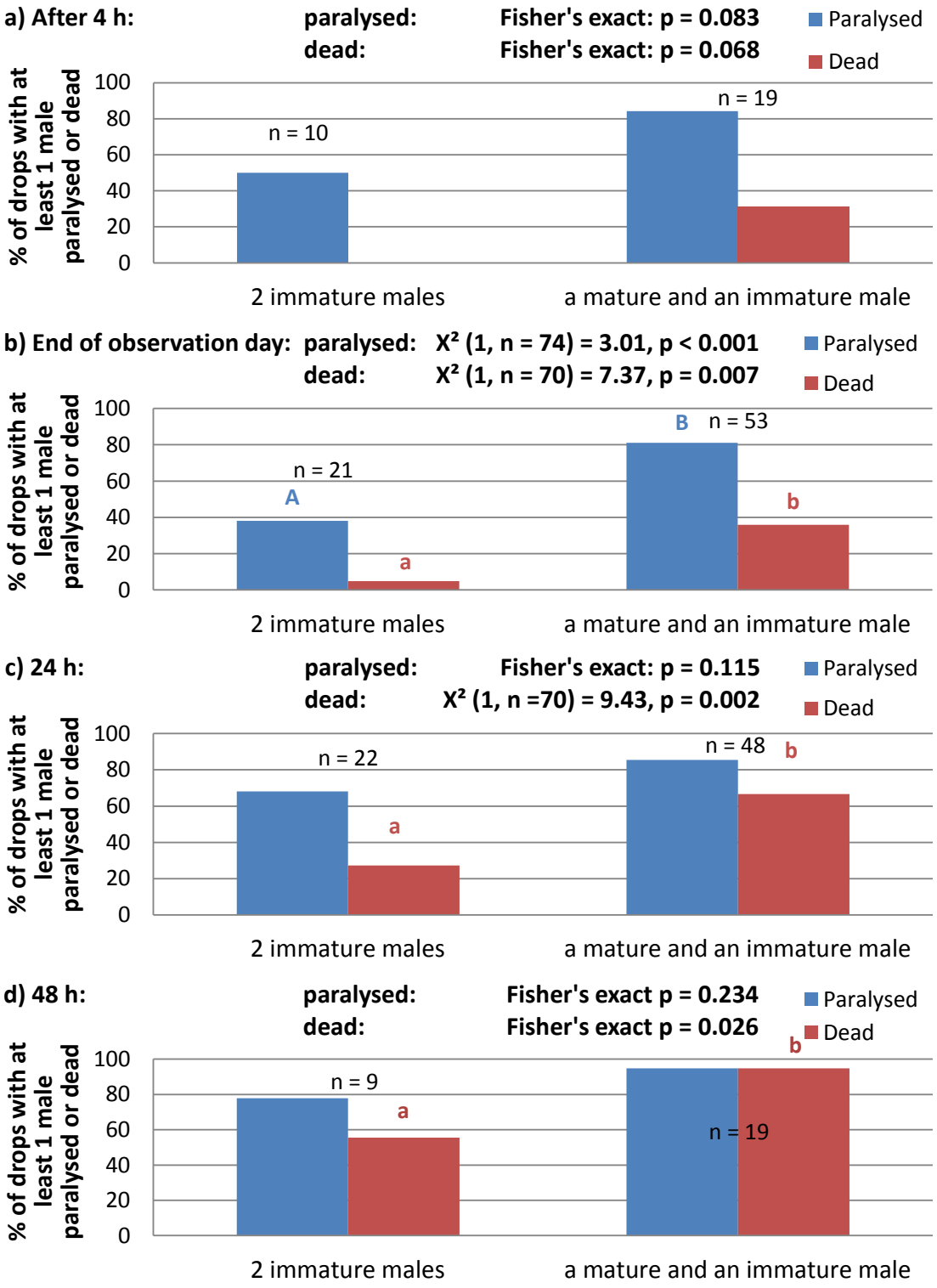

Figure 3.5 **Paralysis and death in drops containing either 2 immature males or an immature and a mature male. Observations were made at 4 time points: a) 4 h after the males were put together in a drop; b) at the end of the observation day; c) about 24 h after the start of the experiment and d) 48 h after the start of the experiment. Within a graph, bars accompanied by no letters are not significantly different.**

3.4.3.3.2. A mature male versus an immature male compared to 2 mature males

Even though 64.3 % of drops with 2 mature males had at least 1 paralysed male at the end of the observation day (Figure 3.6-a) and a mature-immature pair had 84.6 % (Figure 3.6-a), there were no significant differences in the effects of fighting (paralysis or death) between pairs with 1 or 2 mature males (Figure 3.6) at any of the timepoints.

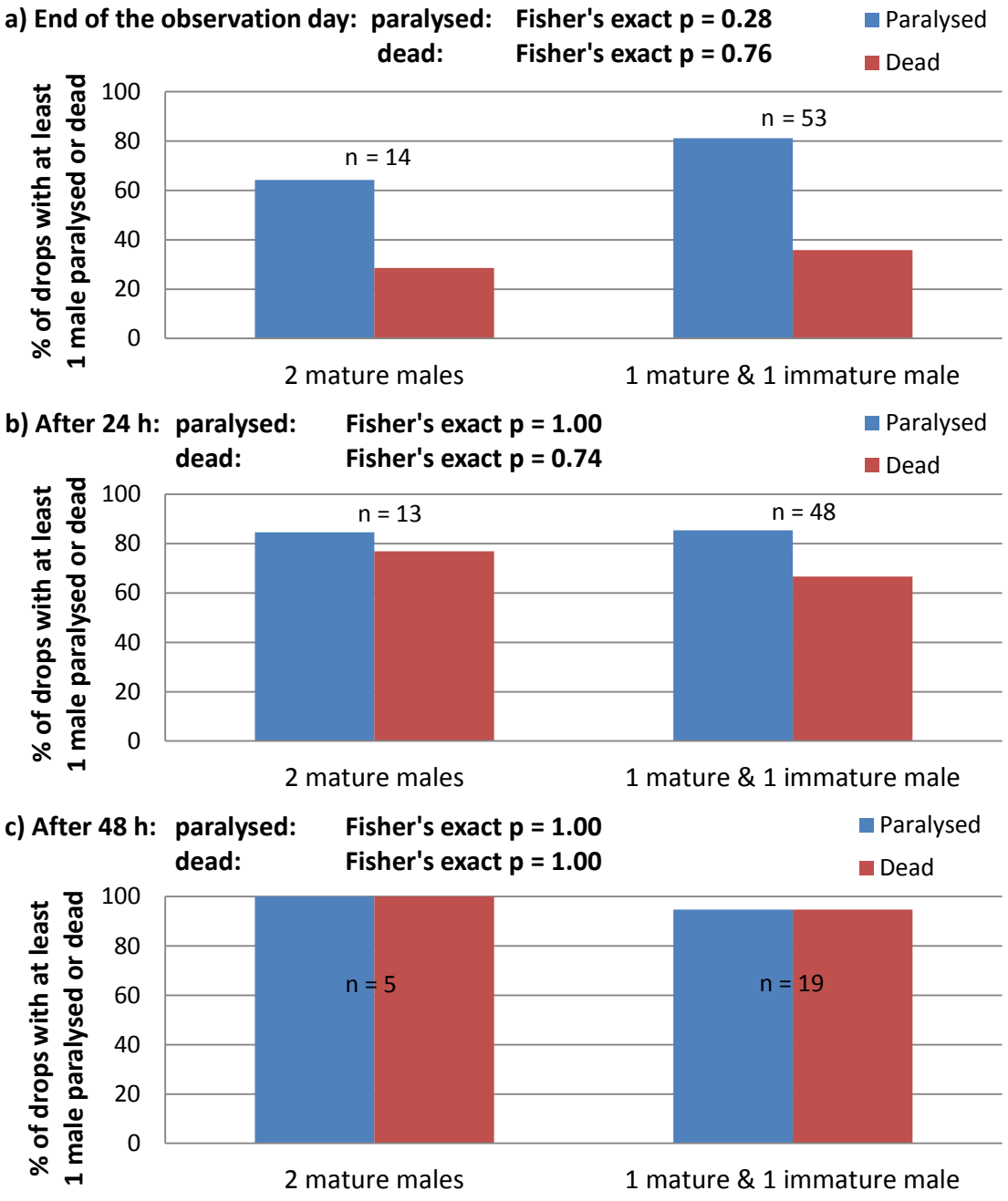

Figure 3.6 Paralysis and death in drops containing either 2 mature males or an immature and a mature male. Observations were made at 3 time points: a) at the end of the observation day; b) about 24 h after the start of the experiment; and c) 48 h after the start of the experiment. Within a graph, bars accompanied by no letters are not significantly different. When possible Chi²-tests with df=1 were used. In the cases where expected numbers were lower than 5, a Fisher's exact test was done.

3.4.3.3.3. Effect of maturity and residency on fighting

Within the 5 min immediately following the introduction of a mature and an immature male in 1 drop, there was no significant difference between the number of drops with fights depending on whether the intruding male was mature or immature (Table 3.1; $p = 0.088$, Fisher's exact test). However, there was a trend ($p < 0.10$) for there to be more fighting when the intruder was mature and the resident immature.

Table 3.1 The combined effect of residency and maturity on the occurrence of fighting in the 5 min immediately following the introduction of 2 males (1 mature, 1 immature) in 1 drop.

Male 1	Male 2	No. of drops without a fight	No. of drops with a fight
Mature intruder	Immature resident	4	5
Mature resident	Immature intruder	0	7
Fisher's exact test: $p = 0.088$			

Subsequent data on paralysis and death were analysed in two ways: mature versus immature (irrespective of residency status, Figure 3.7- left graphs) and resident versus intruder (irrespective of the maturity of each, Figure 3.7- right graphs).

Whether a male was resident or intruding made no significant difference in the number of males paralysed at the different time points (after a) 1st 5 min: $p= 1.00$; b) 4 h: $p = 0.74$; c) at the end of the obs. day: $p = 1.00$; e) after 48 h $p = 1.00$), except for the “after 24 h” time point when more intruding males than resident males were paralysed ($p = 0.033$)(Figure 3.7-d- right graph).

The maturity status of the males had a highly significant effect (after 1st 5 min: $p < 0.01$; all other time points $p < 0.001$) at all the time points tested. N.B. there were more immature males paralysed than mature males (Figure 3.7- left graphs).

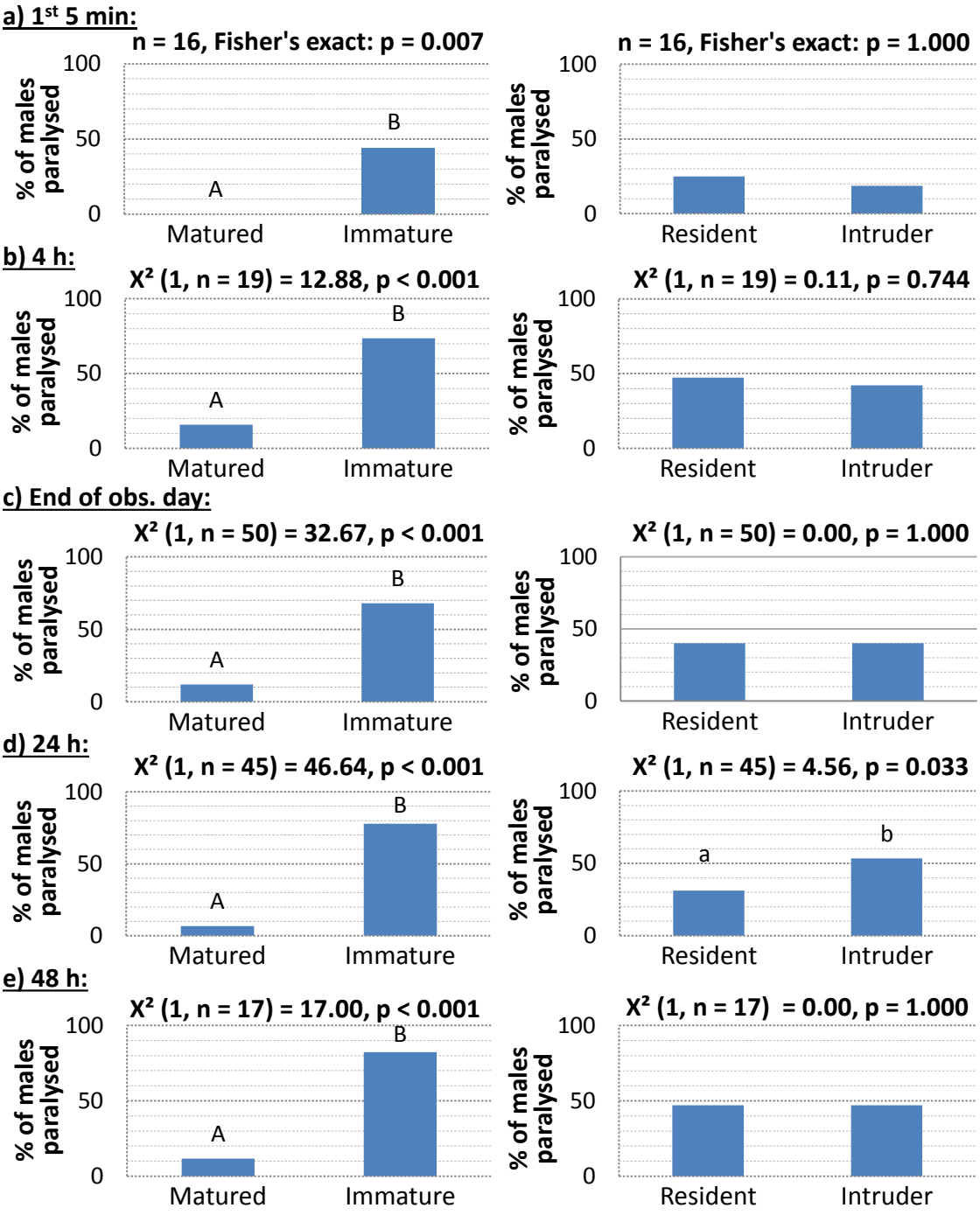

Figure 3.7 **Paralysis and death in males classified by maturity status (left graphs) or by residency status (right graphs). The maturity status of the male made it possible to identify resident and intruding male. Observations were made at 5 time points: a) the first 5 min the males were in a drop together (n=16)r, b) 4 h after the males were put together (n=19), c) at the end of the observation day (n=50), d) about 24 h after the start of the experiment (n=45) and e) 48 h after the start of the experiment (n=17). Within a graph, bars accompanied by no letters are not significantly different. When possible Chi² tests with df=1 were used. In the cases where expected numbers were lower than 5, a Fisher's exact test was done.**

3.4.4. Summary & conclusions

Residency of the male may have had an effect on winning or losing a fight, but the effect of maturity on winning a fight is overwhelming. Whenever at least 1 of the males had had the opportunity to mature, fights, paralyses and death occurred more quickly than when both males were immature. More detailed investigation then showed that a mature male was more likely to paralyse and kill its immature opponent than vice versa, regardless of the residency status of the males.

Having had wrapping experience during mating, mature males might be more confident in their wrapping abilities and/or the wrapping might have trained the muscles used for wrapping and those for protrusion of the spicules. This training would also benefit fighting as this makes use of many of the same movements.

In Chapter 5, the possible involvement of a toxin in fight-induced paralysis is addressed. In case the putative paralysing toxin is a chemical associated with the production of sperm and/or seminal fluid, mature males might physiologically be better prepared for paralysing and killing.

Because mature males have the tendency to be bigger than immature males of the same age (personal observation, not quantified), they may be physically better able to fight. Size is often of importance in determining the contest outcome (Brown *et al*, 2006;Sacchi *et al*, 2009). Moreover, the energy and nutrients these males have put into developing their gonads, and maybe also somatic tissue, would be higher than what the immature males have invested. A higher investment means a higher subjective value of the resource which will result in a more aggressive behaviour. Female *Goniozus legneri* (a parasitoid wasp) owners of contested insect hosts, adjusted their fighting behaviour to the developmental stage of their brood in the host (Bentley *et al*, 2009). The more vulnerable the female owner's offspring still was to conspecific infanticide (from female intruder offspring), the more aggressively the female owner would defend the host. The investment of the female's lifetime reproductive success that it has connected to that particular resource makes it more valuable to her than to an intruder that has not yet invested in that particular insect.

As in Killian's observations on crickets (Killian & Allen, 2008), *Steinernema* males might not have needed actual copulation with the female to gain in aggressiveness. This was not investigated in this thesis, but could easily be done by maturing males through exposing them to females through a semi-permeable barrier (Ebssa *et al*, 2008).

3.5. Effect of presence and reproductive status of a conspecific female on fighting in *S. longicaudum* males

3.5.1. Introduction

In organisms that reproduce sexually, females are normally the physiologically most investing sex (Parker *et al*, 1972). Females produce big, energy rich eggs which are less numerous than the small sperm, which are basically only nuclear genetic material containing gametes, produced by males. Females are then expected to be more careful not to waste these precious eggs, and will normally mate less than males (Clutton-Brock & Parker, 1992). This makes females a limited resource for males (Emlen & Oring, 1977), with varying reproductive values depending on the occurrence of previous mating of the female. Mated females will have fewer unfertilised eggs and will thus produce fewer offspring of the mating male than a mature virgin female (Krebs, 1993). Moreover, in case females mate only once, virgin females become an even scarcer resource. *S. carpocapsae* males are only attracted to virgin conspecific females (Lewis *et al*, 2002) which makes it possible that females might only mate once and which will definitely elevate the competition for virgin females. It is not known whether *S. longicaudum* females mate only once, but if this is the case then there is a higher reproductive value to a male for virgin females.

The presence of a female can increase intrasexual male fighting behaviour (Tachon *et al*, 1999). The reproductive value of the presented female also influences fighting behaviour (Keil & Watson, 2010). In some species (e.g. *Acheta domesticus* crickets) the indication (i.e. chemical cues) that a female is in the vicinity suffices to elevate the fighting behaviour (Buena & Walker, 2008).

Also, since fighting behaviour resembles mating (3.3.3.1), presenting a male with the choice between a male or a female, presents it with a choice between fighting or mating. If fighting occurs under these conditions the possibility that our putative “fighting” is really a case of frustration-mating due to lack of females can be ruled out.

Hypotheses

- Male mating behaviour is dependent on the reproductive value of the available female:
    - males will attempt more mating when the female is of a high reproductive value (big virgin female)
- Fighting behaviour between 2 males is influenced by the presence of a female.
    - more fighting and paralysis when a female is present (Buena & Walker, 2008;Hoem *et al*, 2007;Kruse & Switzer, 2007;Tachon *et al*, 1999)
    - more fighting and paralysis when this female is of a high reproductive value

3.5.2. Materials and methods

In this experiment, pairs of mature males were put together with females of various reproductive status, or no female, and fighting or its outcomes were recorded.

Individual males (2-5 days old) were either put in drops with 2 females of the same age to mate and mature (as in section 3.4.2.1) or were kept single in their original drop. In pairs of males, one was resident and one was an intruder, though the identity of each was not tracked. Where the male would be used as the resident male in a fight, the females were removed from the drop after 24 to 48 h and kept in a vacant drop. Where the male would be used as the intruding male, it was removed from the drop after 24 to 48 h and placed in the observation drop. The mature males were assigned to the following treatments:

- 2 mature males with a mated female (this was 1 of 2 females used for the maturing of another male, the other female from this trio was not reused but kept to check for progeny and thus successful mating of the corresponding male);
- 2 mature males with a large virgin female (same age as the mated females and the males);
- 2 mature males with a small virgin female;
- 2 mature males without a female.

Normally, virgin females of approximately the same age as the mature males used in this experiment were very long, fat and visibly full of eggs. This made it quite difficult to find small

females of around the same age as the males to use in the category “small virgin female”. When a female of 3-6 days old was small, she often appeared to have suffered some developmental delay and did not look completely healthy. In some observation drops, these feeble females were used, but in most drops the females used for this “small virgin female” category were younger than the males and other females used in the experiment.

Immediate observations were normally made for about 30 min during which both fighting and mating attempts were recorded. Observations were also made at the end of the observation day, 24 h after and 48 h after, at which point paralysis and death were recorded. Age of males at observations varied, but 2 males in a drop were always of the same age. The females that were only used for the maturing of males were examined 3-6 days after mating and progeny noted. Males that had been with females for maturing, but had not produced any progeny with this female- even when macrosperm may have been observed - were taken out of the dataset.

3.5.3. Results

When the presence and reproductive status of a female was varied there was no significant difference in the level of fighting between 2 mature males based on both the immediate fighting (Figure 3.8-a; X^2 (3, n = 56) = 2.367, p = 0.500) and the results of fighting in terms of immediate paralysis (Figure 3.8-b; X^2 (3, n = 56) = 1.718, p = 0.633) and paralysis /or death after a longer time period (Figure 3.9; p > 0.3 or number of drops with paralysis or death too low). The drops without females do show the least (though not significant) amounts of paralysis and fighting (Figure 3.8-a & -b) as would be expected based on resource-value theories (Keil & Watson, 2010).

The wrapping of a male around a female was also recorded; this was only interpreted as an attempted mating when it occurred at the female’s vulva. Even though it was not significant (X^2 (2, n = 42) = 3.579, p = 0.167), mature males paired with a big virgin females made mating attempts in 70 % of the drops, only 40 % drops with a small virgin female or a mated female had shown mating attempts (Figure 3.8-c).

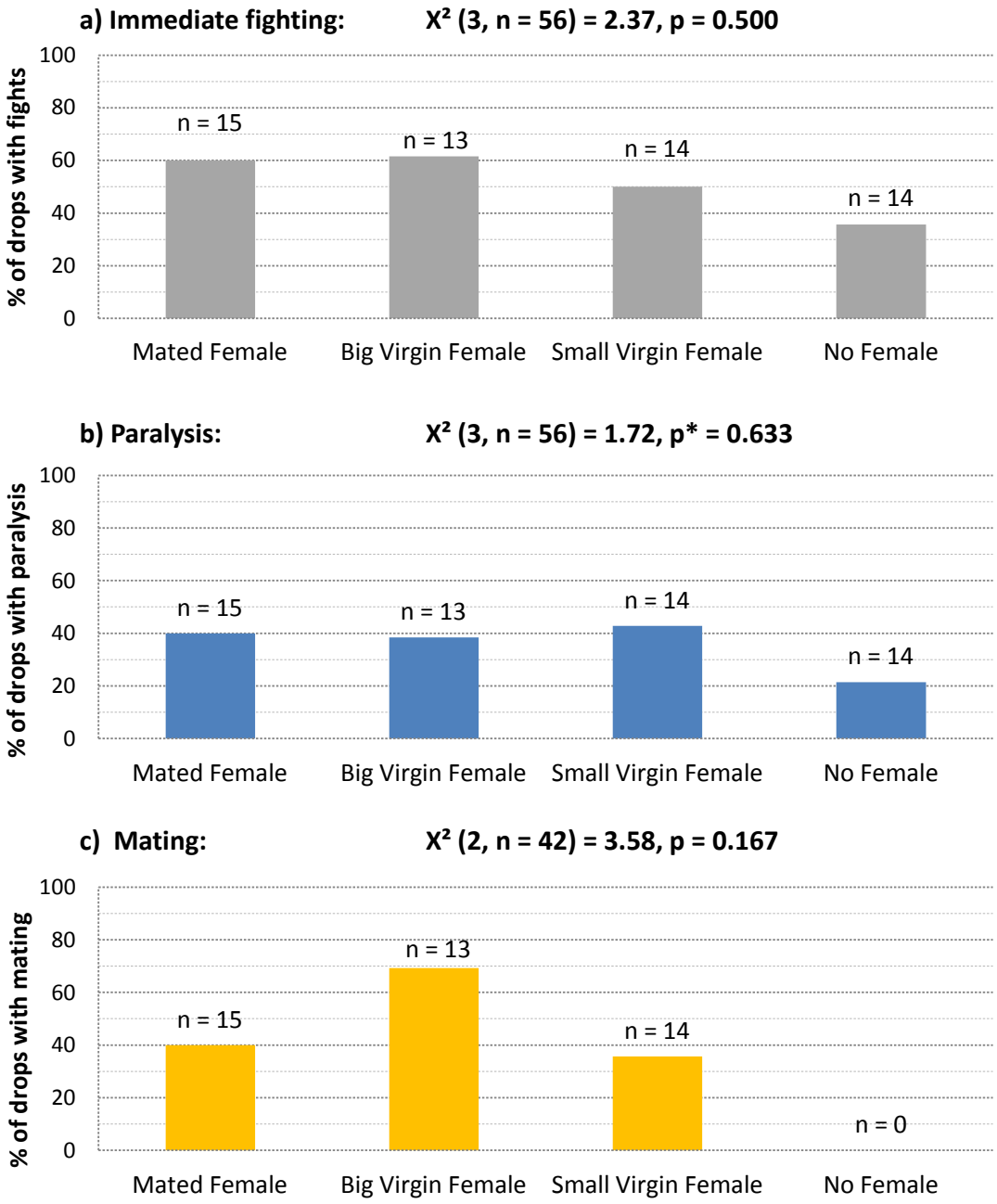

Figure 3.8 Effect of mating status of the female on the behaviour of 2 mature males: a) immediate fighting, b) paralysis of at least 1 male, c) mating. Observations were made over a 30 min period starting directly when all the individuals were placed together in the drop.

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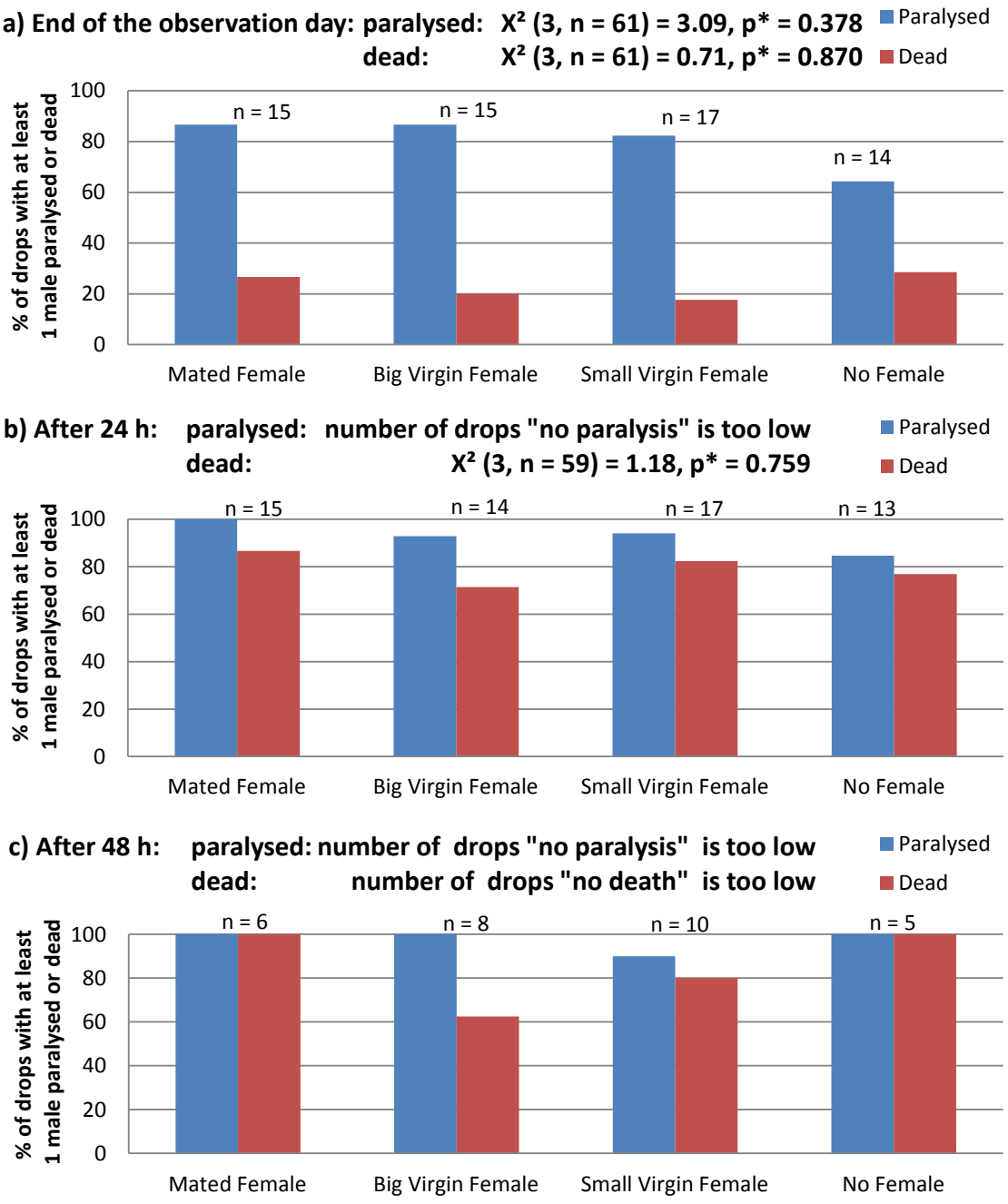


Figure 3.9 Effect of presence and mating status of the female on paralysis of at least 1 male in a drop with 2 mature males: a) at the end of the observation day, b) after +/- 24 h and c) after +/- 48 h. Chi²-tests with df=3 were used.

***: 4 cells with expected counts less than 5.**

3.5.4. Summary & conclusions

Male-male fights occurring when a female was present implies fighting is not just frustration-mating (Field & Waite, 2004).

Despite the lack of significance, fighting, paralysis and mating show the trends that are expected based on theory: more fighting and paralysis when a female is present (Buena & Walker, 2008;Hoem *et al*, 2007;Kruse & Switzer, 2007;Tachon *et al*, 1999) and more attempts for mating when the female is of a high reproductive value (Keil & Watson, 2010). A maximum sample size of 17 is very low and these female choice and female presence experiments should be repeated with higher numbers.

Some of the females used for the “small virgin female” category looked rather unhealthy. This might have had an influence on this experiment and future experiments should aim to only use standard younger females for this category.

3.6. Characteristics of male-sized opponents that elicit fighting behaviour

3.6.1. Introduction

Some animals attack inanimate objects to various degrees. For example, ducklings sometimes direct forceful pecks at a nonliving target (Gaioni *et al*, 1977). This behaviour has been proven to be an aggressive behaviour (Gaioni *et al*, 1977).

Paired rats are known to perform a behaviour named "reflexive fighting" when fighting behaviour is elicited as a reflex reaction to electric shock without any prior specific conditioning (Ulrich & Azrin, 1962). A second moving animal, e.g. a rat, a guinea pig or a hamster is a necessary condition for eliciting the fighting response from a rat stimulated by foot-shock (Ulrich & Azrin, 1962). A rat that was given a foot-shock did not attack a nearby doll, a moving inanimate object or a recently deceased rat (Ulrich & Azrin, 1962). A dead rat only sufficed for eliciting fighting when it was moved about the cage on a stick (Ulrich & Azrin, 1962).

In intrasexual aggression by male Iberian wall lizard, *Podarcis hispanicus*, odoriferous cues seem to be the more important characteristics of the opponent in eliciting an attack, at least at close range (López *et al*, 2002).

Movement and pheromones can thus be important stimuli for eliciting aggressive responses. In this section, stimuli that a male *Steinernema longicaudum* responds to by performing fighting behaviour are identified. By using as an opponent a male substitute or males treated so that they would possess some possible fighting behaviour eliciting factors, but lack others, it would be possible to identify those factors that trigger fighting.

Hypotheses

- Non-intentionally varying factors have no effect on the number of attacks per drop (3.6.3.1).
- An inanimate object of male dimensions (a suture) does not elicit fighting behaviour in a male (3.6.3.2.).
- Males don't wrap all male-sized objects to the same extent. The object needs to have certain conspecific male characteristics like texture (dead conspecific male), movement

(incapacitated conspecific male) or a certain capability to inflict injury (normal conspecific male) to elicit fighting behaviour (3.6.3.2.).

- Presenting a male with a stronger "fight eliciting factor" will affect the male's behaviour to the male sized object (3.6.3.3.1).
- The presence of a male-sized object in a drop can affect fighting between 2 normal males depending on the characteristics of the object (3.6.3.3.2, 3.6.3.3.3 and 3.6.3.3.4.).

3.6.2. Materials and methods

3.6.2.1. Male sized object

Fourteen males were checked for width when aged 2, 5 and 9 days (see Table 3.2); 9 day old males were taken as the upper limit to also account for developmental and environmental differences between batches of males), the age range used in the objects experiments is 2-6 days old. The mean width for males aged 2-9 was between 70-85 µm, this corresponded best with surgical sutures of the 6-0 or 7-0 USP[1] size (see Table 3.3). The 7-0 was deemed the most appropriate, as this corresponded best to the width of males of 2-5 day old males that were used in experiments. The suture was cut to an average male size, the resulting pieces did vary slightly in length, which allowed for pairing up males with a suture of approximately the same size. A piece of the suture was placed in a drop of Ringers and then cut to the appropriate size with a sterile scalpel blade. Clean tweezers or insect needles were used to move the suture of male size into the experiment drop.

SURGIDAC™ undyed polyester sutures were used because they are non-absorbable, sterile, and uncoated. The chemical impact of these sutures should be only minimal and no significant change in strength of the suture should occur.

[1] USP: United States Pharmacopeia, the organisation that defined suture sizes.

Table 3.2 Width (µm) of males aged 2, 5 or 9 days.

Age in days	2	5	9
Mean width in µm	69.05	70.83	85.42
min	50.00	58.33	75.00
max	83.34	83.34	100.00
n	14	6	4
SD	10.56	11.49	10.49
SE	2.82	4.69	5.24

Table 3.3 Diameter in (µm) of conventional USP1 sizes for nonabsorbable sutures. (Source: http://www.meta-biomed.com/english/suture/mepfil.html)

Size	Diameters by USP1 methods (µm)			
USP	Average value		Individual value	
	Minimum	Maximum	Minimum	Maximum
8-0	40	49	35	60
7-0	**50**	**69**	45	85
6-0	**70**	**99**	60	125

3.6.2.2. Incapacitated male

To produce an incapacitated male, a 2-5 day old male was taken out of its drop and was left to dry on a Petri dish for at least 30 sec. During these 30 s, the male was rolled back and forth over a very short distance (less than 1 mm) with the aim to inflict injury. Drops in which the incapacitated male attacked one of the normal males were excluded from the analyses as the purpose of the incapacitated male was to have a live male that was incapable of attacking.

3.6.2.3. Dead male

A dead male was produced by taking a 2-5 day old male out of its drop and leaving it to dry on the lid of a Petri dish for at least 2.5 h. The lid was passed over a Bunsen flame so that the male would suffer lethal dehydration. When the male looked sufficiently dehydrated, a drop of sterile Ringer's was placed on top of it for at least 2.5 h so its body could return to the previous size. Special care was taken to only use males that were definitely dead as some males were able to recover even after repeated cycles of dehydration.

3.6.2.4. Effect of object/opponent

After 3-6 days in the 27 °C incubator, males were randomly assigned to one of the 7 treatments, which can be grouped as follows:

- 2 normal males;
- 2 normal males plus an object (dead male, incapacitated male, suture);
- 1 normal male plus an object (dead male, incapacitated male, suture).

This experiment was done in 10 sets. A set was a group of observations that were performed within 3 days of each other. The difference between sets was the addition or exclusion of extra treatments or extra observation periods so that not all 15 min observation periods and observation time points were included in each set.

Four 15 min observations were made during which the number of attacks and fights were recorded. These periods were (1) 0-15, (2) 16-30, (3) 31-45 min and (4) 1.75-3 h after the start of the experiment. After each 15 min observation period, the males present were checked for paralysis. This was checked again at the end of the day, after 24 h and after 48 h.

Using a mercury thermometer that was adhered to the observation stage, the temperature was checked at the beginning of the 1st observation period for 3 of the 10 sets. In one set, the temperature on the observation stage was also checked at the 4th observation time period.

To produce “male-scented" sutures, in 6 sets, 30 sutures were re-used after they had been in a drop with 1 male for at least 15 min and 3 sutures were re-used after they had been in a drop with 2 males for at least 15 min. Because of the possible presence of male associated chemical stimuli in the drop, any haemolymph that was transferred adhering to the suture might indicate that a male was introduced into the new drop instead of just a suture of male dimensions. It was expected that if male associated chemical stimuli induced fighting behaviour in a male, the “scented” suture would be attacked more often than the “unscented” suture. Scent however did not seem to have any effect on the number of attacks towards the suture (3.6.3.1) and therefore the rest of the sets were only given “unscented” sutures.

The analyses were broken down into fights between 2 normal males and fights between a normal male and an object. The focus was on the number of attacks in a drop, since this encompassed the number of drops with fights, and it was expected that the number of drops with fights would show the same trends. The number of drops with paralysis or death at the end of day, after 24 and 48 h were also recorded and analysed.

3.6.3. Results

3.6.3.1. "Non-target" factors

Because of possible confounding effects of the non-intentionally varying factors (set, temperature on the observation stage of the microscope, age of males) and the additional factor "scent", I first examined the effects of these factors on number of attacks per drop. The rate of development and the number of males that developed, varied a lot over the different sets making it too difficult to assign the same number of observations to all treatment combinations, especially regarding the non-intentionally varying factors. The data for analyses of interaction effects between all factors (controlled and non-controlled) was thus unbalanced and fully factorial analyses were not possible. Therefore, for each treatment, the effect of each of the uncontrolled factors (temperature, set, age and scent) on the number of fights per drop was examined separately using one-way ANOVA. The analysis was conducted separately for each observation time. The results of these tests are given in Appendix section 1.1.

Scent, temperature and age had no effect on fighting in any treatment at any observation time (Appendix tables 1, 2, 3 and 4). Only set had a significant effect on the number of attacks per drop in the 1st 15 min time period for the treatment where only 2 normal males were placed together in a drop ($p = 0.035$, Appendix table 1) and in the 2nd 15 min time period for the treatment where 1 normal male was paired with a dead male ($p = 0.039$; Appendix table 2). A post hoc Tukey's test however, did not detect specific differences between sets within either of these 2 specific treatment-time point combinations. It would therefore be possible to conclude that there was little or no effect of the various non-target factors on the number of attacks in a drop and I next explore the effect of the different planned treatments on the number of attacks per drop and on the number of drops in which attacks occur, ignoring these factors.

3.6.3.2. Attacks by 1 normal male directed against an object

A normal male in its own drop attacked each of the objects that it was presented with. It did not perform a significantly different number of attacks towards the different objects (a dead male, an incapacitated male or an artificial male; all $p > 0.1$). Nor did the number of drops in which attacks occurred vary significantly (all $p > 0.05$, Figure 3.10).

In 4 of 36 drops, a suture of conspecific male dimensions was attacked by a solitary male within the first 15 min (Figure 3.10-a-right graph). In the 2nd 15 min period however, the suture was not attacked in any of the 6 drops (See Figure 3.10-b-right graph).

3.6.3.3. 2 normal males plus an object

In this section I first dealt with attacks of the 2 normal males against the object. Then I looked at the attacks between the 2 normal males. Next, I focussed on drops in which both the other normal male as the object was attacked. In the last part of this section, I investigated the occurrence of paralysis of at least 1 normal male.

3.6.3.3.1. Attacks by 2 normal males directed against an object

Again, when 2 normal males were present, each class of objects was attacked as was observed when there was a solitary male. This time however, there were differences between the classes of objects. During the 1st 15 min, the number of attacks 2 males directed towards a male-sized suture, was significantly less ($F(2, 90) = 4.62$, $p = 0.012$;) than the number of times they attacked a treated (dead or incapacitated) male (Figure 3.11-a left graph). The number of drops in which attacks occurred, was also significantly lower (X^2 (2, n = 93) = 9.764, $p = 0.008$) where the object was a suture (Figure 3.11-a right graph).

During the 2nd (Figure 3.11-b) and last (Figure 3.11-c) observation periods, the number of drops with an attack on the object (right graphs) and the number of attacks per object (left graphs), were not different over the 3 treatments. However, the trend for the suture to be attacked less than the dead or incapacitated male seen in the first observation continued.

3.6.3.3.2. Attacks between 2 normal males

In drops where 2 males either were or were not presented with an object, the number of drops with male-male fighting and the number of times the males attacked each other during the 1st and 2nd 15 min observation periods (see Figure 3.12-a & -b), were not significantly different over the various treatments (all $p > 0.05$).

In the last 15 min period (Figure 3.12-c, right graph), there were significantly (X^2 (3, n = 81) = 11.971, $p = 0.007$) fewer drops with fighting when 2 males were presented with a treated (dead or incapacitated) male than when they were left without an object. Although $p = 0.05$ ($F(3, 77) = 2.72$) in the last observation period (Figure 3.12-c, left graph), a post hoc Tukey's test did not pick up differences in the number of attacks between the 2 normal males due to the presence or absence of the various objects. Figure 3.12-c (left graph) however, shows the same trend for number of attacks as in the number of drops with fights: 2 males fought each more often when they were not presented with a treated male.

The presence of the suture did not significantly influence the number of drops with fights or the number of fights between the 2 normal males (Figure 3.12).

3.6.3.3.3. Did attacks by 2 normal males directed against an object occur in the same drops as attacks against another male?

In Figure 3.13, I classify drops as those in which no attack occurred, those in which only a normal male was attacked, those in which only the object was attacked and those where both normal male and object were attacked. During the first 15 min observation period 13.8 % or 21.9 % of the drops that had attacks towards the incapacitated or dead male (respectively), also had attacks towards the other normal male. In the 2nd and last observation periods the 2 normal males attacked both the object and each other in less than 3.5 - 6.3 % of drops.

The suture was never attacked on its own, it was only attacked in a drop in which a normal male was also attacked. However, the number of drops in which a suture was attacked was low (only occurred in 1 drop).

3.6.3.3.4. Paralysis of at least 1 normal male after the observation period

In this section, I deal with the paralysis of a normal male at the end of each observation period, and paralysis and death at the end of the observation day and after 24 & 48 h.

After the 1st and 2nd 15 min periods the proportion of drops where at least 1 male was paralysed, was significantly different (1st 15 min: $X^2(3, n = 167) = 8.482$, $p = 0.037$; 2nd 15 min: $X^2 (3, n = 150) = 8.335$, $p = 0.040$) over the 4 treatments (Figure 3.14-a & -b). Because of the low numbers of drops in which paralysis had occurred by this stage, the overall significant effects could not be translated into specific differences between treatments. However, inspection of the data showed that the incidence of paralysis appeared to be lower in the presence of a suture or a dead male (0 - 9.4 % of drops) than when an incapacitated male or no additional object was present (17.2 - 20.3 % of drops) (Figure 3.14-a & -b). By the last observation period 28.6 - 40.0 % of drops had a paralysed male (Figure 3.14-c), but there were no significant differences over the 4 treatments ($X^2 (3, n = 81) = 0.934$, $p = 0.817$).

The number of drops in which 1 normal male got paralysed or died 3 - 48 h after the beginning of the experiment, was not influenced by the presence of an object in the drop (Figure 3.15, all $p > 0.5$). However, the incidence of death at the end of the observation day (Figure 3.15-a, right graph) followed the trend in paralysis seen during the earlier observations (Figure 3.14-a & -b), with less death in the suture and dead male treatments than in the other two.

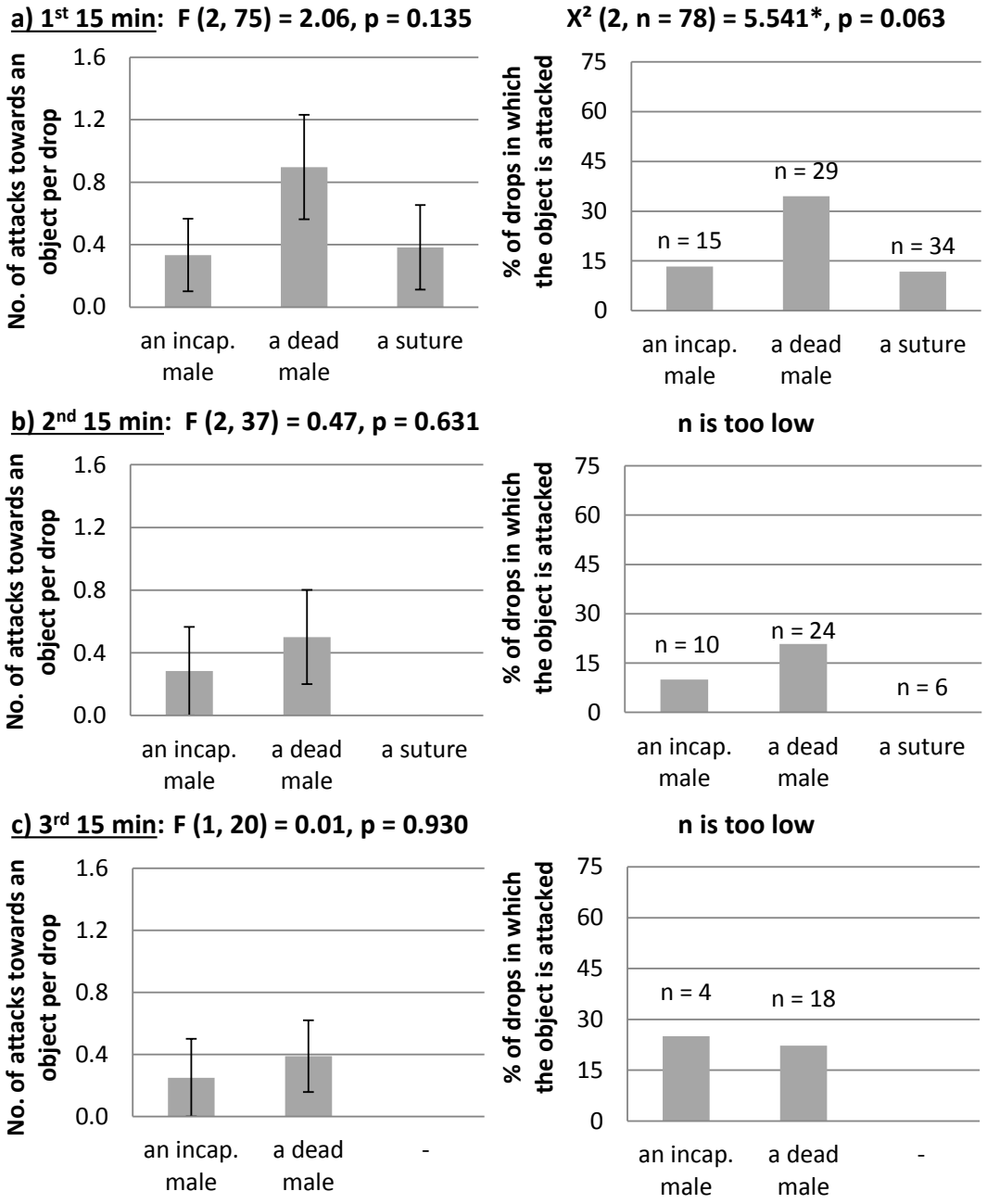


Figure 3.10 Attacks made by 1 normal male towards the object it was placed in a drops with. Left: mean (+/- SE) number of attacks made in a drop; Right: % of drops in which attacks occurred. An object can be an incapacitated male, a dead male or a suture of male dimensions. Observations were made over 3 time periods: a) 0-15, b) 16-30 and c) 31-45 min after the worms and objects were added to the drop. n is equal on left and right graphs. *: 1 cell with expected count less than 5.

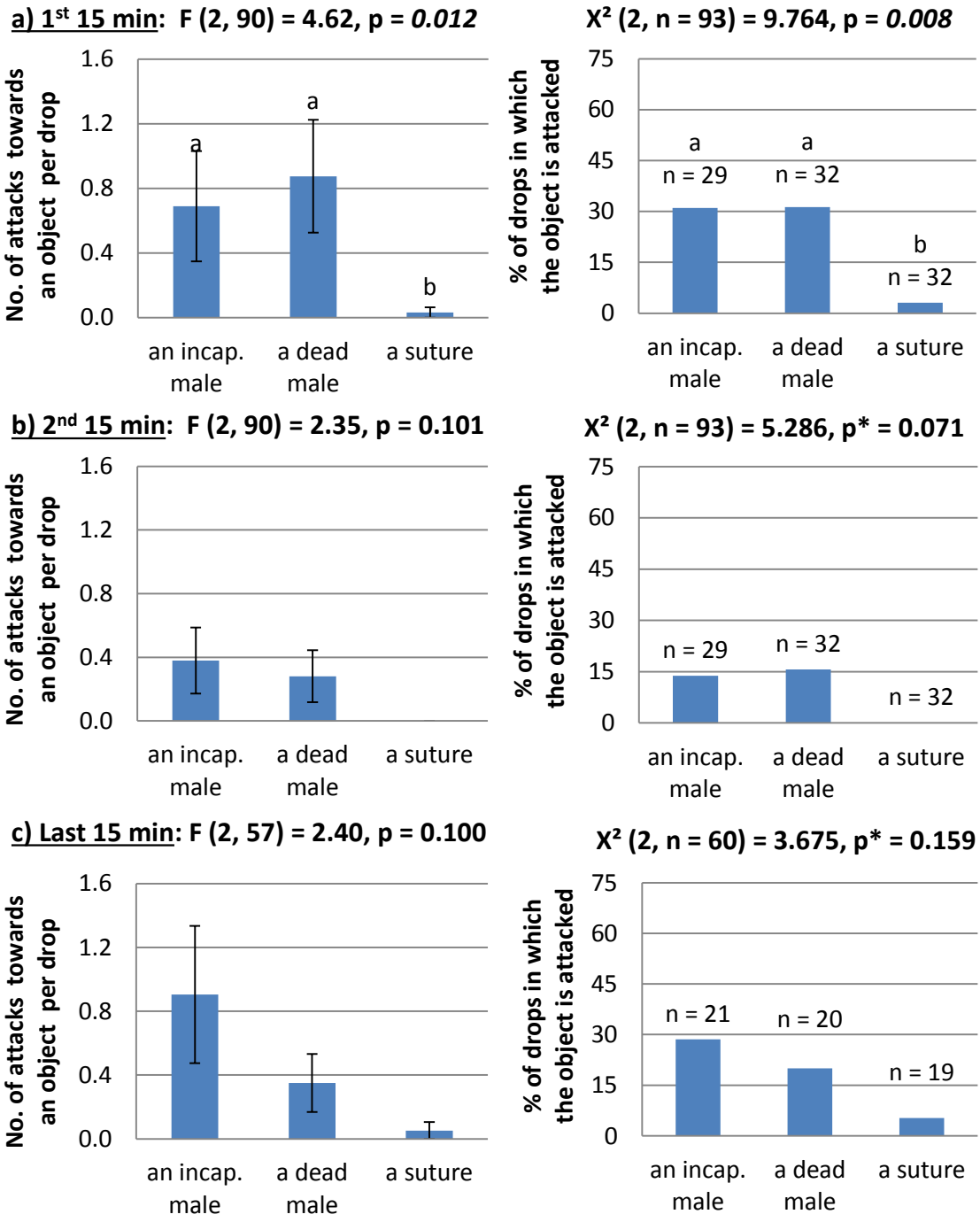


Figure 3.11 **Attacks made by 2 normal males towards the object they were placed in a drops with. Left: mean (+/- SE) number of attacks made in a drop; Right: % of drops in which attacks occurred. An object can be an incapacitated male, a dead male or a suture of male dimensions. Observations were made over 3 time periods: a) 0-15, b) 16-30 min and c) 1.75-3 h after the worms and objects were added to the drop. Within a graph, bars accompanied by the same or by no letters are not significantly different.**

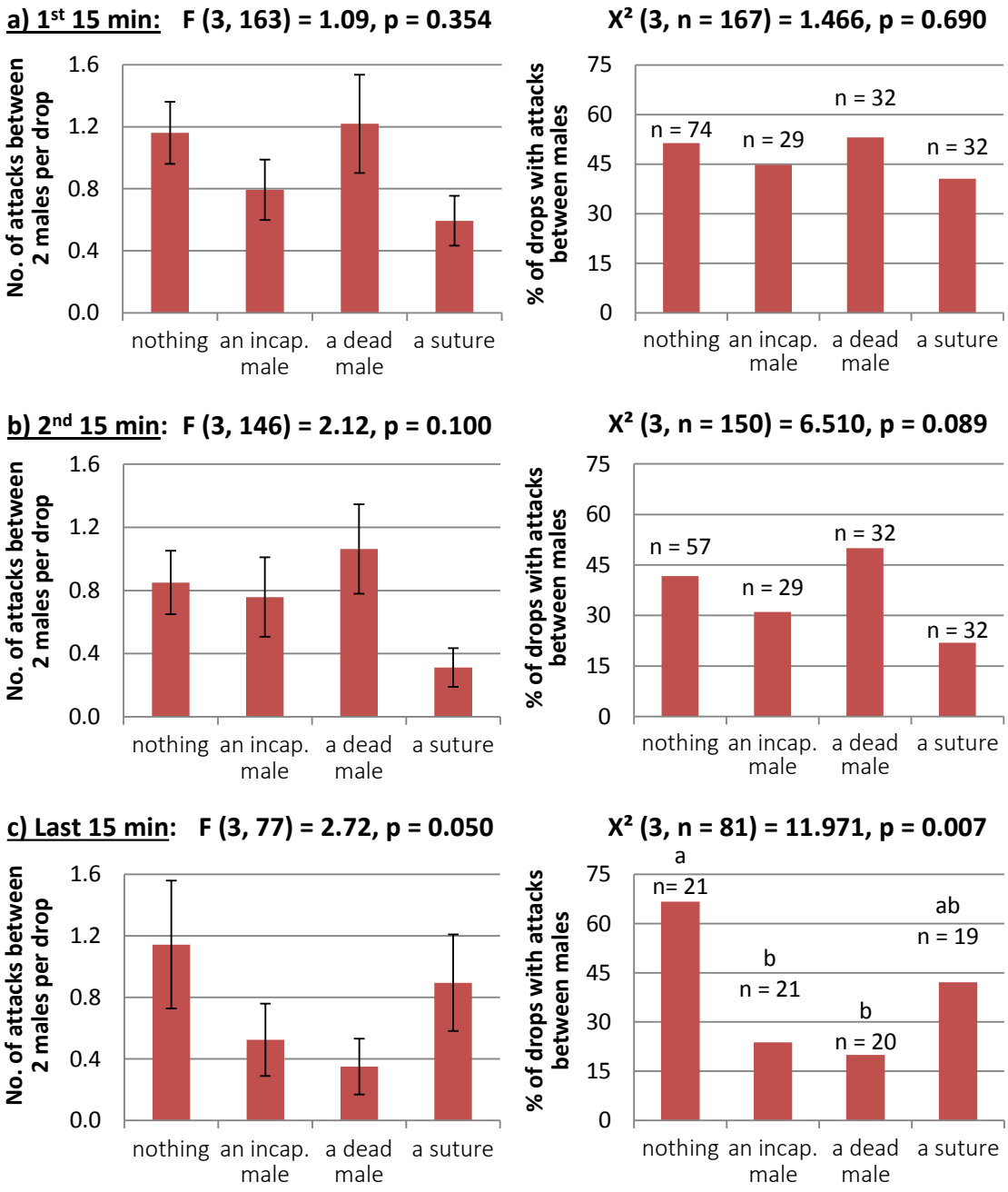


Figure 3.12 **Attacks made between 2 males in drops where 2 normal males were or were not placed together with an object. Left: mean (+/- SE) number of attacks made in a drop; Right: % of drops in which attacks occurred. An object can be an incapacitated male, a dead male or a suture of male dimensions. Observations were made over 3 time periods: a) 0-15, b) min and c) 1.75-3 h after the worms and objects were added to the drop. Within a graph, bars accompanied by the same or by no letters are not significantly different, n is equal on left and right graphs.**

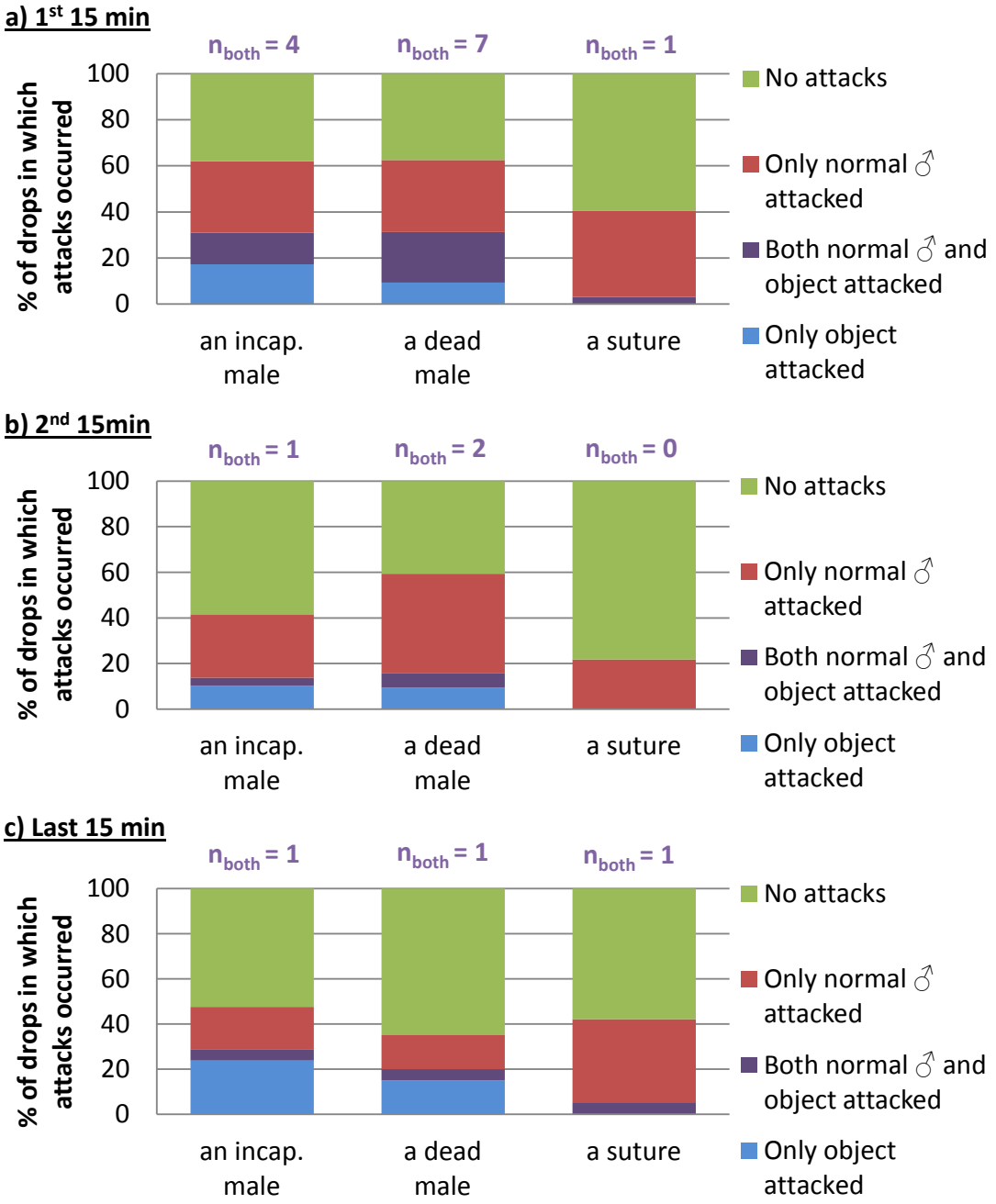


Figure 3.13 **% of drops in which the 2 normal males did not attack, attacked only the other normal male, attacked the other normal male and the object or only attacked the object they were placed in a drop with. Observations were made at the end of 3 time periods: a) 0-15, b) 16-30 min and c) 1.75-3 h after the worms and objects were added to the drop. n_{both}=1 is the number of drops in which both the normal male and the object were attacked.**

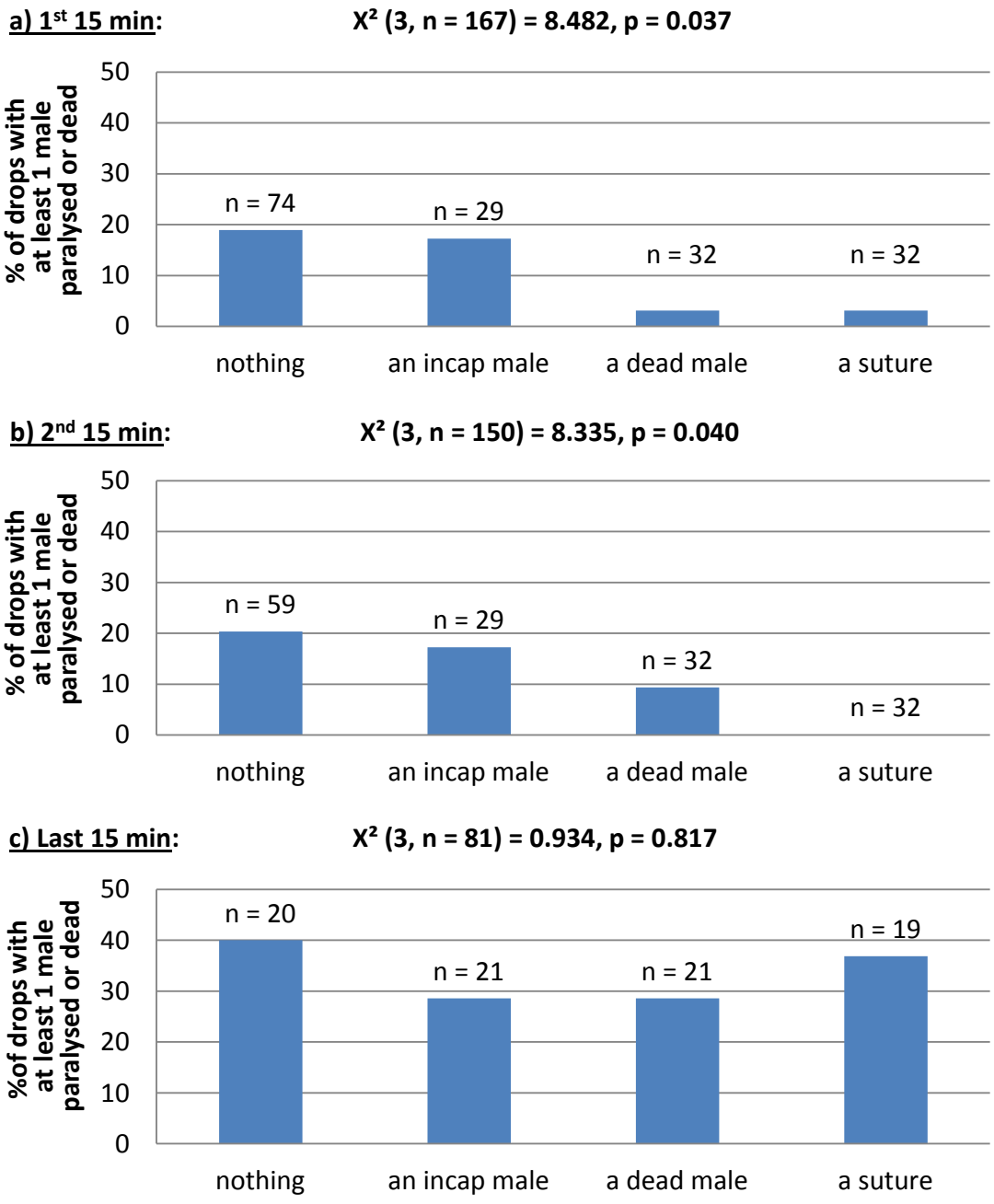


Figure 3.14 **Where 2 normal males were or were not placed together with an object: the % of drops in which paralysis of at least 1 of the normal males occurred at the end of the observation period. An object can be an incapacitated male, a dead male or a suture of male dimensions. Observations were made at the end of 3 time periods: a) 0-15, b) 16-30 min and c) 1.75-3 h after the worms and objects were added to the drop.**

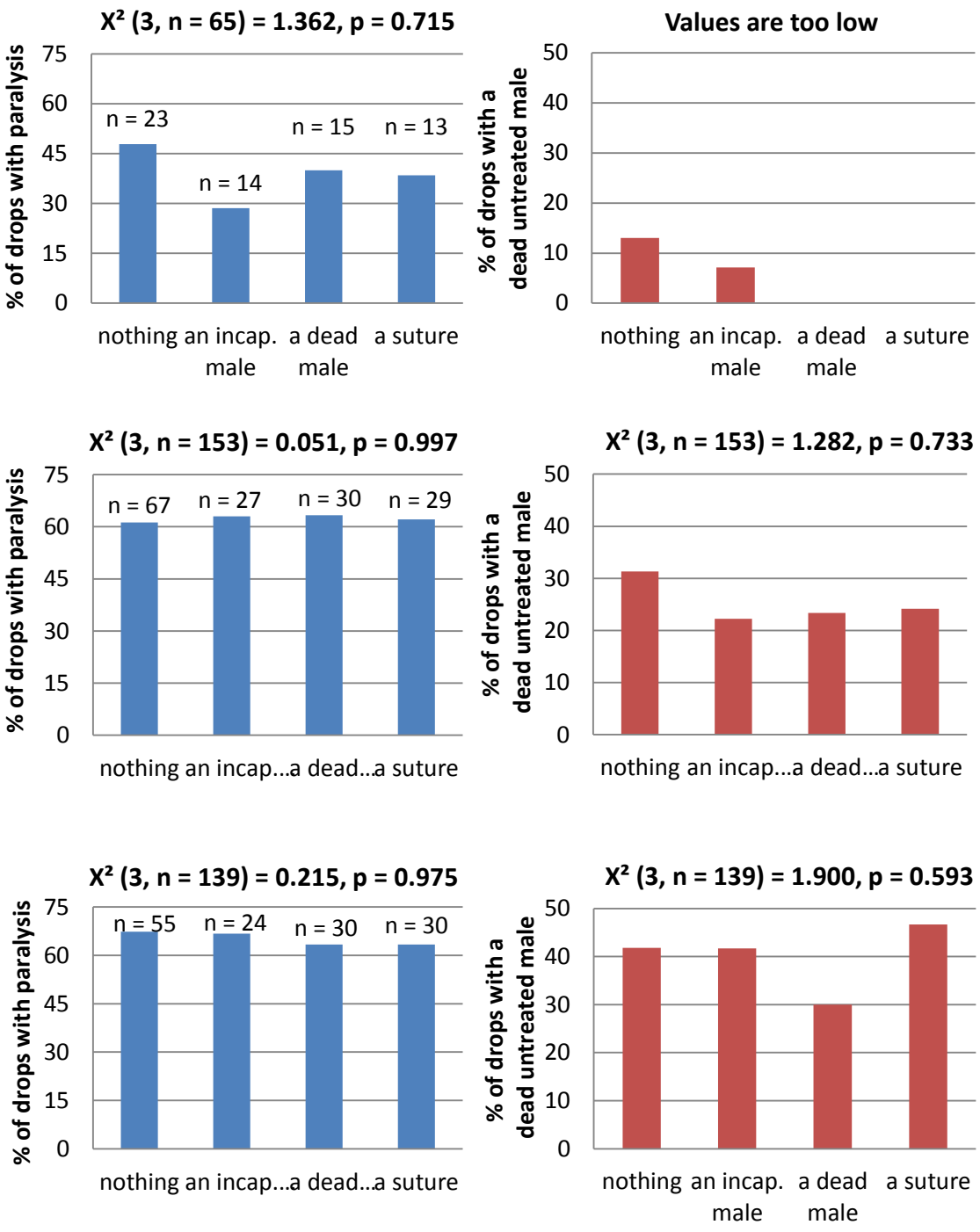


Figure 3.15 **Percentage of drops in which at least 1 normal male is paralysed (left graphs) or dead (right graphs) at a) the end of the observation day, b) after 24 and c) 48 h. n is equal on left and right graphs.**

3.6.3.4. Effect of an object and number of males on number of attacks in a drop

In a drop, attacks can be directed at another normal male or at an object whenever these were present. The treatment can have an effect on these attacks, but also on the total number of attacks in a drop, regardless of the receiver (normal male or object) of the attack. In this section, I focus on the total number of attacks per drop (and drops with attacks), in drops with 2 normal males, 2 normal males plus object, or 1 normal male plus object.

During the 1st 15 min period after the start of the experiment, there was significantly less fighting ($F(2, 243) = 9.55$, $p < 0.001$) by 1 male with an object (suture, incapacitated or dead male) than by 2 males, with or without an object (see Figure 3.16-a, left graph). During the 2nd observation period (see Figure 3.16-b, left graph), 1 male with an object performed significantly less ($F(2, 190) = 3.11$, $p = 0.047$) fighting than 2 males with an object and less (but not significantly) than 2 males without an object. For both these observation periods, the proportion of drops in which attacks occurred was also significantly less (1st 15 min: $X^2(2, n = 245) = 23.534$, $p < 0.001$; 2nd 15 min: $X^2 (2, n = 193) = 9.470$, $p = 0.009$) for 1 male with an object than for 2 males (with or without objects) (Figure 3.16-a & -b, right graphs).

In the 3rd 15 min period, there was no difference (all $p > 0.5$) between treatments (1 normal male with an object and 2 normal males) either in the number of attacks (Figure 3.16-c, left graph), or in the number of drops in which an attack occurred (Figure 3.16-c, right graph). Two normal males with an object were not included at this time period.

When looking over time at the number of attacks per drop and the number of drops in which an attack occurred, there seems to be a big decline (from 0.85 to 0.27, respectively from 41.7 % to 13.6 %) for 2 normal males without an object between the 2nd and 3rd time period (Figure 3.16-b & -c). However, the 22 drops that were observed in the 3rd 15 min time period represent just a subset of those examined in the earlier time periods. This makes it rather difficult to correctly analyse and interpret possible differences over time.

For the total amount of fighting for all seven treatments for the 1st and 2nd 15 min observation periods see Appendix section 1.2 and Appendix figure 1.

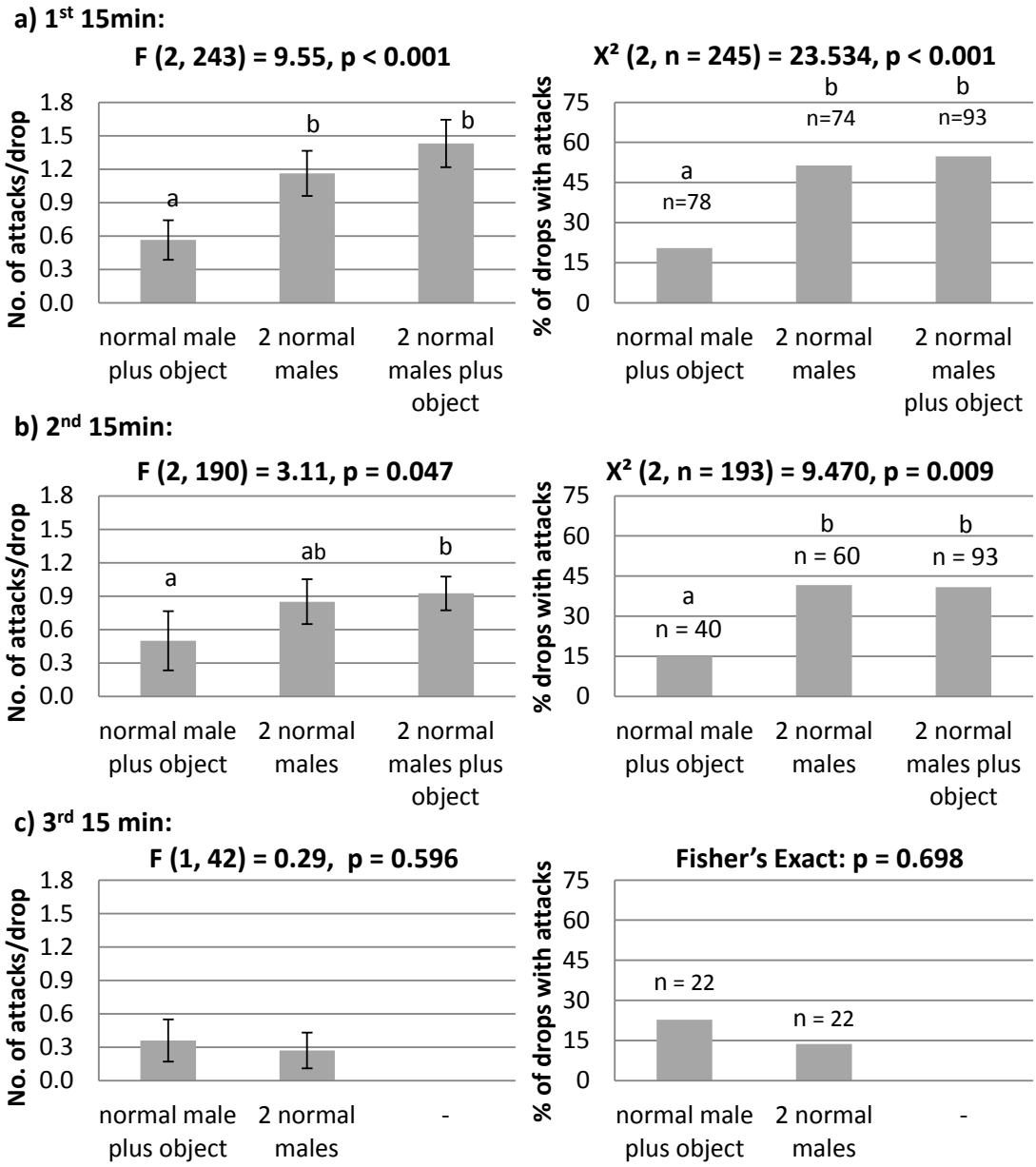


Figure 3.16 **All attacks, regardless of receiver. Left: mean (+/- SE) number of attacks made in a drop; Right: % of drops in which attacks occurred. The treatments were grouped into those where, in a drop, 1 male was placed together with an object, 2 normal males were placed together or 2 normal males were placed together with an object. An object can be an incapacitated male, a dead male or a suture of male dimensions. Observations were made over 3 time periods: a) 0-15, b) 16-30 and c) 31-45 min after the worms and objects were added to the drop. Within a graph, bars accompanied by the same or by no letters are not significantly different. n is equal on left and right graphs.**

3.6.4. Summary & conclusions

With only 1 male present, the nature of the object did not influence the attacks (Figure 3.10). When 2 males were present however, the number of attacks and the number of drops with attacks directed by 2 males towards a male-sized suture during the 1st 15 min (Figure 3.11) was significantly less (resp. $F(2, 90) = 4.62$, $p = 0.012$, $X^2(2, n = 93) = 9.764$, $p = 0.008$) than the number of times they attacked an incapacitated or a dead male. Also, in no event, was a suture attacked without the occurrence of an attack between the 2 males (Figure 3.13).

In the first half hour of the experiment, there were less attacks and less drops with attacks when only 1 male was present than when 2 males were present (Figure 3.16-a & -b, all $p < 0.05$). Thus, a male performed fewer attacks towards an object, than towards a normal male.

In a 15 min period 1.75-3 h after the start of the experiment, there were less drops with attacks between the 2 males when they were presented with an incapacitated or a dead male than when they were left without an object (Figure 3.12-c-right graph, $p < 0.05$). The presence of a suture did not seem to affect the number of attacks between the 2 males. At this same moment in the experiment, paralysis of a normal male (Figure 3.14) did not differ between the treatments where no object was present or where an incapacitated or dead male or a suture was present. Over the following 2 days, the number of drops with at least 1 male paralysed increased in all treatments, without any differences (Figure 3.15). This indicates that the difference in fights between drops with an incapacitated or dead male and drops with no object present might be of a temporary nature.

N.B.: a solitary normal male attacked each of the objects with which it was presented - a dead or incapacitated male, or an artificial male (a suture), and it attacked each of these objects equally (Figure 3.10). This might suggest that any male-sized object will be attacked, or "wrapped", and might call into question whether the observed "wrapping" should be interpreted as an attack. However, although two normal males also attacked all these three classes of object, they tended to attack the suture less frequently than the dead or incapacitated male (Figure 3.11 left graphs). This shows that not all male-sized objects are equally subjected to wrapping and suggests that cues associated with a real male are important. That the number of drops in which sutures were attacked (Figure 3.11 right graphs) was also reduced (not just the overall number of attacks), and that this reduction was evident from the start of the experiment (Figure 3.11-a), indicates that the lower attack rate on sutures is not a result of learning by the normal males through previous attacks. Over all the observational experiments made for this thesis, I observed males heading straight for the

other male and attacking upon first contact. This may indicate that the essential cue for fighting is a diffusible chemical as prior contact with the object is not a prerequisite. Dead males were attacked as frequently as incapacitated ones, both by solitary males (Figure 3.10) and by male pairs (Figure 3.11), showing that the weak movement of an incapacitated male does not make it more likely to be attacked. If anything, there is even a trend for a dead male to receive slightly more attacks than an incapacitated one.

The next question is whether a dead or incapacitated male is attacked as frequently as a normal male. This can be addressed in two ways. Firstly, by comparing the number of attacks made by a solitary male against one of these objects to the number of attacks between two normal males (without an object present). There was a lower level of attack by a solitary male on a treated male (average about 0.3 - 0.9 attacks/drop, Figure 3.10) compared to the attack rate between two normal males (average 0.9 to about 1.2 attacks/drop, "nothing" category in Figure 3.12). However, if we assume that each of the two males is equally likely to initiate a fight, then we might expect two normal males to fight twice as much as a solitary one. Therefore, from this comparison it looks as if a normal male will attack a dead or incapacitated male as much as it will attack another normal male. We see a similar picture when the data for all three classes of object are combined for solitary males and compared with two normal males (Figure 3.16); the attack rate by a solitary male on an object is the same (3rd observation period) or about half the frequency (1st and 2nd observations) as that of two normal males.

The second way to explore whether a normal male is more likely to attack another normal male than a treated (dead/incapacitated) male is to examine what happens when both options are presented simultaneously, i.e. compare the number of attacks between two normal males in the presence of an object with the number of attacks directed by them against the object. If we assume that either male may attack the object, and that either normal male may also attack the other, then the number of attacks between the two should be the same as the number directed against the object. The number of attacks between two males in the presence of an incapacitated or dead male (Figure 3.12) tends to be somewhat higher than the number of fights by the same male pair against the treated male (Figure 3.11), but not greatly so, indicating a slight preference for a male to attack another healthy male rather than a dead or incapacitated male, when given the choice. Thus, normal movement does not seem to be very important in eliciting an attack.

There is no obviously visible difference in the nature of the "wrapping" observed around sutures and males. However, "squeezing" as was observed when a normal male attacked another normal, dead or incapacitated male was not observed when a normal male attacked a

suture. The attacking male thus seems to behave differently when attacking an inanimate object than when attacking a conspecific male.

In conclusion, it would seem that male *S. longicaudum* wrap around any male-sized object, but that where additional cues are involved, chemical cues are more important than movement in eliciting an attack. In combination with O'Callaghan's in vivo experiments (O'Callaghan, 2006), this shows that the artificial circumstances of a haemolymph drop are not the cause of fighting behaviour.

3.7. Effect of stimulation on fighting

3.7.1. Introduction

Physical stimulation has proven to elevate aggressiveness, for example: shaking a subordinate *Acheta domesticus* male cricket, increases its aggressiveness and makes it more likely to win the next contest (Savage *et al*, 2005). Also, subjecting an individual to physical stress (e.g. constant prodding) in the presence of a conspecific can elicit attacks towards the second individual. Administering an electric shock to a rat in the presence of another rat will induce attacking behaviour towards the other individual (Ulrich *et al*, 1966).

The addition of an external controllable stimulus that augments aggression and thus results in more immediate and possibly more severe fighting, would speed up the study of the immediate effects of fighting. In the following experiment, physical stimulation in the form of poking and keeping the 2 opponents in each other's immediate vicinity by moving them are tested as controllable factors that could be used for enhancing immediate aggression.

Hypotheses

- physical stimulation augments the number of attacks a prodded male directs towards a suture of male dimensions;
- physical stimulation augments the number of attacks a prodded male directs towards a male conspecific;
- physical stimulation only has an immediate effect on aggression resulting in an initial higher incidence of paralysis, but as the stimulus is not maintained the effect fades out with time.

3.7.2. Materials and methods

After 3-6 days in the 27 °C incubator, males were randomly assigned to one of the following 4 treatments:

- 1 male and a suture of male dimensions (see section 3.6.2.1) without physical stimulation
- 1 male and a suture of male dimensions with 15 min of physical stimulation immediately following the introduction of the suture

- 2 males without physical stimulation
- Two males with 15 min of physical stimulation immediately following the introduction of the intruding male.

In the stimulation treatments, males that had moved more than a body length away from each other, were pushed closer together by the use of a platinum wire (Ø 0.05 mm). Likewise, when a male and a suture were more than a body length apart, the suture was pushed closer to the male. This stimulation was only applied during the 1st 15 min of the experiment. Observations were made during these 15 min and about 24 h after the introduction of the opponent (suture or male) into the drop. These experiments were done in 3 sets. A set was a group of observations performed within 3 days of each other.

3.7.3. Results

When a male and a suture were placed together in a drop and they were constantly put back in each other's immediate vicinity, the male attacked the suture significantly more than when this stimulation was not added ($F(1, 57) = 62.17$, $p < 0.001$; Figure 3.17-a). When 2 males were placed together in a drop, stimulation also caused a significantly higher number of attacks ($F(1, 83) = 19.33$, $p < 0.001$; Figure 3.17-b). In each case, the difference was highly significant ($p < 0.001$).

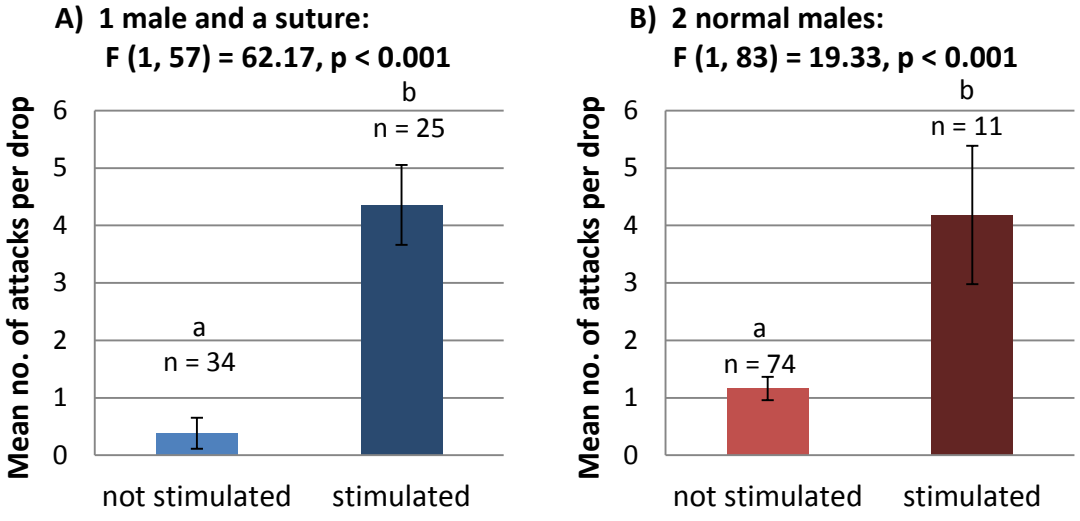


Figure 3.17 The effect of stimulation on the number of attacks in a drop during the 1st 15 min period after the male(s) were put together in a drop with (or without) an object. a) 1 male put together with a suture of male proportions. b) 2 normal males put together in a drop. In each graph: bars accompanied by the same letter are not significantly different from each other.

The effect of this stimulation was only temporary however, the number of attacks per drop (Figure 3.17-b) and the number of drops with attacks (Figure 3.18-1) were significantly higher for stimulated males than for unstimulated pairs, but only for the period during which the stimulation was applied (n = 85, Fisher's exact test: p = 0.002). The number of drops in which at least one male was paralysed (Figure 3.18-2) during stimulation was smaller, but not significantly, for stimulated males than for unstimulated pairs (n = 85, Fisher's exact test: p = 0.063). There was no significant effect of stimulation on paralysis or death at the end of the observation day (at least 3 h after the stimulation ended, see Figure 3.18-3) or after 24 h (Figure 3.18-4).

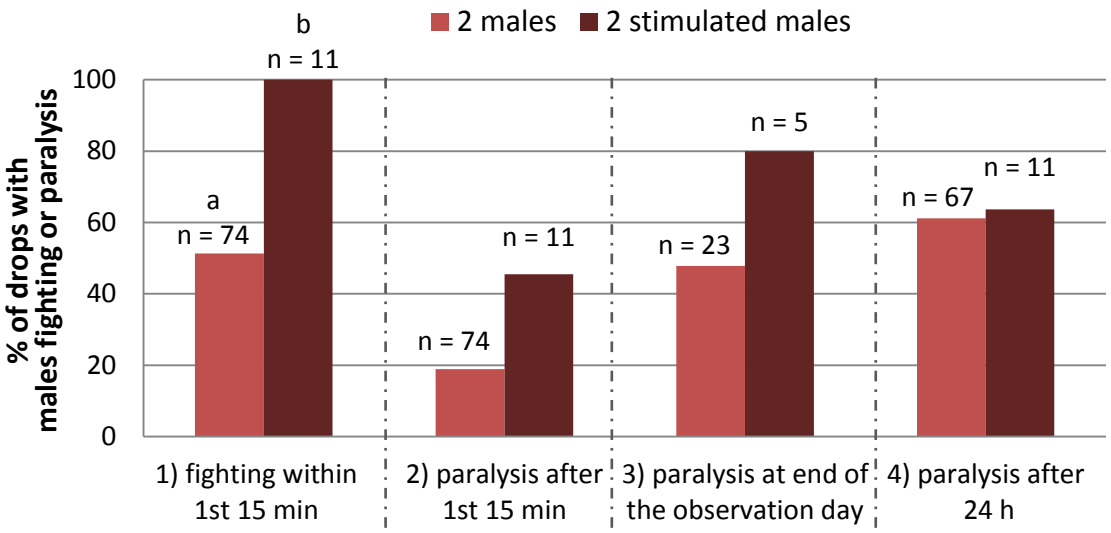


Figure 3.18 The effect of stimulation during the 1st 15 min after the males were put together in a drop, on 1) the number of drops with fights within these 1st 15 min (Fisher's exact test *: p = 0.002*); 2) the number of drops in which at least 1 male is paralysed after these 15 min; 3) the number of drops in which at least 1 male is paralysed at the end of the observation day; 4) the number of drops in which at least 1 male is paralysed after 24 h. Bars within each category accompanied by different letters are significantly different from each other.

3.7.4. Summary & conclusions

When either, a male and a suture or 2 males in a drop, were physically stimulated and kept in each other's immediate vicinity, fighting during the first 15 min was higher than when no stimulation was added, paralysis after the first 15 min was also higher, but not significantly.

There are 2 elements to the stimulation treatment. One is the physical touching of the worm by the platinum wire which might have been interpreted as a physical attack, provoking the male "counter-attack" (though in this experiment, the suture or the other male, and not

the platinum wire, received the counter-attacks). The second element is that the males were not just physically stimulated, but were also pushed closer to each other, which may have increased their exposure to pheromones from each other. However, this is only a factor in the 2-male treatment. The increase in attacks due to stimulation was even more dramatic in the suture treatments, where chemical cues would not be involved. Interestingly, although the number of attacks was lower in the unstimulated male-plus-suture treatment than in the unstimulated 2-male treatment (as expected from the previous experiment), the number of attacks in the 2 stimulated treatments, was very similar, averaging 4 attacks per drop in the 15 min period. Mechanical stimulation is a very powerful inducer of attack behaviour.

Augmentation of the number of attacks due to physical stimulation resulted in more drops with paralysis in the short-term ($p < 0.10$), but not in the long-term. Physical stimulation only has an immediate effect on fighting and doesn't have long-term implications.

3.8. Effect of winning a fight on subsequent fights

3.8.1. Introduction

In many species where conflicts are resolved by fighting, the outcome of a previous fight influences the outcome of future fights (Hsu *et al*, 2006;Hsu & Wolf, 1999;Jennings *et al*, 2004). In general, a victor is more likely to succeed again, whereas a loser is highly likely to lose again (Rutte *et al*, 2006). This can be mediated by a better estimation of its own and/or the opponent's fighting ability (Elias *et al*, 2008;Hsu & Wolf, 2001) or by changing its actual fighting ability (Kasumovic *et al*, 2010).

The aim of this experiment is to describe the effect of a previous victory on subsequent fights.

Hypotheses

- With a victor in a drop, the number of attacks is higher than in drops containing 2 naive males.
- With a victor in a drop, the number of drops with fighting is higher than in drops containing 2 naive males.
- With a victor in a drop, the number of drops with paralysis is higher than in drops containing 2 naive males.

- The total duration of fighting in a drop is higher in drops containing a victor than in drops containing 2 naive males.
- The duration per fight is higher in drops containing a victor than in drops containing 2 naive males.
- These differences diminish over time as 1 of the 2 naive males will become a victor after fighting (see section 3.3).
- Drops containing 2 victors have even higher values for the hypotheses above because both victors perceive their own fighting ability as high and are therefore less likely to give up.

3.8.2. Materials and methods

After 2 days at 27 °C, drops with paired and single males were set up to produce victors (survivor of the first fight) and naïves (males that had not been in contact with a conspecific before) for a second round of fights. Depending on the speed with which the victim in the first fighting drop died, 48-72 h after the first fight, victors were put together with another victor or a naïve male of the same age. There were 3 treatments:

- Victor-Victor
- Victor-Naïve
- Naïve-Naïve

The second fights were always staged in a drop where no fighting had occurred previously. In case of the Naïve-Naïve and Victor-Naïve experiments, the males were put together in the drop of the naïve. Two victors were placed in a drop previously occupied by a naïve but removed male.

Immediately following the pairing of 2 males for the second round of fights, the fighting behaviour of the males was observed for 60 min. The start time and duration of each fight were recorded. For drops in which no fights occurred, the duration of fighting was recorded as 0 s. After about 24 h, paralysis and death were checked. After about 48 h, only dead or alive was scored.

3.8.3. Results

Inspection of the data showed a similar trend for Victor-Victor and Victor-Naïve pairs: 35-45 % of drops had paralysis after 1 h compared to just 10 % of drops with Naïve-Naïve (Appendix figures 2, 3 and 4). Because of the interest in the effect of previous victory on a fight, only 1 victor needed to be present in a drop to show the effect on fighting behaviour. The Victor-Victor and Victor-Naïve treatments were thus grouped into "drops with at least 1 victor".

The grey bars on Figure 3.19 show that there was a trend ($0.1 > p \geq 0.05$) for more fighting within the first hour of a battle when 1 opponent had won a fight before. This fighting then resulted in paralysis (Figure 3.19: blue bars) which occurred in significantly more drops with at least 1 victor (X^2 (1, n = 45) = 5.397, p = 0.020).

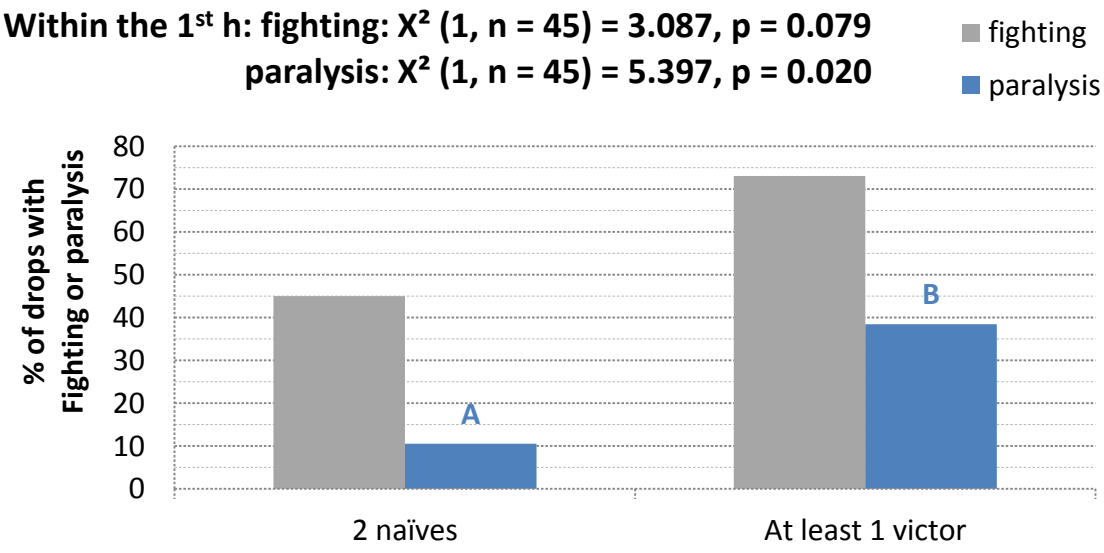


Figure 3.19 The effect of winning a previous fight on the number of drops with fighting (orange) or with paralysis (blue) during the 1st hour after the 2 males were put together in a drop. Bars accompanied by the same, or by no letter are not significantly different from each other. The number of drops with 2 naïves =19, with at least 1 victor =26.

The number of fights in a drop (Figure 3.20-a) was not significantly different ($p > 0.1$) whether 2 naïve males were opposed against each other or when at least one of the opponents had been victorious in a previous fight. Where at least 1 of the opponents was successful in a previous fight, the total amount of time spent fighting (Figure 3.20-b) and the mean amount of time spent in a single fight (Figure 3.20-c) were higher than when both males were naïve (respectively p = *0.042* and p = *0.032*).

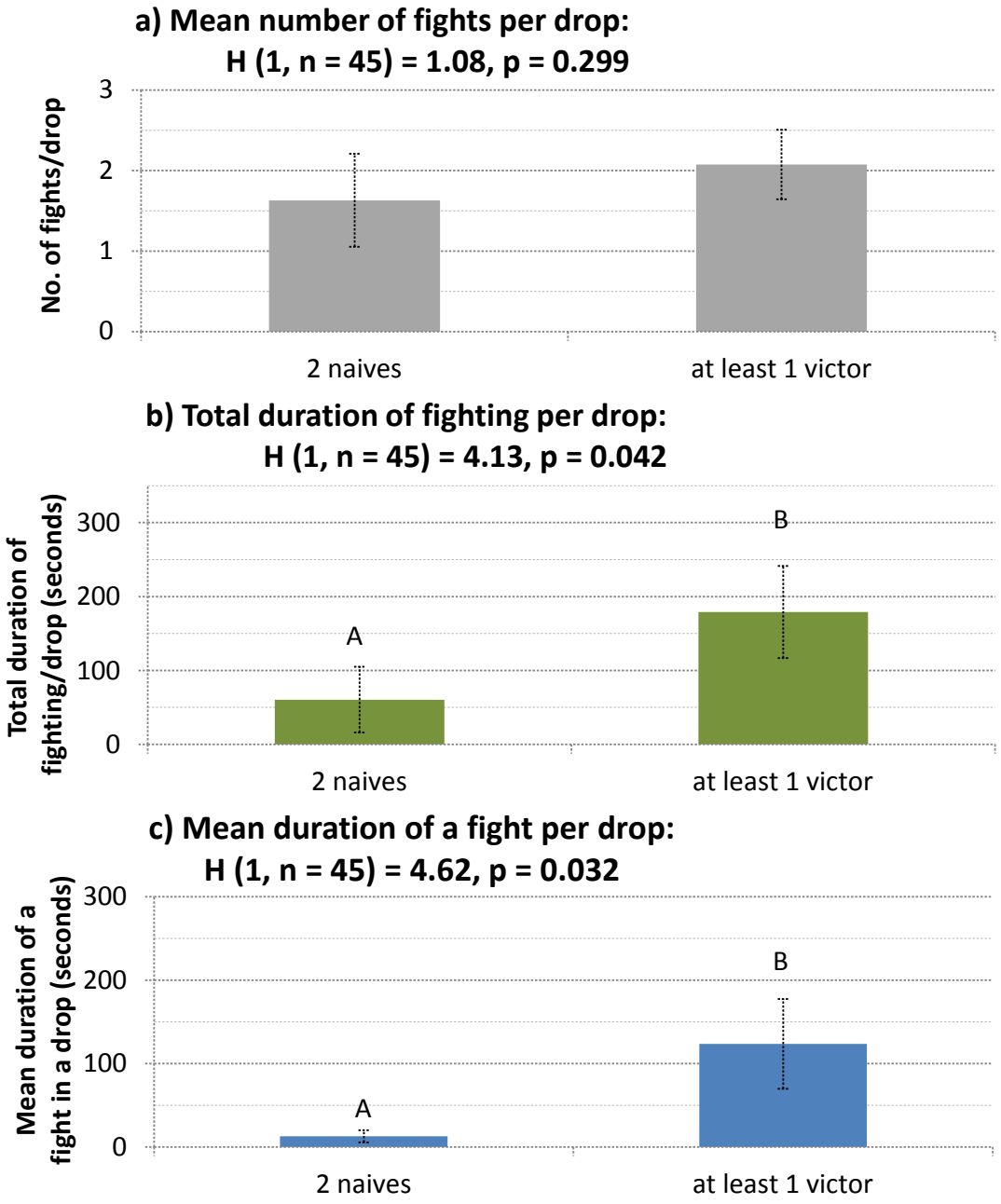


Figure 3.20 **The effect of winning a previous fight on a) the number of attacks per drop; b) the total duration of attacks per drop and c) the mean duration of an attack in a drop during the 1st hour after the males were put together in a drop. Bars accompanied by the same, or by no letter are not significantly different from each other. All values shown are mean+/-SE. Kruskal-Wallis, DF=1.**

The number of drops with paralysis or death after 24 h (Figure 3.21-a) or with death after 48 h (Figure 3.21-b), were higher when at least 1 male was a victor, but this was not significant (all $p > 0.4$).

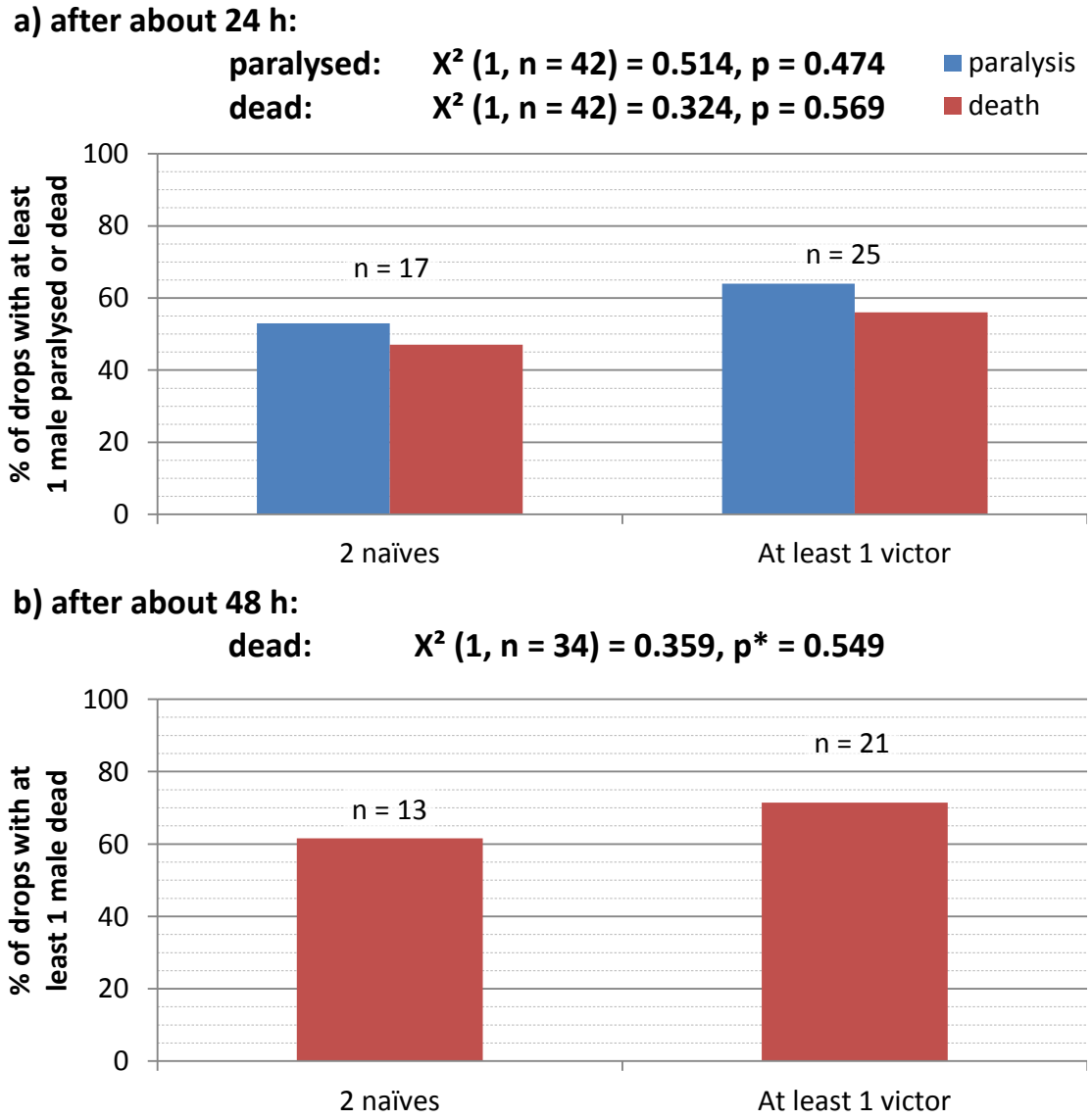


Figure 3.21 The effect of winning a previous fight on the number of drops with paralysis or death a) about 24 h and b) about 48 h after the 2 males were put together in a drop. Bars accompanied by the same, or by no letter are not significantly different from each other. n is the number of drops used for the treatment. n is the number of drops for that treatment.

3.8.4. Summary & conclusions

Comparing the results within the first hour for drops with 2 males of which at least 1 had won its previous fight to drops where both males had not been in contact with a conspecific before, the "at least 1 victor" had significantly more drops with paralysis ($p = 0.20$, a trend for

more drops with fighting (p = 0.079 < 0.100), significantly more time spent on fighting per drop (p = *0.042*) and a significantly higher duration per fight (p = *0.032*). After 24 h, the effect of the presence of a victor was no longer significant. We can then conclude that the presence of a victor elevates the readiness to fight and to paralyse. Because of the difficulties in recognizing individual males and the difficulties in finding an appropriate method to identify males (see section 3.4.1), it was not possible in this setup to investigate whether the victor was the male that initiated more fights and was more able to paralyse its naïve opponent. However, considering the literature on the effect of victory on fighting in other animals (Hsu *et al*, 2006;Jennings *et al*, 2004;Kasumovic *et al*, 2010;Rutte *et al*, 2006), it seems very likely that this is so. In general, a previous battle enables an individual a more accurate estimation of its resource holding potential (RHP) and a victor will then be more ready to fight again.

3.9. Generation & Relatedness

3.9.1. Introduction

In the case of a colony forming insect that occupies habitats that can support a colony for at least a few generations before terminating events prevail (e.g. aphids), Hamilton (1979) suggested that when colony founding is commonly associative, the sex ratio in the first generation (F_0, founder generation) would be about balanced and males would fight, whereas the subsequent generations (F_{n+1}) would show fewer males that are also less prone to fighting. Because *Steinernema* species (except *S. hermaphroditum*) are dicœcious, pathological parasites of insects that disperse through infective juveniles when the insect cadaver can support no more growth, *Steinernema* species like *S. longicaudum* fit Hamilton's prerequisites. Moreover, the first generation of adults in an infected insect has a balanced sex ratio (Alsaiyah *et al*, 2009). The life cycle of *Steinernema* species entail large environmental differences for the different generations. The founder generation of the infection of an insect went through the infective juvenile stage which had dispersed from the cadavers in which they were born (Griffin *et al*, 2005) (their natal cadaver). Subsequent generations of adults have not been through the infective juvenile stage and are not able to disperse out of the cadaver. The size of the population in an insect cadaver also varies extremely with the different generations. Although little is known of the population biology of EPN in nature, the founder generation is expected to normally contain only a few individuals whereas subsequent generations can

number into many thousands individuals (Wang & Bedding, 1996). One of the aims of the following experiments was to investigate the fighting behaviour in the different generations.

The different generations are also characterized by differences in relatedness of the individuals in the cadaver. The degree of relatedness in the founder generation is more likely to be lower than in subsequent generations. Inbreeding is likely to increase as an infection progresses. Fighting a close relative is best avoided as a relative's offspring contributes to an individual's inclusive fitness (West *et al*, 2002). The situation where relatives compete for mating partners in a restricted environment is called local mate competition (LMC) (West *et al*, 2001). LMC is often avoided by a female biased sex ratio so that there are plenty of mating opportunities for each male and contests are not necessary (Nelson & Greeff, 2009). Recognition of close kin including nestmates from less closely related conspecifics including non-nestmates is, theoretically, another way to avoid fighting between relatives (Innocent *et al*, 2011;Nowbahari *et al*, 1999). Lethal fighting between relatives can however occur, e.g. sibling rivalry in ants (Heinze & Weber, 2011) and lethal fighting between sons in *Melittobia australica* (a parasitoid wasp) (Abe *et al*, 2005). The second aim of this section is to ascertain whether fighting in *Steinernema longicaudum* is influenced by the degree of relatedness and if so, in what way?

Hypotheses

- The number of drops with fights is higher for first generation males than for the subsequent generations.
- The number of drops with paralysis is higher for first generation males than for the subsequent generations.
- This difference in fighting behaviour between the generations does not drop off over time.
- The number of drops with fights is higher for unrelated males than for related males.
- The number of drops with paralysis is higher for unrelated males than for related males.
- The difference in fighting behaviour associated with the level of relatedness does not drop off over time.

3.9.2. Materials and methods

To produce bacteria-inoculated haemolymph, 3 drops in which a cadaver-produced infective juvenile had developed into a male - these drops should have been rich in *Xenorhabdus* - were mixed with a few drops of Ringer's solution and about 1.25 ml fresh haemolymph (see section 2.3.2). This solution was thoroughly mixed and used for the production of 25 µl hanging drops on the lid of a Petri dish (see section 2.3.3).

A male and a female aged 3-5 days were placed together in a drop of normal haemolymph so they could mate. These males and females had developed from infective juveniles and so are founder generation nematodes (F_0), hereafter also referred to as 1st generation. They were left at 27 °C for 2-3 days by which time the female had released eggs and these had developed into first stage juveniles.

For the production of 2nd generation (F_1) males, these early juveniles were separated so that each one could develop alone in its own drop, as is the standard protocol in all other experiments. The drop with the gravid female and her offspring was diluted with a drop of Ringer's solution. This diluted drop was then split up in several smaller drops which were diluted even more. This serial dilution made it possible to pick up individual early stage juveniles with a platinum wire (0.100 mm). They were then placed in a bacteria-inoculated drop of haemolymph (see above) and placed at 27 °C for 3-4 days for development into adults.

For the production of sibling 1st generation males, the early juveniles were left to develop in their parental drop with the female present, but the male taken out. When the nematodes were left to develop further like this, the drop became crowded and the production of infective juveniles was stimulated. After several days, infective juveniles were found in the drop. To extract these infective juveniles for the production of single-reared males, a similar serial dilution as above was used. The infective juveniles were then picked up individually with a platinum wire and also reared to adults in inoculated haemolymph (3-4 days). The production of first and second generation males of known lineage is illustrated in Figure 3.22.

When the juveniles, both 1st and 2nd generation, had developed into males, they were paired with either a sibling (of the same parental drop) or a non-sibling male of the same generation. The worms used for this experiment came from 2 cultures of the same strain maintained independently for about 2 years. The two lineages did not differ in aggressive behaviour (data not shown).

In case mortality between males of the 1st and 2nd generations differed, as many males were left single as pairs were formed. Paralysis and death after at least 17 and 37 h were recorded.

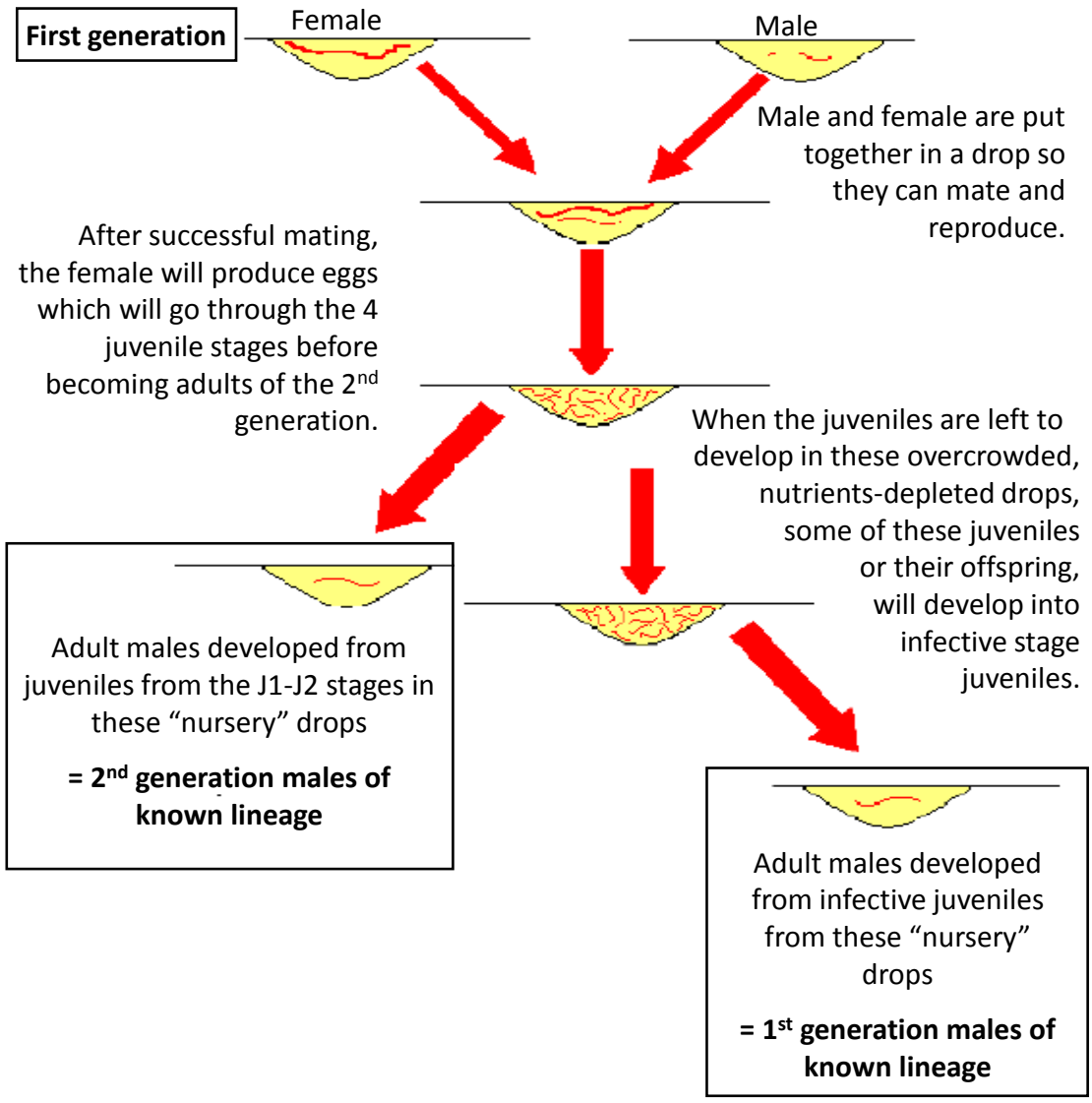


Figure 3.22 The origin of second and first generation males with known lineage.

3.9.3. Results

Because of the experimental setup, it was possible to compare the fighting behaviour of males that had gone through the infective juvenile stage (1st generation, founder generation, F_0) and those that had not gone through the infective juvenile stage before developing into adults (2nd generation, first subsequent generation, F_1). These results are given in 3.9.3.1. It was also possible to compare the fighting behaviour of related and unrelated males in each of the generations (1st generation: 3.9.3.2.1; 2nd generation: 3.9.3.2.2).

3.9.3.1. Generations

Pairs of second generation males performed fighting behaviour and showed significant paralysis and death in their drops relative to single-male drops after 24 h (paralysis: X^2 (1, n = 262) = 13.724, p < *0.001*; death: X^2 (1, n = 262) = 9.024, p = *0.003*) and 48 h (paralysis: X^2 (1, n = 254) = 28.40, p < *0.001*; death: X^2 (1, n = 254) = 28.842, p < *0.001*) but significantly less compared to pairs of first generation males (24 h: paralysis: X^2 (1, n = 179) = 61.016, p < *0.001*; death: X^2 (1, n = 179) = 41.049, p < *0.001*; 48 h: paralysis: X^2 (1, n = 175) = 50.548, p < *0.001*; death: X^2 (1, n = 175) = 45.592, p < *0.001*; Figure 3.23). There was no difference between generations in paralysis and death of singles after either 24 h (paralysis and death: Fishers exact test: p = 1) or 48 h (paralysis: X^2 (1, n = 275) = 0.105, p = 0.745; death: X^2 (1, n = 275) = 0.345, p = 0.557).

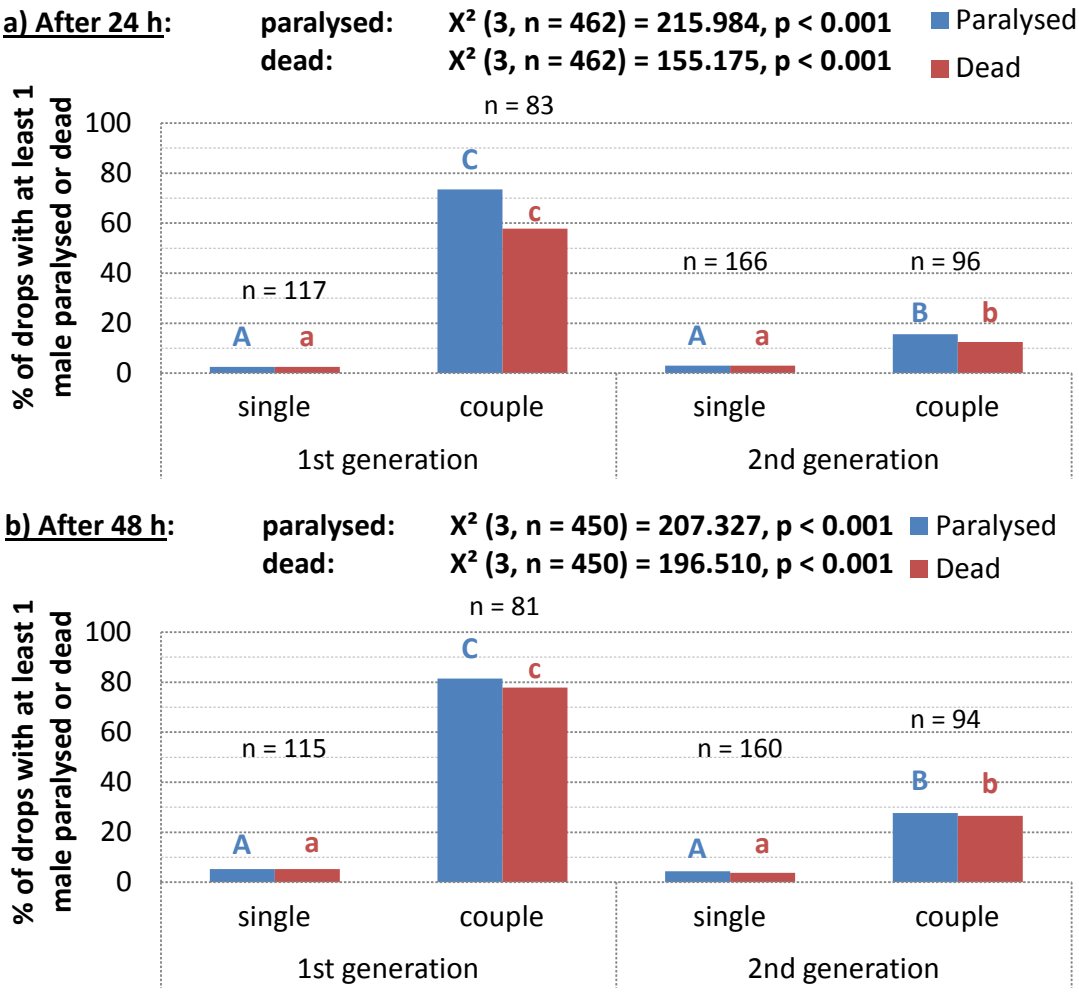


Figure 3.23 Differences in consequences of fighting (paralysis and death) between the first and second generation a) about 24 h and b) 48 h after the males were put together in a drop. Bars accompanied by different letters were significantly different from each other. The capital letters refer to the differences in number of drops with at least 1 paralysed male, the small letters refer to the differences in number of drops with at least 1 dead male.

3.9.3.2. Relatedness

3.9.3.2.1. 1st Generation

After 24 and 48 h, there was no significant difference in the amount of paralysis (24 h: X^2 (1, n = 118) = 0.053, p = 0.818; 48 h: X^2 (1, n = 116) = 0.035, p = 0.852) and death (24 h: X^2 (1, n = 118) = 0.258, p = 0.612 ; 48 h: X^2 (1, n = 116) = 0.167, p = 0.683) occurring in drops with 1st generation males (males that followed the developmental pathway so that they went through the infective juvenile stage before becoming adults) paired with a sibling or a less closely related male (Figure 3.24).

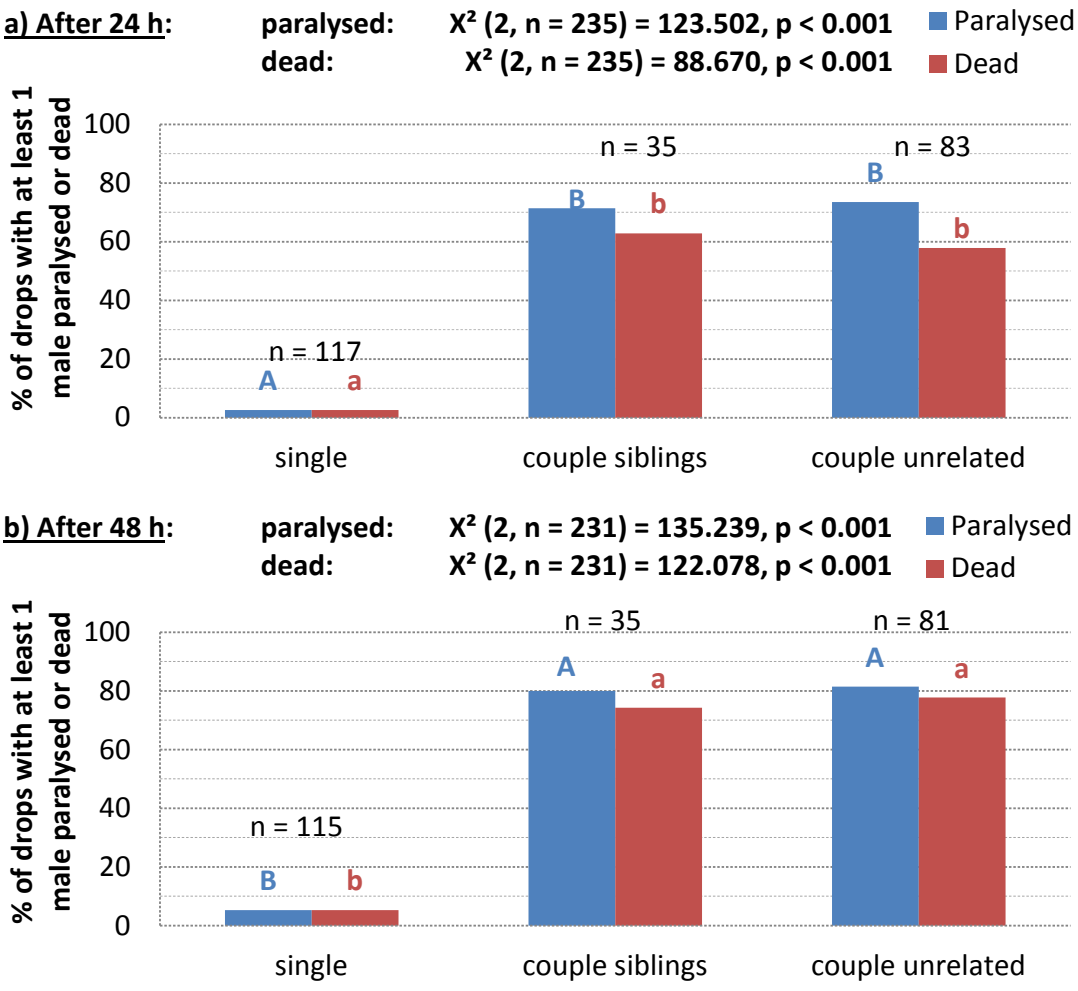


Figure 3.24 Differences in consequences of fighting (paralysis and death) between related (siblings) and unrelated (non-sibling) males of the 1st generation a) about 24 h and b) 48 h after the males were put together in a drop. Bars accompanied by different letters were significantly different from each other. The capital letters refer to the differences in number of drops with at least 1 paralysed male, the small letters refer to the differences in number of drops with at least 1 dead male.

3.9.3.2.2. 2nd Generation

After about 24 h, 2nd generation males that were put together with a sibling did not show any significant difference in paralysis or death (both Fisher's exact test: DF = 1, n = 229, p = 0.143) relative to single male controls, but after 48 h, there was significant paralysis (Fisher's exact test: DF = 1, n = 222, p = *0.035*) and death (Fisher's exact test: DF = 1, n = 222, p = *0.026*) when 2 siblings were put together in a drop. Second generation males that were put together with a non-sibling male, showed significant consequences of fighting (paralysis and death) after 24 h (paralysis: X^2 (1, n = 262) = 13.724, p < *0.001*; death: X^2 (1, n = 262) = 9.024, p = *0.003*) and 48 h (paralysis: X^2 (1, n = 254) = 28.400, p < *0.001*; death: X^2 (1, n = 254) = 28.842, p < *0.001*). After 48 h, there were significantly more drops with non-sibling males that had at least 1 male paralysed or dead than drops with sibling males (paralysis: X^2 (1, n = 156) = 4.773, p = *0.029*; death: X^2 (1, n = 156) = 4.199, p = *0.04*), this difference was not found 24 h after the males were put together in a drop (paralysis: X^2 (1, n = 159) = 2.045, p = 0.153; death: X^2 (1, n = 159) = 0.830, p = 0.362).

Males that had developed without passing through the infective juvenile-stage showed more paralysis and kill when paired with an unrelated male than when paired with a sibling.

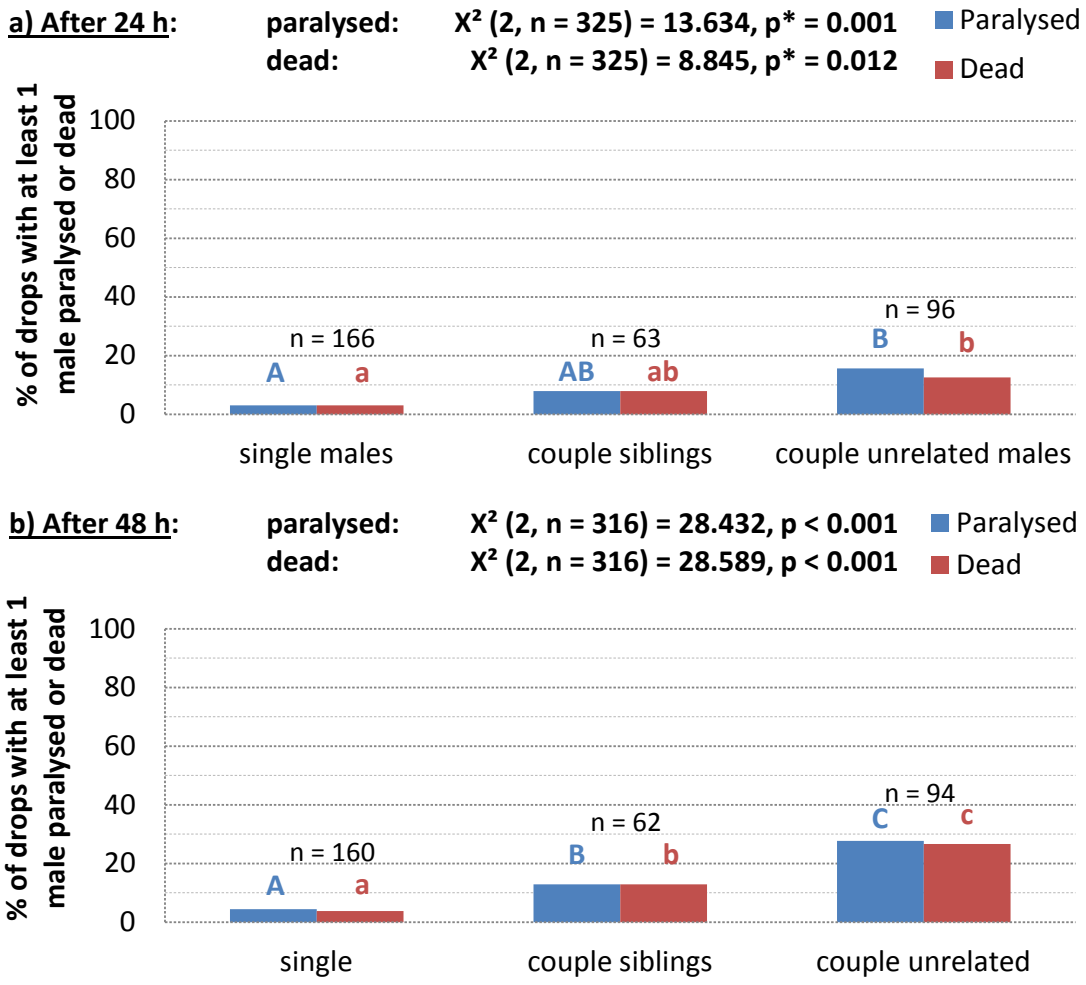


Figure 3.25 Differences in consequences of fighting (paralysis and death) between related (siblings) and unrelated (non-sibling) males of the 2nd generation a) about 24 h and b) 48 h after the males were put together in a drop. Bars accompanied by different letters are significantly different from each other. The capital letters refer to the differences in number of drops with at least 1 paralysed male, the small letters refer to the differences in number of drops with at least 1 dead male. *: 1 cell with an expected count less than 5.

3.9.4. Summary & conclusions

Pairs of first generation males had higher paralysis and higher kill rates after 24 h (resp.: 73% and 58%) and 48 h (resp.: 81% and 78%) than second generation males (24 h: 16% paralysis, 13% kill; 48 h: 28% paralysis, 27% kill). All males developed and were tested under similar conditions, indicating that going through the IJ pathway influences tendency to fight. Fighting thus occurs in both the founder generation and the first subsequent generation and it seems very likely that males of subsequent generations of *Steinernema longicaudum* will also perform fighting behaviour.

These results are in line with the expectations from Hamilton's 1979 theory regarding associative colony-forming insects occupying temporary suitable habitats that can only cater for a few generations: the second generation males fight and paralyse, but to a lesser extent than the 1st generation males and this difference doesn't fade out over time. Hamilton's 1979 theory is thus not only relevant for insects but can also be generalised to include other animals.

Paralysis and death in the first generation male drops was not different between drops with related (24 h: 71% paralysis, 63% kill; 48 h: 80% paralysis, 74% kill) or unrelated males (24 h: 73% paralysis, 58% kill; 48 h: 81% paralysis, 78% kill). This is in contrast to the amounts of paralysis and death within the 2nd generation that were related to the degree of relatedness between the 2 males. Second generation siblings showed paralysis and death only after 48 h (13% paralysis, 13% kill) whereas unrelated males showed paralysis and death already after 24 h (16% paralysis, 13% kill). After 48 h, the number of drops with at least 1 male paralysed or dead were significantly higher when the males were not related (28% paralysis, 27% kill).

Cataglyphis niger ants show aggressive behaviour in graded patterns as a function of their relatedness to the opponent (Nowbahari *et al*, 1999). Founder generation *S. longicaudum* males exhibit a high level of aggression independent of relatedness to their competitor, but the subsequent generation's level of aggression is lower ánd depends on the level of relatedness between the contestants. Males of the non-founder generation of *S. longicaudum* perform less aggressive behaviour towards related than towards unrelated males.

For differential fighting behaviour towards relatives and non-relatives to occur, discrimination of kin and non-kin is necessary (Innocent *et al*, 2011). Recognition involves the perception of a cue or cues signalling relatedness (kin discriminating cue(s)). This recognition can be based on contextual cues (e.g. spatial: female Belding's ground squirrels, *Urocitellus beldingi*, retrieve young when they are placed at their burrow entrance as normally only her own young are in the immediate vicinity of their natal burrow (Mateo, 2004)), prior association (e.g. subordinate females of the Seychelles warbler (*Acrocephalus sechellensis*) assist breeding pairs based on the continued presence of the primary female who previously fed the subordinate (Komdeur *et al*, 2004)), phenotype matching (e.g. *U. beldingi* loose the memory of familiar non-kin after hibernation, yet, they can still discriminate kin from non-kin based on their own odour (Mateo, 2010)) or it can be mediated by recognition alleles (e.g. the social amoebae *Dictyostelium discoideum* rely on the polymorphic genes tgrB1 and tgrC1 to recognize relatives when aggregating to form multicellular fruiting bodies in which 20 to 30 % of the individuals altruistically die while constructing a cellular stalk (Hirose *et al*, 2011))

(Blaustein, 1983;Brown & Eklund, 1994;Gherardi *et al*, 2012;Komdeur *et al*, 2004;Mateo, 2004). These are not likely to be all the mechanisms for recognition and are not mutually exclusive. The utilised recognition mechanism can be situation dependent or even life-stage dependent (Beecher *et al*, 1981;Mateo, 2004). For example, *U. beldingi* mothers use spatial cues until just before the pups leave the nest, use phenotype matching after emergence from the nest to recognize individual young and use prior-association for discriminating among own and unfamiliar young (Mateo, 2004).

In these generation-relatedness experiments, the link to the parental haemolymph drop was kept minimal: non-founder generation males were transferred individually into drops of new haemolymph mixed with drops of unrelated, conspecific infected haemolymph when they were still in a very early juvenile stage. The 2nd generation worms were thus separated at a very early stage in their development which makes prior association as a kin discriminating method unlikely. However careful the juveniles were transferred, co-transfer of some chemical cues or even bacteria (attained in the maternal drop) cannot be ruled out, but considering these amounts would be very minimal, these contextual cues are not likely to have been involved in kin recognition here. Both unrelated and related 2nd generation males developed in separate drops of haemolymph, rendering spatial cues also improbable as a means to identify close kin. Further investigation into the mechanism of kin discrimination would be necessary to confidently conclude that in an insect cadaver, 2nd generation male *S. longicaudum* differentially fight opponents based on their genetic relatedness.

4. Fighting behaviour in the Steinernematidae

4.1. Introduction

Not all *Steinernema spp*. fight, paralyse and kill to the same extent. O'Callaghan (2006) found that males of *S. longicaudum* and *S. carpocapsae* performed intraspecific fighting and killing with death of at least one male in 86 % and 79 %, respectively, of drops with 2 males 24-48 h after pairing. For *S. feltiae* fights however, only 5 % of the drops had death of at least 1 male 24-48 h after pairing in a drop of haemolymph (O' Callaghan, 2006).

This chapter looks into the fighting behaviour of other species in the *Steinernema* genus in order to study the evolution of fighting behaviour within the Steinernematidae. The additional species tested were *S. kraussei*, *S. glaseri* and *S. bicornutum*. *S. feltiae* which was a non-fighter in O'Callaghan's study was alsoincluded. Figure 3.2 in Chapter 3 shows the phylogenetic relationships in the genus and groups species into 5 clades. Apart from clade I, at least 1 species of each of the clades was studied either by me or others in the course of this project: *S. carpocapsae* (clade II), *S. kraussei*, *S. feltiae* (both clade III), *S. bicornutum* (clade IV), *S. longicaudum* and *S. glaseri* (both clade V). This chapter includes results from projects carried out by undergraduate students (Everard, 2006;Foster, 2007;Igoe, 2008) under my supervision during the course of my PhD-study, and designed to be part of the broader study.

4.2. Objectives

This chapter studies the distribution of fighting behaviour within the *Steinernema* genus and thus aims to shed light on the evolutionary origins of fighting in the *Steinernema* genus by including the data from additional species. Other aims were to confirm the fighting ability of *S. feltiae* and to investigate the involvement of species specific strains of *Xenorhabdus bovienii* in paralysis and/or killing.

4.3. Steinernematidae symbiotic with *Xenorhabdus bovienii*: *S. feltiae* and *S. kraussei*

4.3.1. Introduction

In O'Callaghan (2006), *S. feltiae* caused paralysis in only 5 % of drops. *S. kraussei,* another species belonging to clade III, was reported to have 90-100 % of drops with at least 1 male paralysed or dead within 24-48 h (Igoe, 2008)(see section 4.3.3.2). Additional to the close genetic relationship between *S. feltiae* and *S. kraussei* (Nadler *et al*, 2006), both are symbiotically associated with *X. bovienii* (Adams *et al*, 2006). Each *Steinernema* species has a unique symbiotic association with one *Xenorhabdus* species, but, one *Xenorhabdus* species can be the symbiont of several *Steinernema* species, as is the case for *S. feltiae* and *S. kraussei* (Adams *et al*, 2006). The comparison of the fighting behaviour between these 2 nematode species can thus not only inform us of the level of fighting, paralysis and kill of species in clade III but also shed light on a possible influence of the symbiont strain on fighting behaviour.

The tests of intraspecific fighting in *S. feltiae* and *S. kraussei* were repeated but fights by each of these species in drops containing the symbiont of the other were also staged, this to test the hypothesis that the *S. kraussei* symbiont facilitates killing but the symbiont of *S. feltiae* does not.

4.3.2. Materials and Methods

Steinernema kraussei infective juveniles were placed into fresh haemolymph drops as described in 2.3 and put into the 15 °C incubator for 4-6 days to develop into adults. *Steinernema feltiae* infective juveniles were placed into fresh haemolymph drops as described in 2.3 and put into the 20 °C incubator for 4-6 days to develop into adults. The males were then assigned to one of the treatments shown in Table 4.1.

Table 4.1 Treatments to which the 4-6 day old males were assigned in order to investigate the effect of the species specific strains of *Xenorhabdus bovienii* on paralysis and kill.

Males		Drop
No.	Species	Prior occupant
1	*S. feltiae*	*S. feltiae*
2	*S. feltiae*	*S. feltiae*
1	*S. feltiae*	*S. kraussei*
2	*S. feltiae*	*S. kraussei*
1	*S. kraussei*	*S. kraussei*
2	*S. kraussei*	*S. kraussei*
1	*S. kraussei*	*S. feltiae*
2	*S. kraussei*	*S. feltiae*

Each male was rinsed thoroughly in Ringer's solution to reduce the amount of contamination from its own symbiont adhering to the cuticle: the male was taken out of the haemolymph drop it had developed in and was placed in a drop (30 µl) of Ringer's solution on a Petri dish. The male was moved through this drop of Ringer's solution for about 5 s and then lifted out of the drop and placed into a fresh drop of Ringer's where the same washing procedure was repeated. Each male was washed in a total of 4 drops of Ringer's before it was placed in its experimental drop. All manipulations were done with a platinum wire which was flamed and cooled in between the different drops. The drops were then put in an incubator with a temperature suitable to the nematode species, for *S. kraussei* this was 15 °C, for *S. feltiae* this was 20 °C.

After about 3-5, 24 and 48 h, the drops were checked for paralysis and survival.

4.3.3. Results

4.3.3.1. *Steinernema feltiae* in conspecific drops

When 2 *Steinernema feltiae* males were put together in a drop in which they or a conspecific had developed, paralysis or death occurred in 22.5 % of drops within 3-5 h after placing them together (Table 4.2). The number of drops with at least 1 paralysed or dead male increased to 50 % and 55.3 % after 24 and 48 h respectively. Apart from the O'Callaghan (2006) study (see Table 4.3 and Table 4.4), these results are similar to those from the other studies on the occurrence of paralysis or death in drops with singles or couples of *S. feltiae*.

Table 4.2 Summaries of all studies on the occurrence of paralysis or death in drops with 1 or 2 *Steinernema feltiae*. When possible X^2 - tests with df = 1 were used. In the cases where expected numbers were lower than 5, a fisher's exact test was done.

Study	Observation timepoint	Drops with at least 1 dead or paralysed male on total No. of couples		Dead single male controls on total No. of controls		Difference between singles and couples?	
		No.	%	No.	%	χ^2	p
O' Callaghan (2006)	24-48 h:	1/19	5.0	0/14	0.0	-	> 0.9
Everard (2006, unpublished)	24 h:	6/9	67.0	0/9	0.0	-	0.009
	4 days:	3/6	50.0	-	-	-	-
Foster (2007)	1 h:	2/25	8.0	0/51	0.0	-	0.105
	24 h:	9/25	36.0	2/51	3.9	-	< 0.001
	48 h:	13/25	52.0	6/51	11.8	14.485	< 0.001
This study	3-5 h:	9/40	22.5	0/43	0.0	-	< 0.001
	24 h:	24/48	50.0	1/55	1.8	32.372	< 0.001
	48 h:	26/47	55.3	2/54	3.7	33.411	< 0.001
Overall without O' Callaghan's (2006) study	**24 h:**	**39/82**	**47.6**	**3/115**	**2.3**		
	48 h:	**39/72**	**54.2**	**8/119**	**7.6**		

Table 4.3 Statistical analysis of the differences between all the studies that have been performed on the fighting behaviour of *S. feltiae* males at the 24 h timepoint. Statistics on the single drops were not relevant because the expected values were too low. A general X^2-test on the number of coupled males drops with at least 1 paralysed male after 24 h showed a significant difference between the studies: χ^2 (3, n = 101) = 14.428, p = *0.002*, 1 cell with exp. count < 5). After further analysis, the differences were due to differences between the O' Callaghan (2006) study and all other studies. When possible X^2 tests with df = 1 were used. In the cases where expected numbers were lower than 5, a fisher's exact test was done.

24 h Studies		Difference between these studies? χ^2	p
Foster (2007)	This study	1.301	0.254
Foster (2007)	Everard (2006, unpublished)	-	0.139
Foster (2007)	**O' Callaghan (2006)**	**-**	*< 0.05*
This study	Everard (2006, unpublished)	-	0.476
This study	**O' Callaghan (2006)**	**11.647**	*< 0.005*
Everard (2006, unpublished)	**O' Callaghan (2006)**	**-**	*< 0.005*

Table 4.4 Statistical analysis of the differences between all the studies that have been performed on the fighting behaviour of *S. feltiae* males at the 48 h timepoint. Statistics on the single drops were not relevant because the expected values were too low. A general X^2-test on the number of coupled males drops with at least 1 paralysed male after 48 h showed a significant difference between the studies: χ^2 (2, n = 91) = 14.667, p = *0.001*. After further analysis, the differences were due to differences between the O' Callaghan (2006) study and all other studies. X^2-tests with df = 1 were used.

48 h Studies		Difference between these studies? χ^2	p
Foster (2007)	This study	0.072	0.788
Foster (2007)	**O' Callaghan (2006)**	**10.870**	*< 0.005*
This study	**O' Callaghan (2006)**	**14.024**	*< 0.001*

Over these 4 studies, the incubation temperature and period varied, as did the handling method (using a platinum wire or a microcapillary tube attached to a mouth respirator), the amount of haemolymph transferred with the nematodes and the timing of the observation time points. However, as these parameters also varied between the 3 studies of which the results are not statistically different from each other, these parameters are unlikely to be responsible for the statistically significant differences of these 3 studies with O' Callaghan's (2006) study (Everard (2006): Fisher's exact test, n = 28, p < *0.005*; Foster (2007):Fisher's exact test, n = 44,p < *0.05*; this study: X^2 (1, n = 67) = 11.647, p < *0.005*). The strain of *S. feltiae* that was used was different between O' Callaghan (2006), *S. feltiae* strain UK76, and the other 3 studies which used the Irish 4CFMO isolate.

Table 4.5 The methodological differences between all the studies that have been performed on the fighting behaviour of *S. feltiae* males.

Study	Rearing and fighting temperature	Observation timepoints	Handling method	Relative amounts of haemolymph transferred with the nematodes	*S. feltiae* strain
O' Callaghan (2006)	23 °C	24-48 h	Microcapillary tube attached to a mouth respirator	Substantial	UK76
Everard (2006, unpublished)	27 °C	24 h and 4 days	Microcapillary tube attached to a mouth respirator	Substantial	4CFMO
Foster (2007)	20 °C	1, 24, 48 h	Platinum wire	Minimal	4CFMO
This study	20 °C	3-5, 24, 48 h	Platinum wire and rinsing of the nematode in Ringer's solution (4x)	Very minimal, almost none	4CFMO

The attached CD contains a recording of a fight between 2 *S. feltiae* males from the 4CFMO strain (*S. feltiae* Fight.AVI). It also contains a recording of an injured and paralysed *S. feltiae* 4CFMO male from a different fighting couple (the males from Figure 4.1; *S. feltiae* Injured male.AVI).

The occurrence of a paralysing fight between 2 *S. feltiae* males, could be observed h after the fight occurred by the consequences of the fight. These consequences are the same as for *S. longicaudum* fighting: the victim can be paralysed (varies from not being able to move a certain part of the body to complete immobility) and will most likely eventually die. Seven out of 9 paralysed *S. feltiae* males, showed puncture wounds or even a ruptured body wall from which the internal organs were protruding (see male 1 in Figure 4.1). The degree of loss of contents varied from only a small group of cells to almost the whole digestive tract and the complete gonad. Puncture wounds were also observed after fights between 2 *S. longicaudum* males (see Chapter 5), but the occurrence of ruptures and protrusion of internal organs were not observed as often as for *S. feltiae*. Male 2 in Figure 4.1, was capable of moving normally while male 1 was only able to move the tip of its head and had a rupture in its lower body through which a large part of its intestine and about one third of its reproductive tract were protruding.

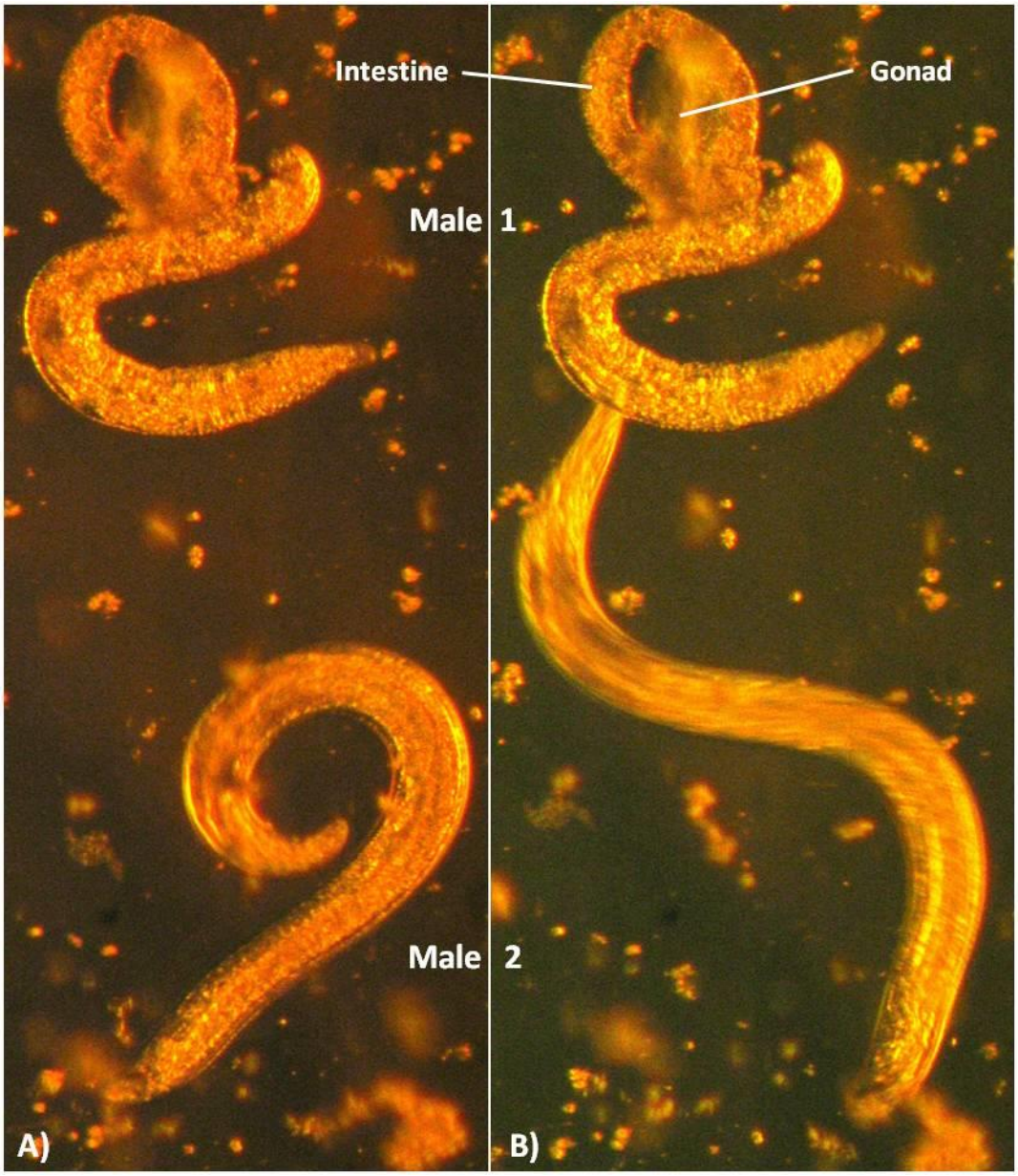


Figure 4.1 Pictures A and B are taken from a timeseries about 5 h after the 2 *S. feltiae* males were placed together in a drop. Picture A was taken a couple of seconds before picture B. This timeseries shows movement in the bottom male (male 2: had its upper body coiled in A) and was in an undulating movement in B)) and the lack of movement in the top male. Male 1 male was only capable of moving its head a little bit and had suffered a rupture of its body wall through which its digestive and reproductive tract protruded.

4.3.3.2. *Steinernema kraussei* in conspecific drops

When 2 *Steinernema kraussei* males were put together in a drop in which they or a conspecific had developed, paralysis or death occurred in 35 % of drops within 3-5 h after placing them together (see Table 4.6). The number of drops with at least 1 paralysed or dead male increased to 60.8% and 61.2% after 24 and 48 h respectively. These results are significantly different from those in Igoe's studies (2008) (see Table 4.6) on the occurrence of

paralysis or death in drops with singles or couples of male *S. kraussei* (Fisher's exact test, 24 h: n = 63, p < *0.05*; 48 h: n = 62, p < *0.05*).

Igoe (2008) reported that in 75 % of 12 drops with 2 *S. kraussei* males, fighting was observed within the first 10 min after the males were put together in a drop.

The attached CD contains a recording of a fight that occurred between 2 *S. kraussei* males (*S. kraussei* Fight.AVI). The recording started with one male wrapped around the head of its victim with its tail. At first the coil looked rather tight and the victim was only moving its head a little. The coil loosened and the attacker let go of its victim. The attacker then slowly started to move more and increasingly showed it could move all parts of its body fluently. The victim however showed only slight head movement.

The attached CD also contains a recording of a male that was injured and paralysed after a fight had occurred between it and another *S. kraussei* male (*S. kraussei* Injured male.AVI). The victim has suffered a wound in its upper body through which content of its intestine or even a part of its intestine has spilled out. The male moved slowly and in rather short bouts.

Table 4.6 Summaries of all studies on the occurrence of paralysis or death in drops with 1 or 2 *Steinernema kraussei*. When possible X^2 - tests with df = 1 were used. In the cases where expected numbers were lower than 5, a fisher's exact test was done.

Study	Temperature used for rearing and fighting	Observation timepoint	Drops with at least 1 dead or paralysed male on total No. of couples		Dead single male controls on total No. of controls		Difference between singles and couples?	
			No.	%	No.	%	χ2	p
Igoe (2008)	15 °C	2 h:	7/12	58.3	0/12	0.0	-	*< 0.010*
		24 h:	11/12	91.7	0/12	0.0	20.308	*< 0.001*
		48 h:	12/12	100.0	0/12	0.0	24.000	*< 0.001*
This study	15 °C	3-5 h:	14/40	35.0	0/29	0.0	12.734	*< 0.001*
		24 h:	31/51	60.8	1/49	2.0	39.360	*< 0.001*
		48 h:	30/49	61.2	1/48	2.1	39.002	*< 0.001*

The occurrence of a paralysing fight between 2 *S. kraussei* males, could be observed h after the fight occurred by the consequences of the fight. These consequences are the same as for *S. feltiae* fighting: the victim can be paralysed, could suffer a puncture wound with possible protrusion of the internal organs and will eventually die. For *S. kraussei* 10 out of 14 paralysed males showed puncture wounds or even a ruptured body wall from which internal organs were protruding (see Figure 4.2). The degree of loss of content varied from only a small group

of cells to almost the whole digestive tract and the complete gonad protruding. Male 1 in Figure 4.2, was not capable of any movement and had internal organs protruding from a puncture wound in the tip of its tail. Male 2 was capable of all normal movements.

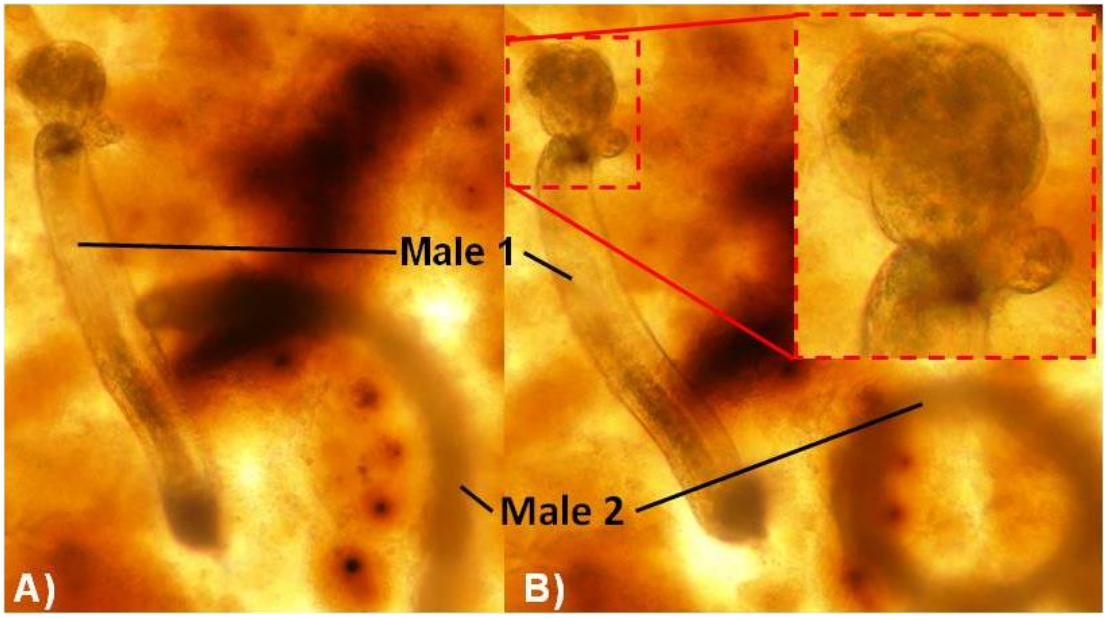


Figure 4.2 Pictures A and B are taken from a timeseries about 5 h after the 2 *S. kraussei* males were placed together in a drop. Picture A was taken a couple of seconds before picture B. This timeseries makes it possible to see the ability of normal movement of male 2 (stretched in A and coiled up in B) and the lack of movement of male 1. Male 1 was not capable of any movement and had internal organs protruding through a wound in its tail, see the insert in B (2.3x the little red square).

4.3.3.3. *Steinernema kraussei* and *Steinernema feltiae* in each other's drops

The health (measured as "paralysis") and mortality of single *S. kraussei* and *S. feltiae* males (see Figure 4.3) did not differ whether they were put in a conspecific or a heterospecific adult nematode's drop. Paralysis and kill also did not differ when 2 conspecific males (see Figure 4.4) were put together in a conspecific or a heterospecific adult nematode's drop.

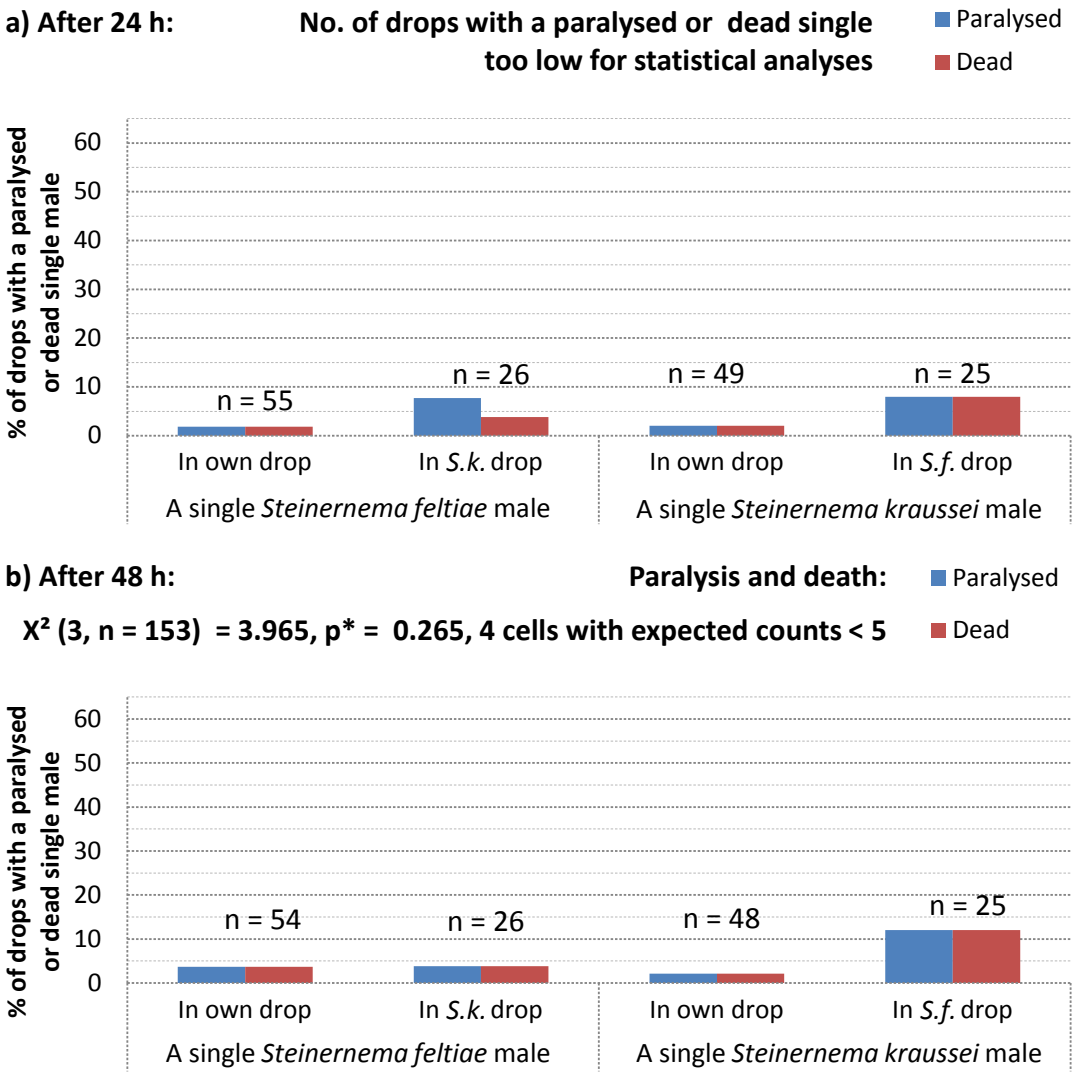


Figure 4.3 Paralysis and death in drops containing either 1 *S. feltiae* or 1 *S. kraussei* male, either replaced in it's own drop or in a vacated drop of the other species. Observations were made at 3 time points: about 3-5 h (not shown in the figure, no single males died or got paralysed), a) 24 h and b) 48 h after the start of the experiment. *S.f.* = *Steinernema feltiae*; *S.k.* = *Steinernema kraussei* . At each time point, there were no significant differences found. "n" is the number of drops for that treatment.

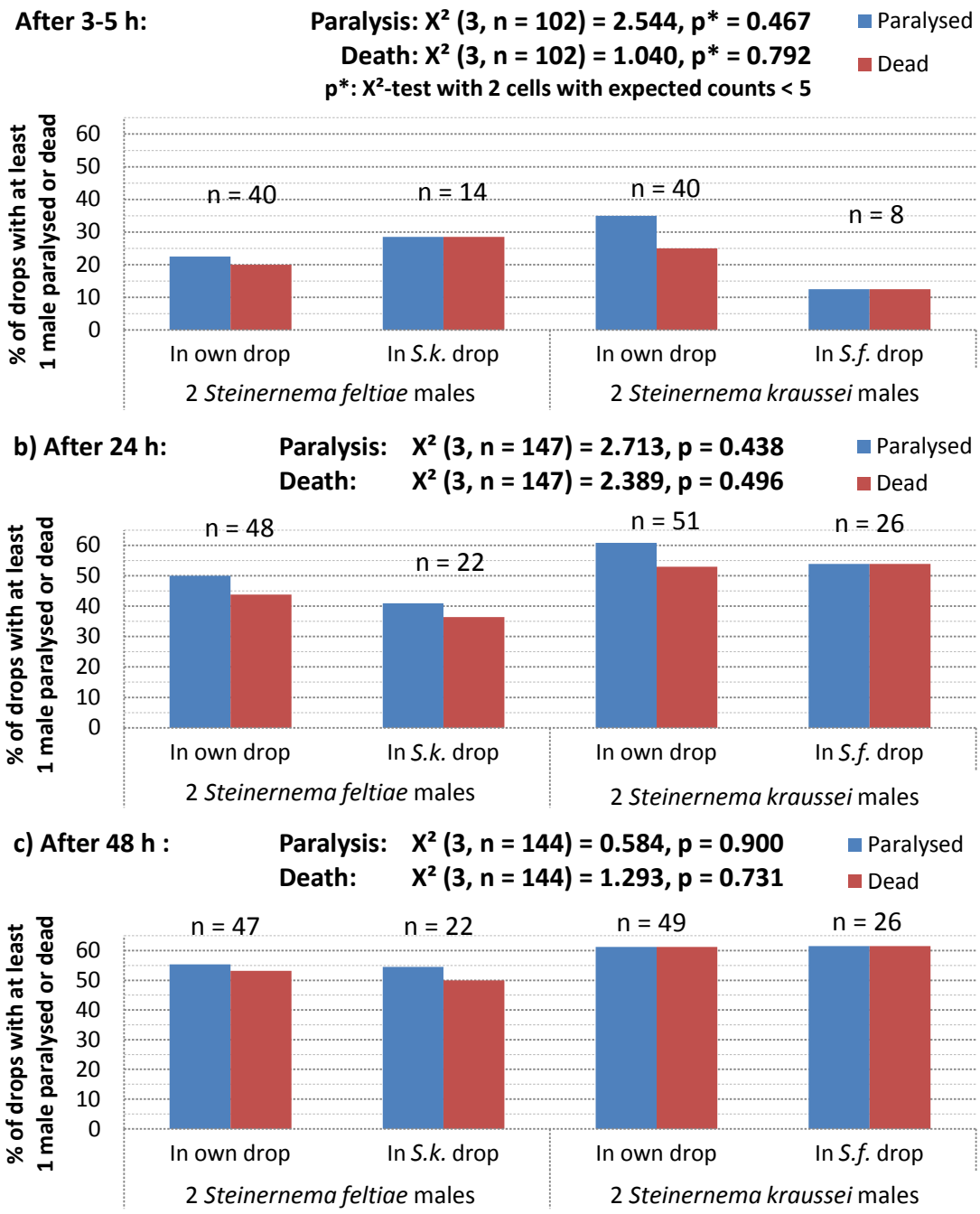


Figure 4.4 Paralysis and death in drops containing either 2 *S. feltiae* or 2 *S. kraussei* males and placed for fighting in one of their own drops, or in drop of the other species. Observations were made at 3 time points: about a) 3-5 h, b) 24 h and c) 48 h after the start of the experiment. *S.f.* = *Steinernema feltiae*; *S.k.*= *Steinernema kraussei* . At each time point, there were no significant differences found. "n" is the number of drops for that treatment.

There were no visual differences between males that were paralysed or died in a heterospecific's drop compared to males that were paralysed or died in a conspecific's drop.

4.3.4. Summary & conclusion

Both *S. kraussei* and *S. feltiae* performed fighting behaviour which could wound, paralyse and kill the victim. For both species, there were no drops with 2 males paralysed or dead. The number of drops with a dead or paralysed male increased over time, but fighting, paralysis and death could be observed about 3 h after the males were put together.

Whether the males of these species were teamed up with a conspecific in the drop of a conspecific or in the drop of a heterospecific adult did not have an effect on paralysis or death. Both in a conspecific drop as in a heterospecific's drop, a paralysed male often (7/9 for *S. feltiae*, 10/14 for *S. kraussei* and 3/5 in a heterospecific's drop) showed wounds through which internal organs could be seen protruding.

Paralysis and death also didn't differ significantly between *S. feltiae* and *S. kraussei* over time.

4.4. *Steinernema glaseri*

4.4.1. Introduction

In O'Callaghan (2006), *S. longicaudum* showed a high paralysis and kill rate that was not matched by other investigated species (*S. feltiae*, *S. carpocapsae)*. The question arose whether this aggressiveness was also found in more closely related species. *Steinernema glaseri* belongs to the same clade as *S. longicaudum*. In case the level of observed fighting, paralysis and kill is similar to that of *S. longicaudum*, the "more aggressive" trait might thus be a characteristic for clade V.

4.4.2. Materials and Methods

Infective juveniles were placed into fresh haemolymph drops as described in Chapter 2.3 and put into the 20 °C incubator for 4-6 days. Males were then either replaced in their drop singly or teamed up with another *S. glaseri* male of the same age. The drops were checked for paralysis and survival after about 1, 24 and 48 h. In between the observation time points, the drops were put in the 20 °C incubator.

4.4.3. Results

The development of infective juveniles into adults was poor: after 6 days at 20 °C, only 20 males were obtained from 80 drops in which an IJ had been inserted. A second batch did not show more development (6 males out of 27 infected drops after 7 days of incubation). Thus, taking into account time constraints, further experiments with *S. glaseri* were abandoned.

With regards to paralysis and death, the data from Foster (2007) (see Table 4.7) and this study (see Table 4.7) differ significantly at the 48 h observation time point (Fisher's exact test: n = 23, 24 h: p = 0.16, 48 h: $p < 0.05$). In both studies, paralysis and death were already observed after 1 h, but only in Foster's 2007 study did they increase over time. Contrary to Foster (2006), this study did not find a significant difference in the incidence of death between the single male drops and the paired males (see Table 4.7). Foster (2007) observed immediate fighting (within 10 min after the males were put together) in 29.4 % of 17 male-male couples. These fights lasted anywhere between 30 s and two min and resulted in paralysis or death in over 80 % of drops after 48 h. Over all experiments that were done with *S. glaseri*, paralysis

and kill were observed in 60.9 % of drops after 24 h and in 69.8 % after 48 h. The studies don't differ significantly at the 24 h observation timepoint (Fisher's exact test: n = 23, p = 0.16), so when analysing the sum of the 2 studies for the 24 h observation timepoint, 3.6 % of single drops contained a dead male and 60.9 % of the coupled drops, which would indicate fighting in *S. glaseri* took place and resulted in significant paralysis and death after 24 h (X^2 (1, n = 51) = 19.968, p < *0.001*).

Table 4.7 Summaries of all studies on the occurrence of paralysis or death in drops with 1 or 2 *Steinernema glaseri*. When possible X^2 - tests with df = 1 were used. In the cases where expected numbers were lower than 5, a fisher's exact test was done.

Study	Rearing and fighting temperature	Observation timepoint	Drops with at least 1 dead or paralysed on total No. of couples		Dead single male controls on total No. controls		Difference between singles and couples?	
			No.	%	No.	%	χ^2	p
Foster (2007)	20 °C	1 h:	6/17	35.3	0/21	0.0	-	*< 0.010*
		24 h:	12/17	70.6	0/21	0.0	21.665	*< 0.001*
		48 h:	14/17	82.4	0/21	0.0	27.382	*< 0.001*
This study	20 °C	1 h:	2/6	33.3	0/7	0.0	-	> 0.100
		24 h:	2/6	33.3	1/7	14.3	-	> 0.500
		48 h:	2/6	33.3	1/7	14.3	-	> 0.500
Overall	**-**	**1 h:**	**8/23**	**34.8**	**0/28**	**0.0**		
		24 h:	**14/23**	**60.9**	**1/28**	**3.6**		
		48 h:	**16/23**	**69.8**	**1/28**	**3.6**		

The attached CD contains a recording of a fight that occurred between 2 *S. glaseri* males (*S. glaseri* Fight.AVI). Figure 4.5.A-C shows these 2 males after the fight. The recording started with one male wrapped around the tail of its victim with its tail. During the fight, the victim was only moving the tip of its head and the tip of its tail. After the fight, most of its body stayed in a cramped coil (see Figure 4.5A-B, male 1). Male 2 in Figure 4.5A and B was moving normally and was thus the victor of the fight. Figure 4.5C is a picture of the dead victim about 24 h after the males were put together.

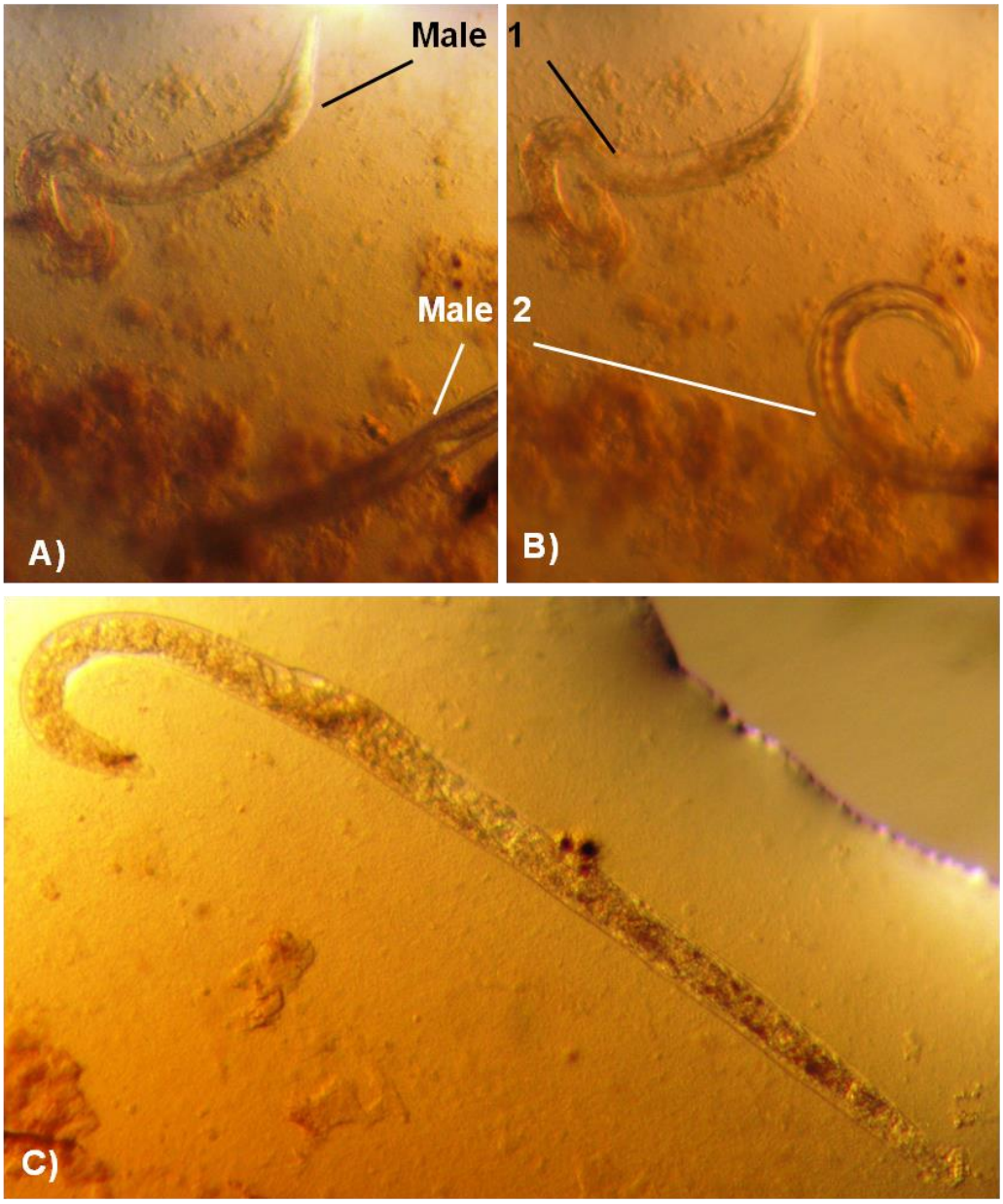


Figure 4.5 Pictures A and B are taken from a timeseries about 5 h after the 2 *S. glaseri* males were placed together in a drop and just after a fight had ended. Picture A was taken a couple of seconds before picture B. This timeseries shows normal movement of male 2 (stretched in A and coiling its tail in B) and the lack of movement of male 1 (the observed victim of the previous fight). C) One of the males was dead abour 24 h after they were placed together in a drop.

Although only 2 drops with paralysis/death were observed, injuries as those that were often the results of fights in *S. feltiae* and *S. kraussei* were not observed for *S. glaseri* fights. The consequences of a serious fight were paralysis characterised by the inability to move the body or parts thereof.

4.4.4. Summary & conclusions

Fighting in *Steinernema glaseri* frequently resulted in paralysis or lead to death of (at least) one of the 2 males. In this study 33 % of the paired male drops had at least 1 male paralysed or dead compared to 0-14 % in single male drops and over both studies 35-70 % of the paired male drops had at least 1 male paralysed or dead compared to 0-3.6 % in the single drops. In Foster (2007), the number of drops with a dead or paralysed male increased over time so that after 48 h just less than 70 % of the drops had a dead or paralysed male. Males were observed to start fighting within the first 10 min the males were placed together (Foster, 2007).

Due to significant differences in the results for *S. glaseri* in this study and the 2007 study by Foster (Fisher's exact test: n = 23, 24 h: p = 0.16, 48 h: $p < 0.05$), more experiments on the fighting of *S. glaseri* will need to be performed to gain insight in this species' fighting behaviour.

4.5. *Steinernema bicornutum*

4.5.1. Introduction

Steinernema bicornutum is a species belonging to clade IV.I used *S. bicornutum* strain IRA7 which was isolated from soil samples in Iran (Kary *et al*, 2009;Kary *et al*, 2010). Igoe (2008) also studied this strain and a second strain 27-1 which was recovered in Ireland (Harvey, 2010).

At the molecular level, *S. bicornutum* Tallosi et al., 1995, shows differences in more than 15% of rDNA nucleotides - in a 715 bp long fragment of ribosomal gene (ITS1 + 5.8S + ITS2) - with the closest species of the genus, *S. ceratophorum*, which was described from China (Ivanova & Spiridonov, 2003;Tallosi *et al*, 1995;Jian *et al*, 1997;Spiridonov *et al*, 2004;Kary *et al*, 2010).

4.5.2. Materials and Methods

Infective juveniles were placed into fresh haemolymph drops as described in Section 2.3. Drops with infective juveniles were put into the 23 °C incubator. The infective juveniles normally developed into adults after 3-4 days in the incubator.

4.5.3. Results

In this study the development from IJ to adult nematodes in hanging haemolymph drops was not sufficiently high for setting up fighting experiments: fewer than 2 % of IJs developed to adult (see Table 4.8).

Table 4.8 Development of *S. bicornutum* strain IRA7 in this study.

Batch	Nr of days of development	Total number of drops	Drops with an adult		Drops with a male	
			Number	%	Number	%
1	13	261	2	0.77	1	0.38
2	8	108	5	5.00	3	3.00
Overall	**8-13**	**369**	**7**	**1.90**	**4**	**1.08**

Igoe's (2008) study did have development of the infective juveniles in hanging haemolymph drops and he could thus test the occurrence of fighting, paralysis and death in drops containing 2 males and compare it to the survival rate of single males. In 21 drops (see Table 4.9), over the 2 strains, he did not observe fighting within the first 10 min the males were put together. After 24 h, all paired and singles males were still moving normally. Only after 48 h together, one *S. bicornutum* strain IRA7 male in a two-male drop had died. Mortality of single males was still zero (Table 4.9).

Table 4.9 Summary of Igoe's 2008 study on the occurrence of paralysis or death in drops with 1 or 2 *Steinernema bicornutum*. As the expected numbers were lower than 5, a fisher's exact test was done.

Strain	Rearing and fighting temperature	Observation timepoint	Drops with at least 1 dead or paralysed on total No. of couples		Dead single male controls on total No. of controls		Difference between singles and couples?	
			No.	%	No.	%	χ2	p
27-1	20 °C	10 min:	0/15	0	0/15	0	-	-
		24 h:	0/15	0	0/15	0	-	-
		48 h:	1/15	6.7	0/15	0	-	> 0.5
IRA7	23 °C	10 min:	0/6	0	0/6	0	-	-
		24 h:	0/6	0	0/6	0	-	-
		48 h:	0/6	0	0/6	0	-	-
Overall	**20-23 °C**	**1 h:**	**0/21**	**0**	**0/21**	**0**	**-**	**-**
		24 h:	**0/21**	**0**	**0/21**	**0**	**-**	**-**
		48 h:	**1/21**	**4.8**	**0/21**	**0**	**-**	**> 0.5**

4.5.4. Summary & conclusions

S. bicornutum is the only species studied so far that performed no intraspecific fighting behaviour with serious consequences like paralysis, wounding or death (Igoe, 2008). After 48 h, *S. bicornutum* had only 1 drop (4.8 %) out of 21 drops with 2 males in which at least 1 male was paralysed or dead (Igoe, 2008), whereas the other tested species (see Figure 4.7) had 47.6 % to 89.6 % of drops with 2 males in which at least 1 male was paralysed or dead. Although this conclusion is based solely on the work of an undergraduate, he worked under my close supervision [Moreover, the same student also recorded high levels of paralysis/death in *S. kraussei* (Section 4.3.3.2) and so clearly was adequately familiar with the condition to record it if it was seen].

However a total sample size of 21 is not very large, more of these experiments with *S. bicornutum* need to be performed to confirm or reject the current very low level of aggression status of *S. bicornutum*.

4.6. Comparison of fighting in *Steinernema* species

Based on the results from this study, *S. longicaudum* was the fiercest fighter. This was evident from the number of drops with at least 1 paralysed male after 24 and 48 h (Figure 4.6Table 4.7). *S. feltiae* and *S. kraussei* showed similar levels of paralysis, but were not significantly different from the level of paralysis in *S. glaseri* (Figure 4.6). Though, given the statistically significant difference between this study's *S. glaseri* results and Foster (2007) (see section 4.4.3; Fisher's exact test: n = 23, 24 h: p = 0.16, 48 h: p < *0.05*) combined with a higher number of drops (Table 4.7) and the absence of death in the single male drops (Table 4.7), more weight was given to the *S. glaseri* results from Foster (2007) (Table 4.10).

Due to poor development in this study, Igoe (2008) with *S. bicornutum* could not be repeated and comparisons of the fighting behaviour other species with *S. bicornutum* are based on Igoe's (2008) results (Table 4.10). The results on the fighting behaviour of *S. kraussei* obtained by Igoe in his 2008 study are significantly different from the results of the experiments in this study (Fisher's exact test, 24 h: n = 63, p < *0.05*; 48 h: n = 62, p < *0.05*). Because this study's sample size was a lot higher than in Igoe's (respectively n = 51 and n = 12), only the results from this study were used for comparisons between species (Table 4.10).

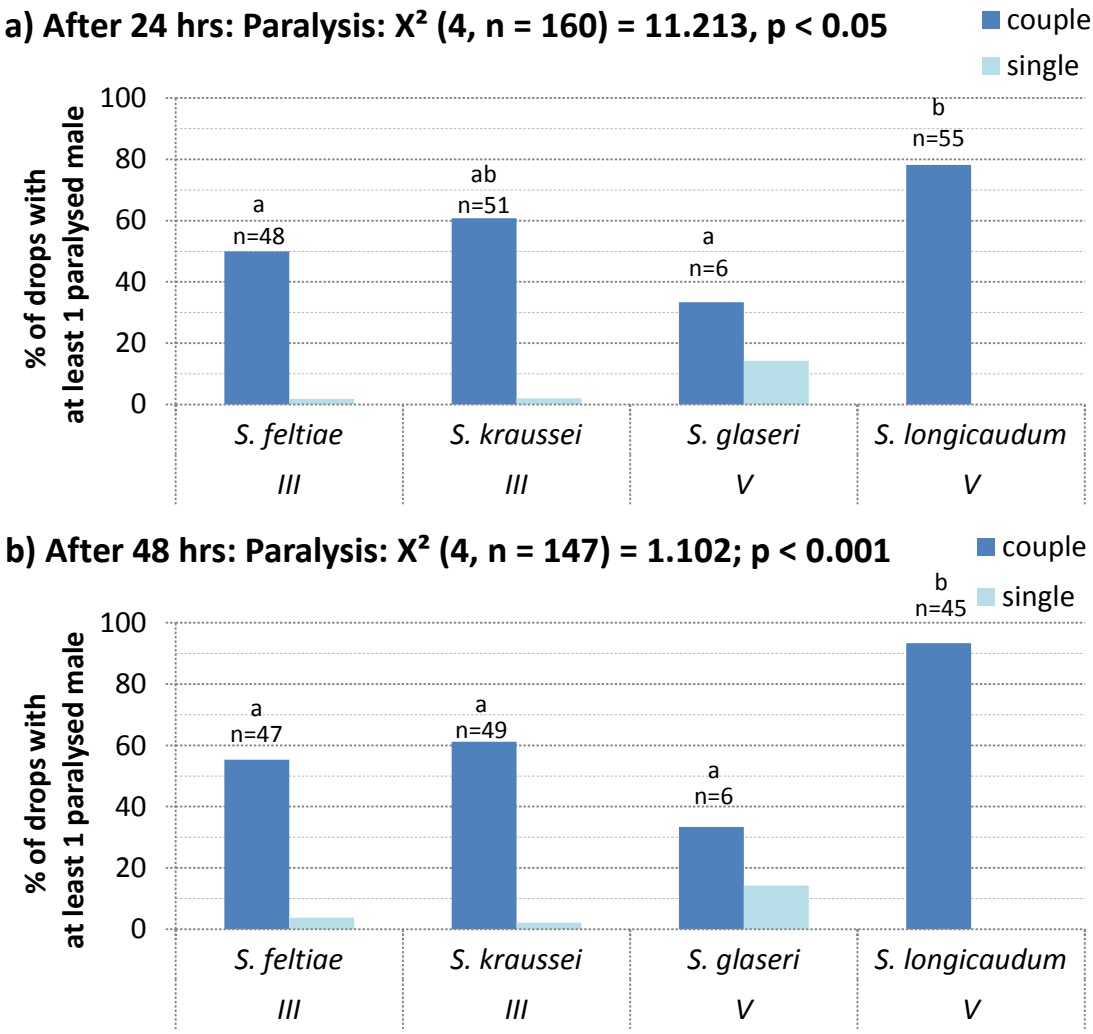


Figure 4.6 **Paralysis or worse in drops containing 2 conspecific males or a single male control over all *Steinernema* species investigated for fighting behaviour in this study. Observations were made about a) 24 h and b) 48 h after the males were put together in a drop. These data have already been shown earlier in this chapter or in Figure 5.22 for *S. longicaudum* (see Chapter 5). Roman numerals represent the clades enumerated by Spiridonov *et al*. (2004). Within a graph, bars accompanied by the same letters are not significantly different. "n" is the number of drops for that treatment.**

Table 4.10 All studies of which data are incorporated in Figure 4.7. These results were already shown in Table 4.2, Table 4.6, Table 4.7 and Table 4.9. Studies significantly different from other studies on the same species and with a smaller sample size were rejected for Figure 4.7.

Species	Study
S. bicornutum	Igoe (2008)
S. carpocapsae	O'Callaghan (2006)
S. feltiae	Foster (2007), Everard (2006, unpublished) & this study
S. glaseri	Foster (2007)
S. kraussei	this study
S. longicaudum	O'Callaghan (2006), Everard (2006, unpublished) & this study (Chapter 3)

Figure 4.7 summarises the results of the studies summed up in Table 4.10 and shows that the fighting behaviour of *S. bicornutum* differed significantly from all the other species investigated (Appendix table 5 and Appendix table 6; percentages of drops with at least 1 male paralysed after 24 and 48 h in 2 male *S. bicornutum* drops: respectively 0 and 4.8). After 24 and 48 h together, *S. feltiae* proved to be significantly more pugnacious than *S. bicornutum* (Appendix table 5 and Appendix table 6) but had significantly less drops with a paralysed or dead male than *S. carpocapsae* and *S. longicaudum* (Appendix table 5 and Appendix table 6). *S. glaseri* only differs significantly from *S. bicornutum* in numbers of drops with at least 1 paralysed after 24 and 48 h (Appendix table 5 and Appendix table 6). After 48 h, the significance of the difference between *S. feltiae* and *S. glaseri* depends on the view on application of the sequential Bonferroni adjustment (Moran, 2003) (Appendix table 5 and Appendix table 6). The lack of significant difference with other species (like *S. feltiae*) is highly likely an artefact of the small sample size of *S. glaseri* experiments (Appendix table 5 and Appendix table 6). The statistical difference between *S. feltiae* and *S. carpocapsae* after 48 h is also debatable when applying the sequential Bonferroni adjustment (Appendix table 5 and Appendix table 6). This could be caused by the presentation of the data in O'Callaghan's (2006) study in which only combined results for 24 and 48 h are given. This combination might have resulted in a false increased shift for the percentage of 2-male drops with at least 1 male paralysed after 24 h and a false decreased shift for after 48 h. *S. kraussei* showed significantly less paralysis/death after 24 and 48 h than *S. longicaudum* (Appendix table 5 and Appendix table 6) as did *S. feltiae* which belongs to the same clade and even has the same symbiotic bacterium, *X. bovienii*, as *S. kraussei* (Appendix table 5 and Appendix table 6). *S. carpocapsae*

and *S. longicaudum* did not differ significantly in the amount of paralysis (Appendix table 5 and Appendix table 6).

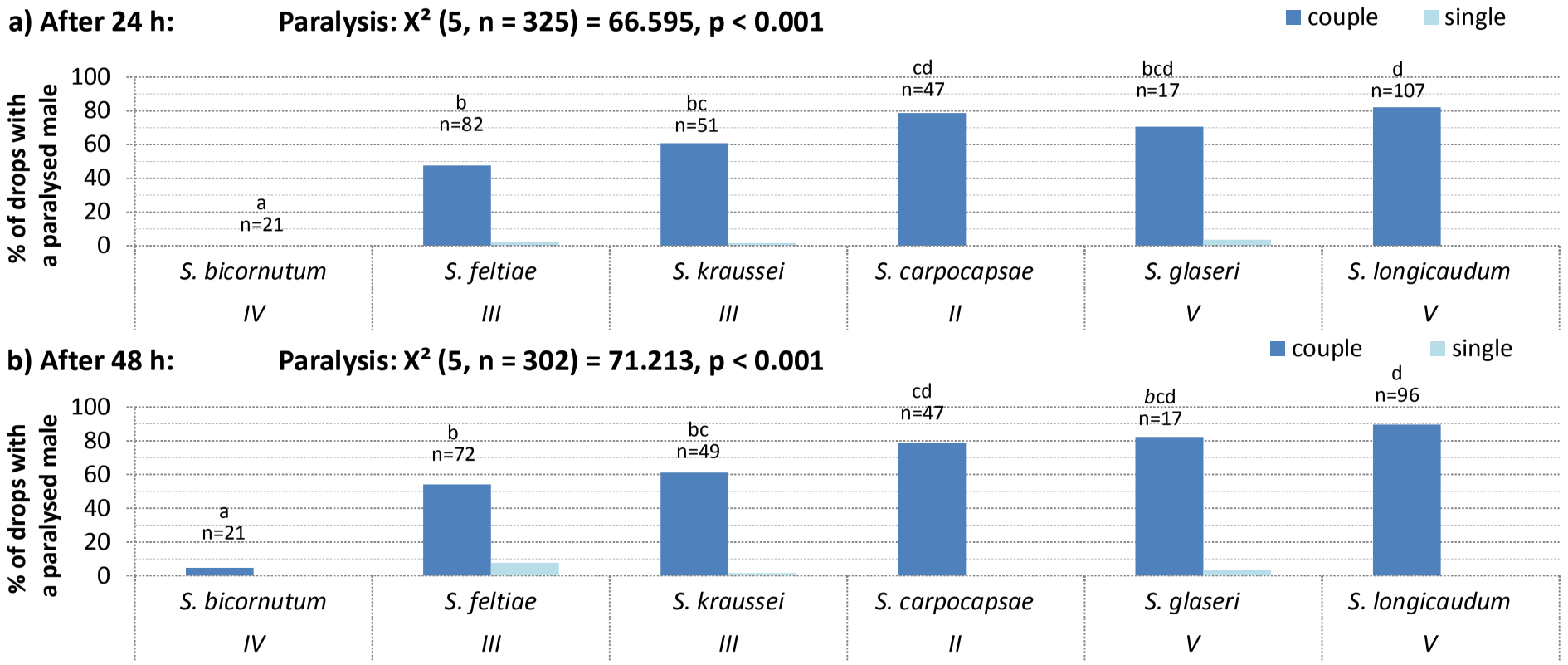


Figure 4.7 **Paralysis or worse in drops containing 2 conspecific males or a single male control over all *Steinernema* species investigated for fighting behaviour so far. Observations were made about a) 24 h and b) 48 h after the males were put together in a drop. This figure incorporates already shown results from all studies as listed in Table 4.10. Roman numerals represent the clades enumerated by Spiridonov *et al.* (2004). Within a graph, bars accompanied by the same letters are not significantly different. "n" is the number of drops for that treatment.**

Figure 4.7 is the basis for placing the studied *Steinernema* species on a gradient of aggression as is shown in Figure 4.8. *Steinernema* species in which less than 20 % of the 2-male drops show paralysis or death of at least 1 of the males after 48 h are almost not aggressive. Species with 40-60 %, 60-80 % and 80-100 % of drops with at least 1 male dead or paralysed after 24-48 h are respectively moderately, highly and very highly aggressive (Figure 4.8). At the present time, no species with a low level of fighting behaviour (20-40 % of drops with at least 1 male dead or paralysed after 24-48 h) have been investigated/found (Figure 4.8).

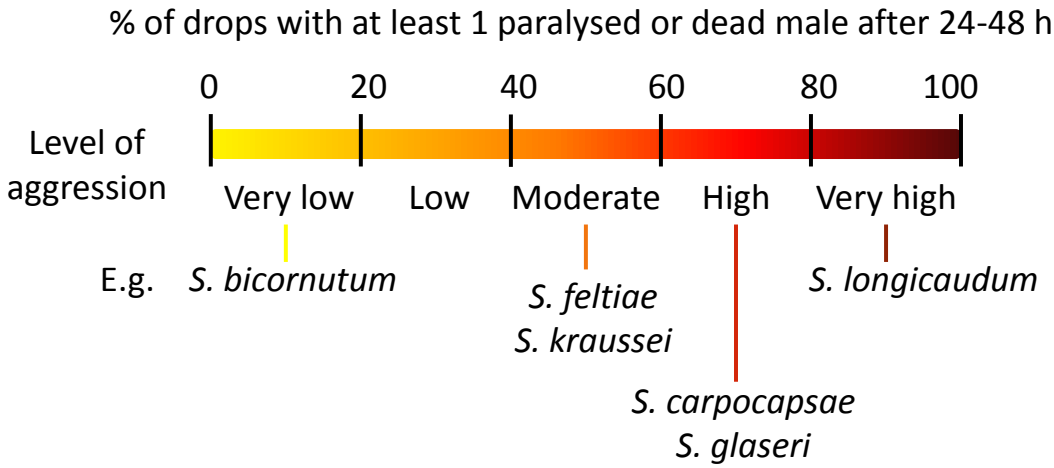


Figure 4.8 Aggressiveness scale of *Steinernema* species based upon the percentage of drops with at least 1 paralysed or dead male after 24-48 h.

4.7. Conclusions

Six species of the *Steinernema* genus have so far been studied for fighting behaviour, only one out of these showed less than 10 % of drops with signs of paralysis 48 h after 2 males were placed together in a drop. In order to cover as much as possible of the *Steinernema* genus, these six species were chosen out of clades II to V of the *Steinernema* genus (no species of Clade I was readily available). The occurrence of significant paralysis ($p < 0.05$) in more than 40 % of 2 male drops in five out of the six studied species, covering 3 clades, reflects how widespread fighting behaviour is and suggests fighting, paralysis and kill are ancestral traits that have been conserved in most of the species. It is more likely that a “No fighting”-strategy developed once than that fatal fighting evolved separately in 3 different clades. Clade IV, the clade to which *S. bicornutum* (the only very low level of aggression species thus far found) belongs, is thus a very interesting group of species for further studies. *Steinernema bicornutum* was observed with visible sperm in its gonads even when the male was reared in complete isolation (personal observations), as opposed to *S. longicaudum* in which males only produce

large spermatozoa after several hours with a conspecific female (Ebssa *et al*, 2008). *Steinernema bicornutum*'s significantly different fighting behaviour (all p < *0.001*, Appendix table 5 and Appendix table 6) might indicate a different mating strategy that allowed for and maybe even promoted less aggressive competition. However, the total numbers for *S. bicornutum* studied are very low and this research thus needs to be repeated for this species. Aiming for better development of *S. bicornutum*, future studies could try different *S. bicornutum* strains as they might develop better in hanging drops. Rearing *S. bicornutum* in hanging drops co-infected with infective juveniles from other *Steinernema* species would not be preferable as it would rather result in less fit adults (Sicard *et al*, 2003) and/or yield a lower number of *S. bicornutum* adults (Koppenhofer *et al*, 1995;Sicard *et al*, 2006). This could be due to interspecific fighting that would paralyse and/or kill the *S. bicornutum* juveniles and adults (O'Callaghan, 2006). Also, development of *S. bicornutum* with non-native symbionts could be detrimental to the nematode as it might not have resistance to specific virulence factors produced by the non-native bacteria. (Sicard *et al*, 2003;Sicard *et al*, 2004;Webster *et al*, 2002) or as the non-native symbionts produce toxins against the native symbiont (Fodor *et al*, 2010).

S. bicornutum should also be observed for longer periods and under varied conditions to assess whether wrapping occurs but causes no paralysis or death, whether the *S. bicornutum* males need longer for the effects of fighting to become observable or whether the species doesn't even fight at all. It might even be possible to see what characteristic of the fighting behaviour was lost first: wrapping or paralysis and killing.

The results of this study are significantly different from the Foster (2007) study for *S. glaseri* (see section 4.4.3, Fisher's exact test: n = 23, 24 h: p = 0.16, 48 h: p < *0.05*). Both studies had low numbers due to poor development of *S. glaseri* infective juveniles in hanging drops of haemolymph and these experiments for *S. glaseri* need to be repeated. But even with this small sample size we can conclude that *S. glaseri* males fight and even do so pretty quickly: in both studies *S. glaseri* males were observed fighting within 1 h of being placed in a drop together with another male. Even though more evidence is needed, clade V might be a fierce fighter- clade.

The comparison of species and their placing on the gradient of aggressiveness needs to be interpreted with caution, as it is based on data pooled from various studies. Although all were done in the same laboratory, and most (except O'Callaghan, 2006) were either done by myself or under my close supervision, there could be inter-experimenter differences. In particular, the differences between *S. longicaudum, S. glaseri* and *S. carpocapsae* are small and non-significant (Appendix table 5 and Appendix table 6), so assigning them to different categories

of aggressiveness is done cautiously. The difference between the clade III species (*S. feltiae* and *S. kraussei*) and *S. longicaudum* is more robust, being based on significant differences in my own data (Figure 4.6) as well as the pooled data (Figure 4.7) (all $p < 0.005$; Appendix table 5 and Appendix table 6).

This chapter confirms the fighting ability of *S. feltiae* by means of three different studies. The 3 studies in this chapter, used the *Steinernema feltiae* isolate 4CFMO (Dillon, 2003;Dillon *et al*, 2006) whereas O'Callaghan (2006) reported only a very small amount of fighting in *S. feltiae* isolate UK76. O'Callaghan's (2006) sample size for *S. feltiae* was however not very large and thus more research into a possible difference in fighting behaviour between these two *S. feltiae* strains (and perhaps other, more recently isolated strains) should be done. The strain used by O'Callaghan has been in laboratory culture for more than 20 years (C. Griffin, pers. Comm.) and may have lost the ability to fight, while the 4CFMO strain was isolated more recently (Dillon, 2003). Another difference between these 2 strains is the habitat-type from which they were isolated. *S. feltiae* UK76 was isolated from grassland – the most recorded habitat type for this species (Stock *et al*, 1999) – in the UK, whereas *S. feltiae* 4CFMO was isolated from woodland in Ireland (Dillon, 2003;Dillon *et al*, 2006). Different habitat types could imply different insect hosts (Mrácek & Becvar, 2000) and/or soil type (Mrácek *et al*, 2005) which can influence infection behaviour (Griffin *et al*, 2005;Griffin, 2012).Thus, as habitat type can alter juvenile behaviour, it might also influence adult behaviour.

Different *Steinernema* species might also use different methods for eliminating their competitor during a fight. Chapter 5 treats experiments on the mechanisms of fighting, paralysis and killing in *S. longicaudum*. In this chapter the differences in type of wounding by different species has already been touched upon. Victims of fighting in *S. kraussei* and *S. feltiae* showed ruptured cuticles combined with protrusion of internal organs in 7 out of 9 injured *S. feltiae* victims and in 10 out of 14 injured *S. kraussei* victims. O'Callaghan (2006) also reported that females/hermaphrodites that died in the presence of a heterospecific male (either *S. longicaudum*, *S. Steinernema* sp. INA S3 or *Steinernema* sp. Macau) had signs of a damaged and/or ruptured body wall, but did not give the frequencies of this type of injury. She also noted that *S. longicaudum* and *S. carpocapsae* males that had died in the presence of another male usually appeared shrunken in size and/or with a damaged body wall, compared to males that had died alone which had intact body walls. This type of injury was not seen here for *S. glaseri* fight victims, suggesting differences in fighting strategies might be possible. However, it is more likely that this is due to the fact that there were only two fight victims in my

experiments on *S. glaseri*, and Foster (2007) did not record the appearance of fight victims for this species.

O'Callaghan (2006) reported a higher mortality rate for *S. carpocapsae* and *S. feltiae* females with a punctured cuticle that were placed in a drop that was previously occupied by a *S. longicaudum* nematode than those that were placed in a drop that was previously occupied by a conspecific. A different species of symbiotic bacteria might thus be detrimental to an injured nematode. Even a different conspecific strain of the symbiotic bacteria can negatively influence the parasitic success (emergences of juveniles) of a nematode (Sicard *et al*, 2004). Conspecific pairing of 2 S*. feltiae* or 2 *S. kraussei* males, two species with a high incidence of ruptured cuticle after fighting, in a drop previously occupied by the congeneric other species, did not affect the mortality rate. This showed the strain of the symbiotic bacteria *X. bovienii* doesn't seem to have an effect on the incidence of paralysis or death of conspecifically paired *S. feltiae* or *S. kraussei* males. N.B.: the question of whether bacteria are implicated at all in paralysis and death is dealt with further in Chapter 5.

Intraspecific male fighting has never been reported in the intensively studied *Caenorhabditis elegans* or in other *Caenorhabditis* species. *(Caenorhabditis* nematodes are placed in the order Rhabditida, like the Steinernematidae, but these 2 families are not very closely related to each other: Blaxter et al. (1998) place them in separate clades of the Nematoda]. Intraspecific male competition does exist in *Caenorhabditis* nematodes but is expressed through sperm competition (Lamunyon & Ward, 1998;Singson *et al*, 1999;Timmermeyer *et al*, 2010). Increasing sperm competition is probably translated into larger sperm size (Lamunyon & Ward, 2002;Lamunyon & Ward, 1999): hermaphroditic species which have a low number of males and thus less opportunity for sperm competition have smaller sperm than gonochoristic species where males are more common and thus experience higher sperm competition. Variation in competition for reproduction occurs both within the *Steinernema* and *Caenorhabditis* genera, but respectively through intraspecific male combat and sperm competition.

5. Cause of paralysis and death in *S. longicaudum*

5.1. Introduction

The cause of paralysis and death resulting from fighting in *Steinernema longicaudum* is the topic for this chapter.

Keeping in mind that frequency of paralysis and death differs between the *Steinernema* species (Chapter 4) and that the investigations in this chapter only cover *S. longicaudum*, it might very well be that the mechanism of paralysis and/or kill differs between the different *Steinernema* spp. and that some of the items discussed here are not applicable to other Steinernematidae.

This chapter starts with the examination of the injuries sustained by victims in a fight (5.3). Next, attempts were made to inflict similar injuries and observe the subsequent occurrence of paralysis and/or death (5.4, 5.5 and 5.6). In the experiments in 5.4, only locally applied external pressure was used to mimic the physical forces applicable when the attacker wraps its body around its victim, whereas in 5.5 a needle was used to mimic the possibility of stabbing the victim with the spicule. In 5.6 the aim was to inject worms with different media (with and without bacteria) to mimic the possible use of the spicules for the injection of a toxin.

In 5.7, the involvement of bacteria and the existence of limiting requirements of the medium in which the nematodes fight are investigated. The symbiotic bacteria naturally received most attention, the interdependency between *Xenorhabdus* bacteria and *Steinernema* nematodes has closely linked their evolution (Adams *et al*, 2006;Ciche *et al*, 2006;Lee & Stock, 2010b;Lee & Stock, 2010a) and might have influenced the evolution of the fighting behaviour. *Xenorhabdus* spp produce a range of bioactive metabolites into the medium, including molecules with antibiotical, insecticidal and nematicidal properties (see section 1.2.2). Xenorhabdidae also produce antibiotic factors against other *Xenorhabdus* species which are likely to be of a different nature than the antibiotics produced against competing bacteria from other genera (Fodor *et al*, 2010). *X. ehlersii*, the symbiont of *S. longicaudum* shows weak antibiotic activities against non-related bacteria, but is quite active

against related species (Fodor *et al*, 2010). Toxins produced by the bacteria and secreted into the medium could diffuse into the nematode through wounds sustained during fighting. Ehlers (1990) puts forward the possibility that *Xenorhabdus* bacteria produce chemicals toxic for *Steinernema* nematodes and combines it with the suggestion that the *Steinernema* species naturally associated with that particular *Xenorhabdus* species/strain is able to metabolize these toxins, rendering the nematode insensitive to these particular toxins. However, lesions could render a nematode susceptible to these otherwise metabolised toxins.

5.2. Definition of used terms

- An **injury** is hurt, harm, damage, or loss sustained so that the worm is no longer fully healthy or in good condition.
- A **puncture** is the **small** perforation, hole or wound made by piercing with a **needle or spicule.**
- A **wound** is an injury to the body that typically involves laceration or breaking of the cuticle and usually also damage to underlying tissues.
- A **lesion** denotes a localized, well defined, pathological, abnormal change in the structure of an organ or in tissue due to injury or disease thus including punctures, ruptures, and wounds.
- A **crush-rupture** is used to denote a wound caused by the pressure of a wire that involved the tissue to be broken apart or an organ to be burst open.

5.3. Examination of *S. longicaudum* fight victims

5.3.1. Introduction

O'Callaghan (2006) noted that female *Steinernema* that died in the presence of a male of another *Steinernema* species often had "signs of a damaged and/or ruptured cuticle". In this section, the victims of male-male fighting in *Steinernema longicaudum* are examined.

Hypotheses

- Within 20 min of putting 2 males together in a drop and agitating them regularly, a fight, whether or not with immediate paralysis, will have occurred in the majority of these drops. After these 20 min several 2- males drops will contain a male with visible injuries or paralysis as a result of fighting.
- Within 24 h after 2 males were put together in a drop, the majority of drops will show at least 1 male with signs of paralysis, a damaged and/or ruptured cuticle or other injuries whereas the majority of the single male controls will show no injuries or paralysis.
- The injuries sustained by males in 2-male drops will be visibly different from injuries of single control males.

5.3.2. Material and Methods

Infective juveniles were placed into fresh haemolymph drops as described in 2.3 and put into the 27 °C incubator for 4-5 days. Males were then teamed up with another *S. longicaudum* male of the same age (naïve couples) or left alone in their original drop (single male controls). The age of males at observations varied, but 2 males in a drop were always of the same age.

Immediate observations in ambient room temperature and room humidity were made for 20 min or until an attack with the possibility of injury was observed. When the observed drop showed signs of drying out, about 25 µl of 100 % Ringer's solution was added. During the observations, the males were regularly disturbed by a piece of platinum wire, used as an aggressor as per earlier chapter (see section 3.7), in order to keep both males within nematode length of each other and to keep both males active and stimulated for fighting (see section 3.7). When a male coiled on itself it was attempted to uncurl it by stretching it with the wire.

Every time the wire was out of the drop for more than 10 seconds, it was passed through the flame of a Bunsen burner. The wire was allowed to cool before it contacted another male.

In the case of a special event like a fight that resulted in paralysis or a possible injury of the victim, both males were transferred to a drop of Ringers on a microscope slide. The drop was placed in the middle of a square made of high vacuum grease (Dow Corning High vacuum grease # 5054). Efforts were made not to let the drop of Ringers touch the grease. This was possible most of the times. The line of grease made it possible for a cover slip to be placed upon the drop without crushing the nematodes. The cover slip facilitated observations at 10x40 and 10x100 (oil immersion). Apart from 1 couple, all males were put back together in their original fighting drop and observed again after 24 h.

Images were captured as specified in 2.5.

5.3.3. Results

5.3.3.1. Single male controls

Eight males were left as single controls in their original drop. Of these, 2 died when they were 6 days old, which was 48 h after the other males from the same batch were teamed up. The other 6 single males survived for more than 72 h after the beginning of the fight staging (after this they were not checked again). The 2 single control deaths came from the same batch and at the age of 5 days both males showed a clear space in the tip of their tail (Figure 5.1) which neither of them was moving.

Figure 5.1 Single *S. longicaudum* male with a clear space just before the spicules, observed 24 h after the other males were put together. The male was not moving its tail. The inset is a blowup (1.7x the main image) from a different picture.

5.3.3.2. Immediate observations:

Out of 12 couples, 9 fought within 20 min of the start of the experiment (Table 5.1). In 2 out of these 9 couples (drops 1 and 9), immediate paralysis after a fight was observed. One of the males that showed paralysis immediately after an observed fight, showed no visible injuries (drop # 1). In 5 out of these 9 couples visible injuries were seen at the end of the 20 min observation:

- 1 victim had suffered a punctured body wall (drop #12; Figure 5.2);
- 1 victim had a punctured body wall together with a punctured digestive tract at the same spot which allowed for loss of content of the digestive tract (drop # 8; Figure 5.3). The contents of the digestive tract were also clearly separated over the

whole width of the male at this spot. This indicated that the fighting wrap had caused a constriction in the internal organs.

- 3 victims (drops # 6, 7 & 9) showed a separation of the contents of the digestive tract as in Figure 5.4 & Figure 5.5 (both drop # 7). No puncture wounds were observed. This indicated that the fighting wrap had caused a constriction in the internal organs, but no effective piercing had happened. One of these three victims (drop # 9) had shown impeded movement immediately after the fight.

Table 5.1 Summary of the observations made 20 min after pairs of naïve *S. longicaudum* males were put together in a drop where they were continuously stimulated to elicit fighting so that injuries could be examined.

	Did fighting or paralysis occur within 20 min?		**Were injuries visible after the observed fighting?**
Drop	Fighting	Paralysis	
1	Yes	Yes	No
2	Yes	No	No
3	No	-	-
4	No	-	-
5	Yes	No	No
6	Yes	No	Constriction
7	Yes	No	Constriction (Figure 5.4 & Figure 5.5).
8	Yes	No	The victim had a punctured body wall, a punctured digestive tract with loss of content and results of a constriction (Figure 5.3).
9	Yes	Yes	constriction
10	*No*	-	-
11	Yes	No	No
12	Yes	No	The victim had suffered a punctured body wall (Figure 5.2).

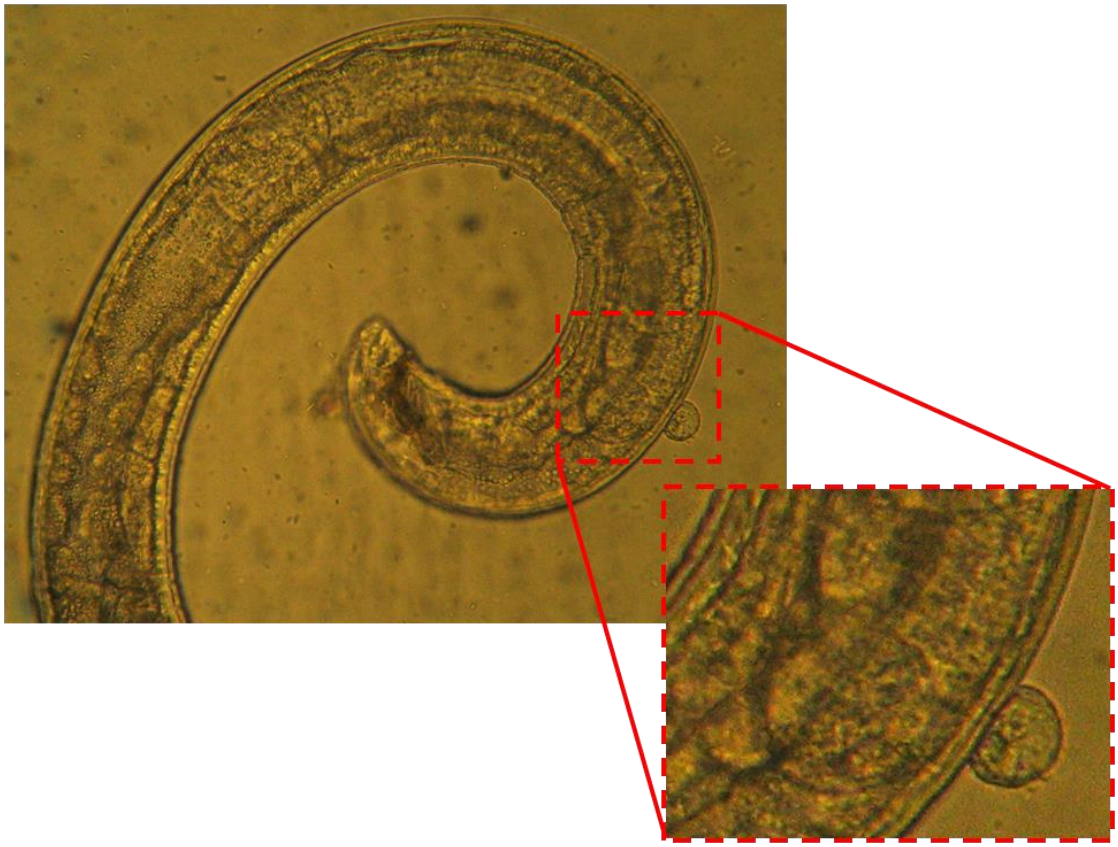


Figure 5.2. ***S. longicaudum*** **male with pierced body wall observed immediately after fighting with another male (drop # 12). The males in this drop fought after 2 min (tail wrapped around the head of the other male), 4 min (tail wrapped around the head of the other male), 4 min (tail wrapped around the middle of the anterior body of the other male) and 5 min (tail wrapped around the posterior part of the other male). This last fight resulted in the victim coiling back onto its attacker. This fight broke up but started again immediately for a couple of times. After this last fight one of the males appeared to have a puncture wound resulting in the formation of a "bubble". This bubble was located at the place on the posterior body of the victim where wrapping was observed. The inset is a blowup (2.6 x) from the red square on the original image.**

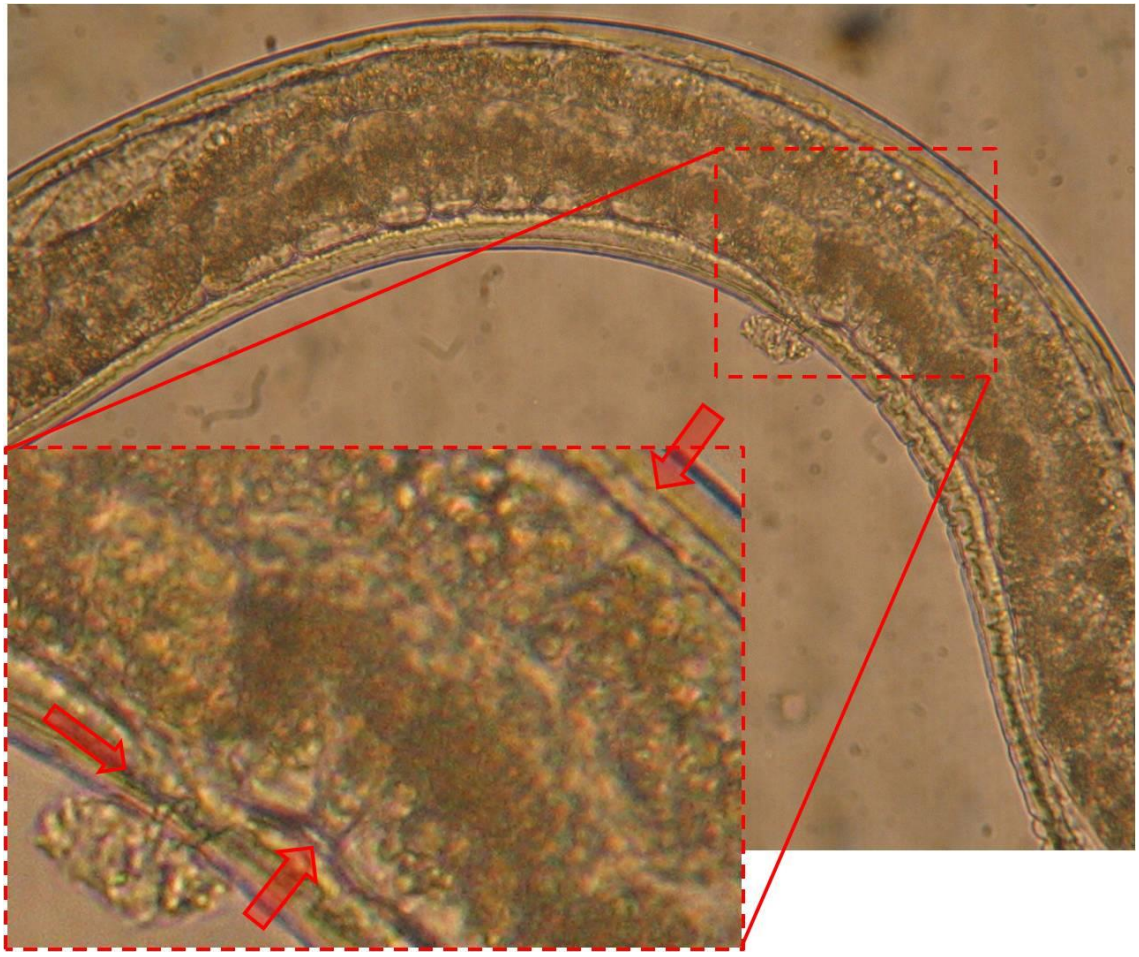


Figure 5.3 S. longicaudum male with punctured body wall, punctured digestive tract and constricted digestive tract, observed immediately after fighting with another male (drop # 8). The males in this drop fought after 1 (this fight lasted 3 min), 4, 5 and 6 min. After this last fight, a constriction of the digestive tract of the victim was visible at low power magnification. Under high power magnification it became clear that the victim had also suffered a pierced digestive tract and body wall (left red arrow) where the constriction (2 red arrows on the right) was observed. The inset is a blowup (2.7 x) from the red square on the original image.

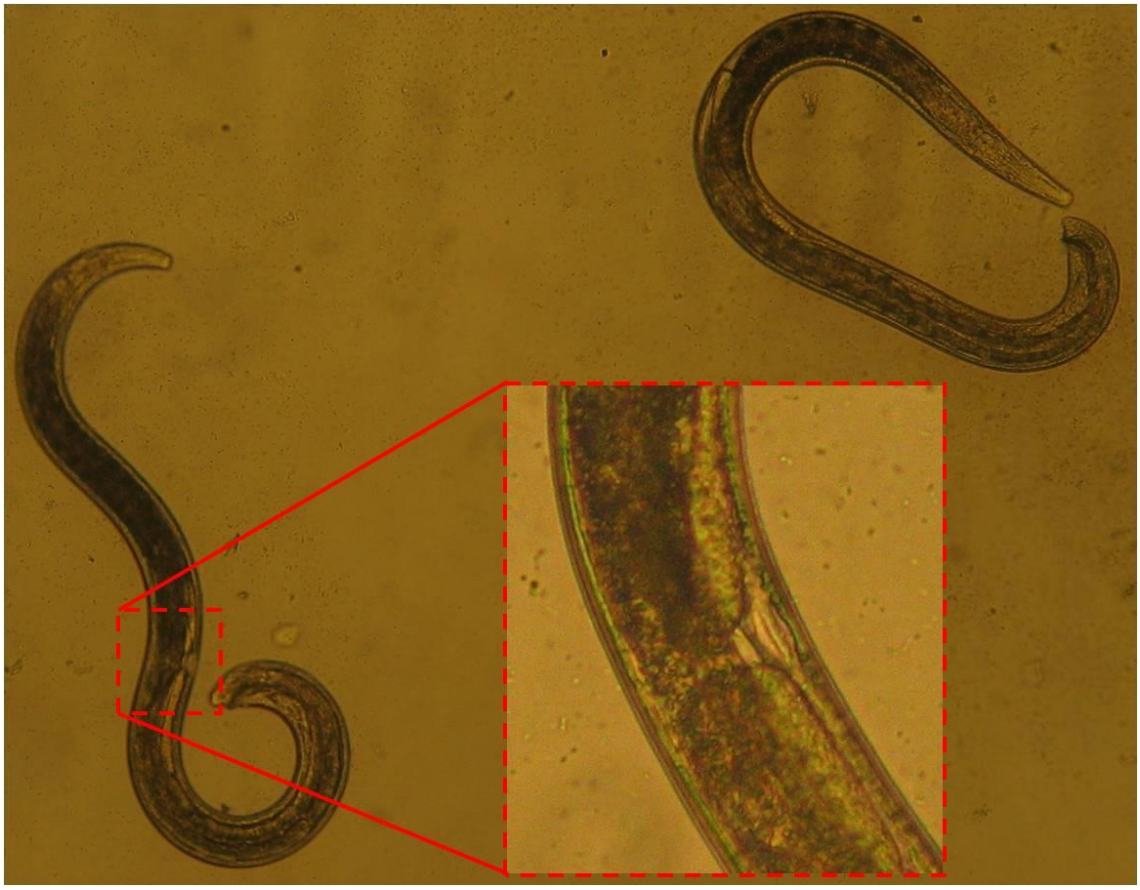


Figure 5.4 ***S. longicaudum*** **male with constricted internal organs but no obvious puncture, observed immediately after fighting with another male (drop # 7). The males in this drop fought after 1 (tail wrapped around the head of the other male), 3 (the attacking male –upper right– squeezed the victim –bottom left–) and 21 min. The last fight showed a separation of the contents of the digestive and gonadal tracts of the victim at the point where the other male had wrapped its tail around the victim (worm on left of main image). The attacker is shown in the upper right of the picture. The inset is a blowup (3.4x the main image) from a different picture taken.**

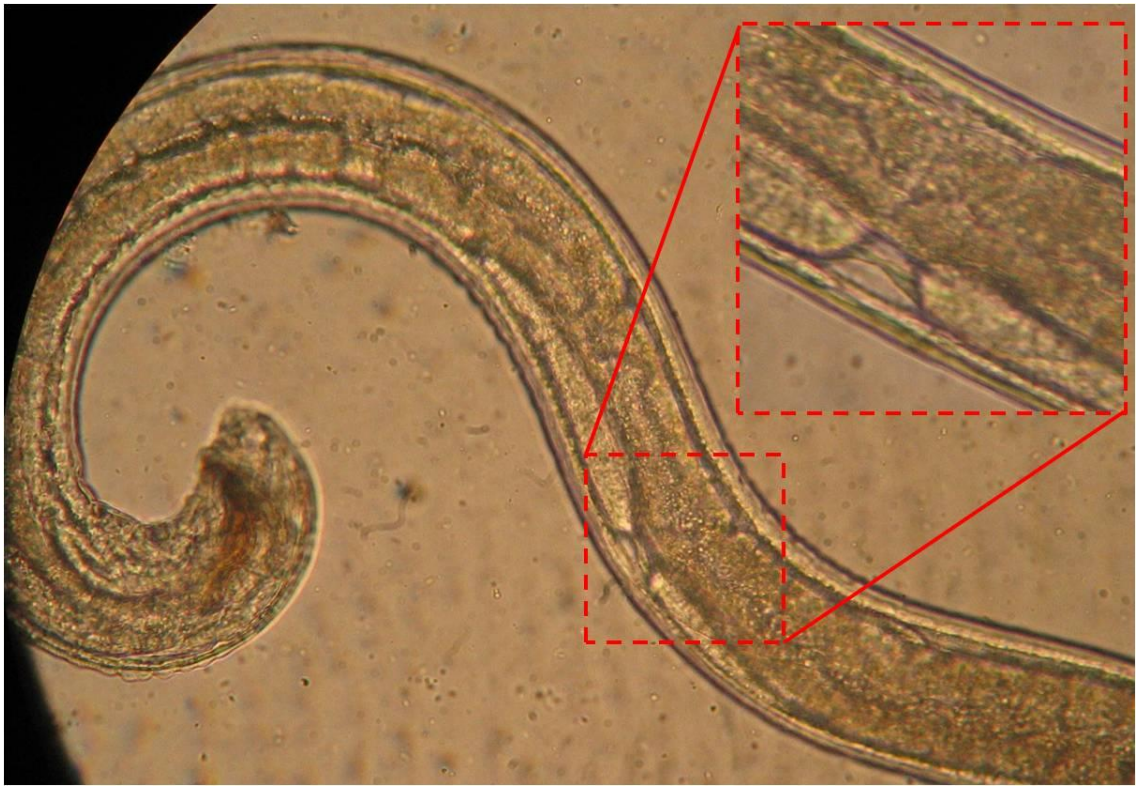


Figure 5.5 Another close up of the victim of the same drop (# 7) as in Figure 5.4. *S. longicaudum* male with constricted internal organs but no obvious puncture, observed immediately after fighting with another male. The males in this drop fought after 1 (tail wrapped around the head of the other male), 3 (the attacking male –upper right– squeezed the victim –bottom left–) and 21 min. The last fight showed a separation of the contents of the digestive and gonadal tracts of the victim at the point where the other male had wrapped its tail around the victim. The inset is a blowup (1.7x the main image) from a different picture.

5.3.3.3. Observations 24 h after the males were put together in a drop

After 24 h, at least one of the males in 8 out of 11 drops was paralysed or dead (Table 5.1):

- 3 drops had both males moving normally;
- 1 drop had 1 male moving normally and 1 male that was alive, but not moving normally;
- 1 drop had 1 male moving normally and 1 male that was barely alive;
- 5 drops had 1 male moving normally and 1 dead male;
- 1 drop had 1 male that was barely alive and 1 dead male.

In 5 of these 11 drops, the victims (still) showed visible injuries 24 h after having been put together with another male:

- In one of the drops in which both males were moving normally, the victim had a puncture wound (drop # 12; no picture; same injury as shown 20 minutes after the males were put together in a drop).
- In 2 of the drops in which 1 male was moving normally and the other male was dead, the dead male showed a wound in its body wall and ruptured internal organs which allowed for loss of content of the digestive tract (drop # 6, Figure 5.6; drop # 8, Figure 5.7). The victim in drop # 6 was previously seen only to have an internal constriction, but no wound in the body wall.
- In the drop were 1 male was barely alive and the other male was dead (drop #9), the dead male showed separation of the contents of the intestines, but without a wound in the body wall, this reflects the injuries he had shown 20 min after the 2 males were put together.
- In the drop with 1 normally moving male and 1 living but abnormally moving male, the victim's spicule and/or the surrounding tissue was damaged. The male's spicules were sticking out of the cloaca and the tissue of the male's tail looked unhealthy (drop # 7; Figure 5.8). A worm in this drop was previously described as having separated content in its intestine, but this was not observed at this time.
- Additionally, in one of the drops where both males were able to move normally when touched, one male had an abnormal appearance to the outer tissues of the posterior part of its body and kept this part more or less rigid (drop # 11; Figure

5.9, Figure 5.10 and recording "*S. longicaudum* Fight injuries.AVI" on the attached CD), like the single males in 5.3.3.1. Both males of this pair came from the same batch as the 2 abnormal single males (5.3.3.1 and Figure 5.1) and the abnormal male of the pair was also observed dead 48 h after pairing.

- **Table 5.2 Summary of the observations made 24 h after pairs of naïve *S. longicaudum* males had been continuously stimulated for 20 min to elicit fighting in order to obtain injuries from fighting.**

Drop	Did paralysis or death occur and were any injuries visible 24 h after the males were placed together in a drop?
1	One of the males was moving normally, the other male was dead. No injuries were observed.
2	One of the males was moving normally, the other male was barely alive. No injuries were observed.
3	One of the males was moving normally, the other male was dead. No injuries were observed.
4	One of the males was moving normally, the other male was dead. No injuries were observed.
5	Both males were moving normally. No injuries were observed.
6	One of the males was moving normally, the other male was dead. The dead male had suffered a wound in its body wall and ruptured internal organs (Figure 5.6).
7	One of the males was moving normally, the other male was alive but moving abnormally and had suffered spicule lesion (Figure 5.8).
8	One of the males was moving normally, the other male was dead. The dead male had suffered a puncture wound and loss of content of the internal organs (Figure 5.7).
9	One of the males was barely alive, while the other male was dead. The dead male showed separation of the contents of the intestine, but had no wound or rupture of the cuticle.
10	*The drop had fallen.*
11	Both males were able to move normally. One of the males had an abnormal appearance to the tissue of its posterior body and was not moving this part of its body much (Figure 5.9 , Figure 5.10 and recording "S. longicaudum Fight injuries.AVI" on the attached CD).
12	Both males were moving normally. One male showed a puncture in its body wall.

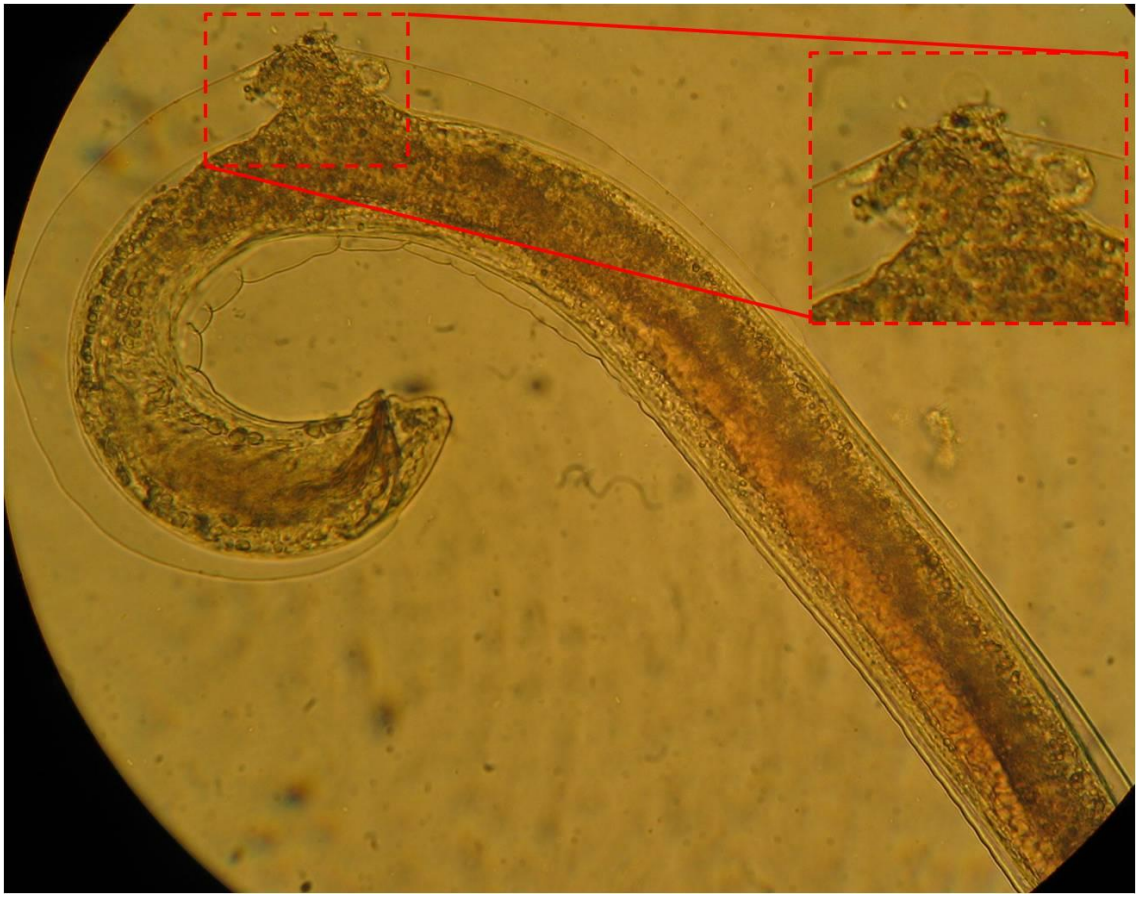


Figure 5.6 ***S. longicaudum*** **male with ruptured internal organs, observed 24 h after fighting with another male (drop # 6). These males had fought 5 min after being put together, when these males were then observed under high power magnification, the results of constriction were visible. The next day, when this picture was taken, one of the males was dead showing a puncture of the cuticle. He had also suffered a severe rupture of the internal organs witch resulted in loss of contents of the intestine into the space between the cuticle and the hypodermis. The inset is a blowup (2.1x) from the red square on the original image.**

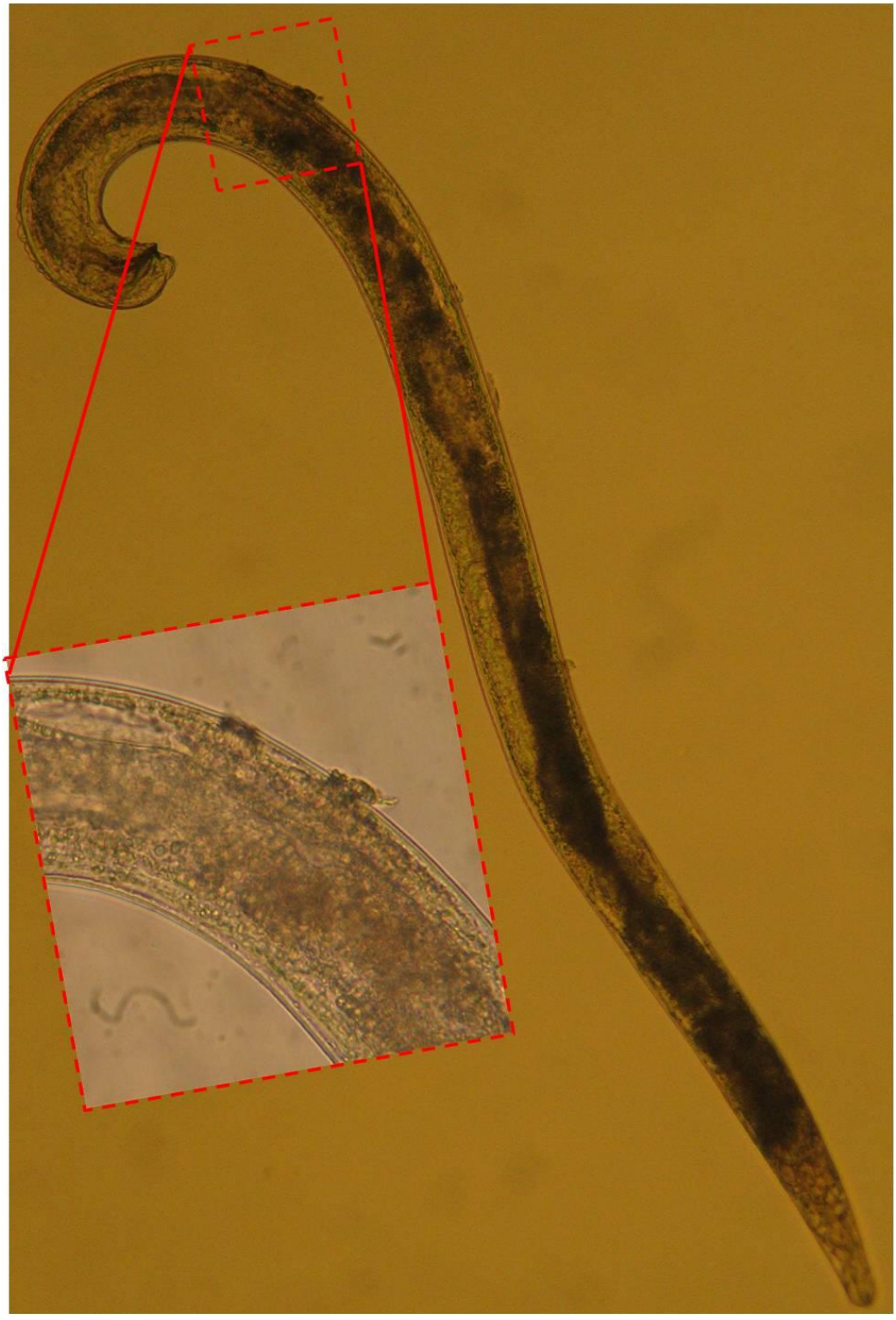


Figure 5.7 ***S. longicaudum*** **male with punctured internal organs and body wall, observed 24 h after fighting with another male (drop # 8). This is the same victim as in Figure 5.3. About 24 h after the start of the fight, the victim still showed a pierced digestive tract and body wall under high power magnification. The inset is a blowup (2.4x the main image) from a different picture.**

Figure 5.8 ***S. longicaudum*** **male with spicule lesions, observed 24 h after fighting with another male (drop # 7). About 24 h after the start of the fight, one of the males was moving abnormally and had sustained damage to its spicule and/or the surrounding tissue.**

A) shows the normally moving male and a detail of its posterior end. The inset is a blowup (3.4x) from the red square on the original image.

B) shows the abnormally moving male and a detail of its posterior end. This is the same victim as in Figure 5.4 and Figure 5.5. The male's spicules are constantly protruded combined with damage to the tissue surrounding the cloaca. The inset is a blowup (4.5x the main image) from a different picture.

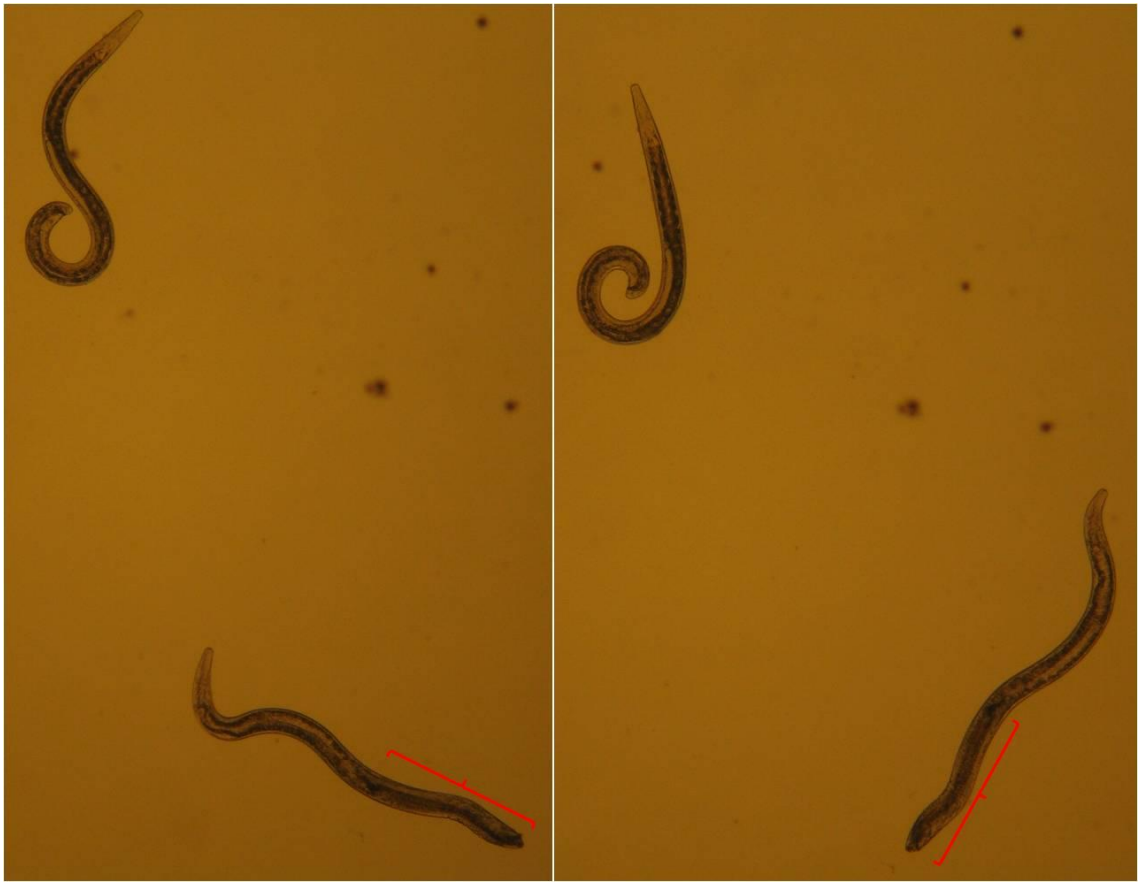


Figure 5.9 Two still frames in sequence of 2 *S. longicaudum* males observed 24 h after fighting (drop # 11). The male in the upper left of the pictures was moving completely normally, but the male in the lower right corner showed an abnormal appearance to the tissues of its posterior body and was not moving the part of its body flanked by the red bracket. Also see the recording "*S. longicaudum* Fight injuries.AVI" on the attached CD.

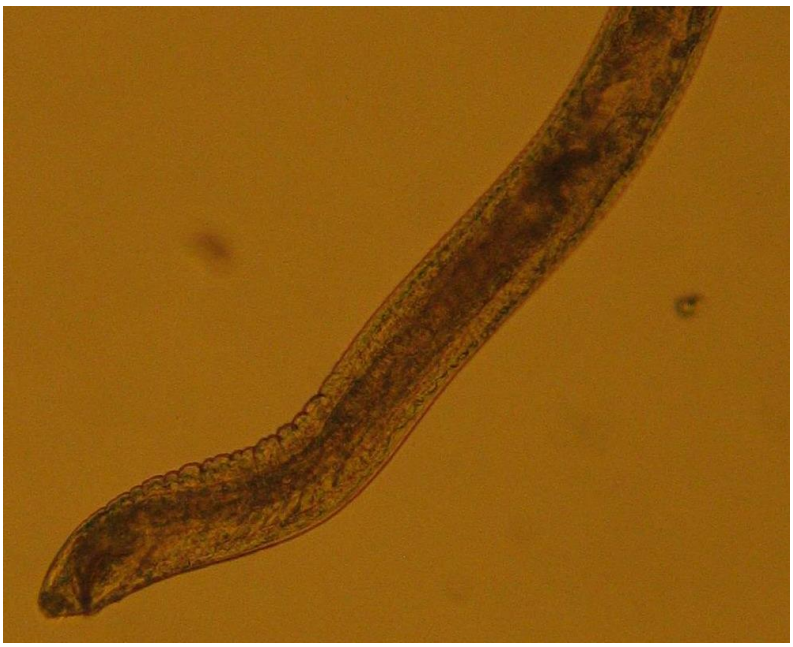


Figure 5.10 Another picture of the injured male of drop # 11 with more detail of the abnormal looking posterior part of the male's body.

5.3.3.4. Comparison of injuries in different species of *Steinernema*

Fighting in *S. feltiae* and *S. kraussei* (see section 4.3) resulted in similar injuries to *S. longicaudum* (see sections 5.3.3.2 and 5.3.3.3), however the incidence of a ruptured body wall in injured males was higher (Figure 5.11), although not significantly. Pairwise comparisons of the proportions of injured males that had suffered a ruptured body wall at the 12-24 h observation timepoint showed a significant difference between *S. longicaudum* and *S. kraussei* (Fisher's exact: p = 0.044, n = 40). It needs to be noted that the *S. longicaudum* data below were obtained from drops in which the males had experienced regular agitation within the 20 min after the males were paired, whereas the data of *S. feltiae* and *S. kraussei* were obtained from drops in which the males were put together and then left untouched for 24 h.

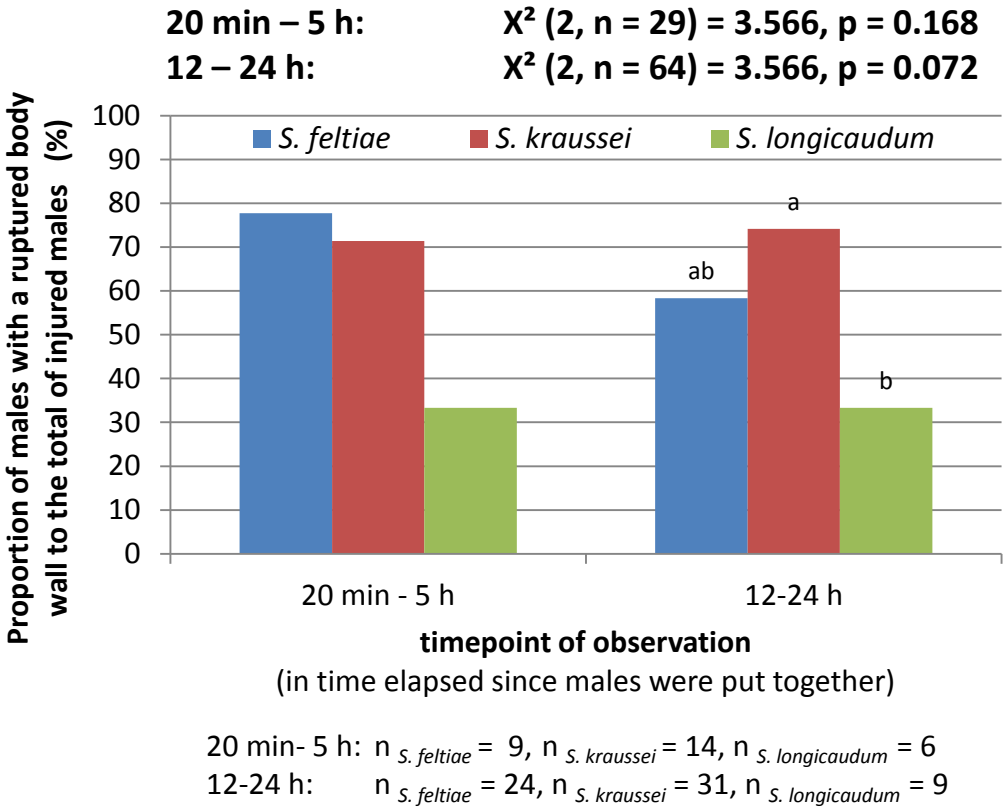


Figure 5.11 The injured or dead males with a ruptured body wall as a percentage of the total number of injured or dead males compared between 3 *Steinernema* species at 2 time points after pairing. Bars accompanied by no letter, or by the same letter are not significantly different from each other. Raw data and statistics in Appendix table 7.

5.3.4. Summary & conclusions

Of the single male controls, none showed injuries similar to those observed after fighting in 2-male drops. Only one 2-male drop had a male that showed injuries similar to those observed in the 2 single controls. The time pattern of injury and death of these 3 males was also alike and because all 3 males also came from the same batch, the injuries and subsequent death were likely caused by something unrelated to fighting.

Stimulated fighting of 2 male *S. longicaudum* CB2B resulted in different types of injuries. The victims sustained effects resulting from physical constriction of internal organs to the loss of content from internal organs protruding through a wound in the body wall. Stimulated fighting also resulted in tissue damage, for instance damage to the tissues surrounding the cloaca.

The most commonly observed injury immediately after a fight had taken place in the stimulated circumstances was disruption of the contents of internal organs such as the digestive tract due to constriction of the organ during fighting (observed in 4 worms). Constricted organs tended to burst within 24 h. Where the constriction was accompanied by a puncture of the body wall, the contents were extruded to the exterior of the worm (see Figure 5.7), but in some cases the ruptured internal organs were contained mainly within the cuticle that had become separated from the epidermis (see Figure 5.6).

About 24 h after the males were introduced to each other, the most commonly observed injuries were disruption of internal organs and puncture or rupture wounds of the body wall (4 worms including drop #9).

Perforation of the body wall alone was not followed by immediate paralysis; one male with a puncture wound even survived without paralysis for more than 24 h, showing that a lesioned cuticle or body wall does not inevitably result in entry of paralysing or lethal toxins or bacteria from the surrounding medium or even injection of a toxin by the attacker. The content protruding through this perforated cuticle or body wall still seems to be enclosed in a bubble by a membrane. In case diffusion through this membrane is not a possibility, this will be no real entry point of toxins or bacteria from the medium.

Paralysis immediately following a fight was seen in only two worms, one of which had a constriction but no visible puncture, and the other had no visible injuries. Thus, the 2 victims that showed immediate paralysis after a fight had not sustained any visible puncture wounds and one of them showed only the results of constriction. It seems contradictory to the

hypotheses of injection of a toxin using the spicules that paralysis occurred without the body wall being punctured.

It needs to be noted that the males in this experiment were constantly mechanically disturbed and this could have affected fighting and its resulting injury. Infliction of harm within the first 20 min of these stimulated fights occurred at a ratio about 5 times what would be expected in half an hour of normal fighting based on the results from 3.3.1 (about 10 %). Abnormal fighting by more agitated nematodes does not seem unlikely. In this light, differences between type of fighting injuries sustained by *S. longicaudum, S. kraussei* and *S. feltiae* will be more distinct when the frequency of ruptured cuticle injuries resulting from normal male-male *S. longicaudum* fights will be considered. Nota bene, the significantly higher proportion of ruptured cuticle injuries resulting from normal male-male *S. kraussei* fighting compared to stimulated male-male *S. longicaudum* fighting already shows the differences in injuries sustained from fighting in these 2 species and suggests differences in the mechanisms of fighting behaviour and wounding. Repeating the comparison of injuries between *S. feltiae* and *S. longicaudum* with only non-stimulated fights might show a significantly higher proportion of ruptured cuticle injuries in S. feltiae, indicating differences in the mechanisms of fighting and wounding might also exist between *S. feltiae* and *S. longicaudum*. In order to unravel the evolution of fighting behaviour in Steinernematidae, the fighting, killing and paralysis mechanisms of different species should also be examined in more detail.

Death in this experiment with stimulated *S. longicaudum* males could be the result of damaged internal organs, or entry of bacteria and/or toxins from the medium through puncture wounds in the body wall or by injection of a toxin by the attacker. However, four of the males that were dead or close to dead after 24 h had no visible lesions and one of the males that showed paralysis immediately after a fight did not show any visible lesions.

5.4. Mimicking of injuries and paralysis: Crushing

5.4.1. Introduction

In the previous experiment, the most common injury immediately after a fight was disruption of the contents of internal organs due to constriction of these organs during a fighting wrap. After 24 h, dead males without puncture wounds, but with these constriction injuries were observed. If paralysis is not caused by a toxin, either injected by the attacking male or originating from the medium, the pressure and constriction caused by a wrap might be the cause of paralysis and maybe even of subsequent death. In the following experiments, it is attempted to reconstruct similar constriction injuries by applying local pressure to male *S. longicaudum*.

Hypotheses

- Applying localised pressure on a male will cause disruption of the contents of internal organs in the majority of males and will lead to impeded movement of these males within 24 h after the pressure was applied.
- The majority of males that showed disruption of the contents of internal organs or paralysis within 24 h will have died within 72 h after the pressure was applied.

5.4.2. Material and Methods

Male *S. longicaudum* CB2B were reared in separate drops as described in section 2.3. Single males of 5 days old were used in their own drops for these experiments.

Several methods and instruments were tried out to produce wounds comparable to those that had resulted from fights between 2 male *S. longicaudum* CB2B. The normal movement of a *Steinernema* nematode made it quite difficult to precisely crush it. Therefore, 2 methods were tried out to minimise the possibilities of movement of the nematode. There were disadvantages associated with each method. Just placing the worm in a shallow part of the drop seemed not to suffice: the male could easily wriggle out from underneath the instrument used. Because of this, the method was changed to pulling the nematode out of its drop where it would stick to the surface of the Petri dish. This was effective but held the risk of desiccation of the nematode. This risk of desiccation was however preferred over not applying enough pressure or applying it to the wrong part of the body of the nematode. Adult nematodes had already proven to be able to survive quite a bit of desiccation (see sections 3.6.2.2 and 3.6.2.3:

production of incapacitated and dead males). Eight worms were treated in the shallow part of the drop, and five were treated outside the drop.

The immobilised worm was then briefly subjected to pressure applied to the posterior half of the worm's body. Table 5.3 lists the instruments used to apply pressure, and their characteristics. To reduce the risk of applying too much pressure, the pressure was applied only once, for 4 seconds or less. This however, enhanced the risk of not wounding the nematode due to incomplete contact of the wire with the nematode caused by bending of the wire or due to insufficient pressure or insufficient exposure to the pressure.

Table 5.3 Instruments tried out for mimicking the lesions sustained by *Steinernema* males during fights as a result of externally applied pressure on the male.

Material used for injuring the nematode	Characteristics of the material used
An insect dissecting needle	Very hard and sturdy
Platinum wire with diameter 0.2 mm	Easily distorted
Platinum wire with diameter 0.1 mm	Even more easily distorted
Tip of glass pipette pulled out in a flame to obtain a very thin glass rod.	The thin glass rod did not distort but did yield under pressure so that the application of pressure was visible but also very variable.

The immediate effects on the nematode were recorded, as were the outcomes 24 and 72 h after the application of local pressure. These observations were carried out as described in section 2.4.2.

5.4.3. Results

In eight out of 13 males, movement slowed down after the application of pressure (Table 5.4). Out of these, 3 males had sustained a bend in their body; four males had suffered a punctured body wall through which the intestines were protruding (Figure 5.12) and two males showed the separation of content of the digestive tract as was observed in victims of stimulated male-male fighting in section 5.3.3. However, none of the treated males showed real paralysis defined as the inability of moving part(s) of the body.

Six out of 13 males were dead or in very bad shape after 24 h. The other 7 males showed no or very limited longer term effects despite the application of local pressure on the posterior part of the body.

Table 5.4 Summary of the immediate observations and of the observations 24 h after the application of pressure on the posterior body of *S. longicaudum* males trying to mimic the wounding and paralysis occurring in *Steinernema* male-fights.

Instrument used to apply local pressure	No. of worms	Immediate consequences	Consequences 24 h later
Platinum wire of 0.10 mm diameter	5	All 5 males slowed down in movements.	Though 2 males were dead within 24 h, none showed visible damage.
Platinum wire of 0.20 mm diameter	4	2 males slowed down in movements.	1 male showed no visible effects. 1 male showed visible separation of internal organs due to constriction (Figure 5.14).
		2 males showed protruded entrails through a crush-ruptured body wall (Figure 5.12).	1 male was dead (Figure 5.15). 1 male was in very bad shape (recording " *S. longicaudum* Crush injuries.AVI "on attached CD; Figure 5.16).
Glass rod	1	Visible separation of internal organs due to constriction.	Moving normally after 24 h, but dead by 72 h.
Dissecting needle	3	1 male showed no visible effects.	No visible effects
		2 males showed protruded entrails through a crush-ruptured body wall (Figure 5.13).	Both males are dead.

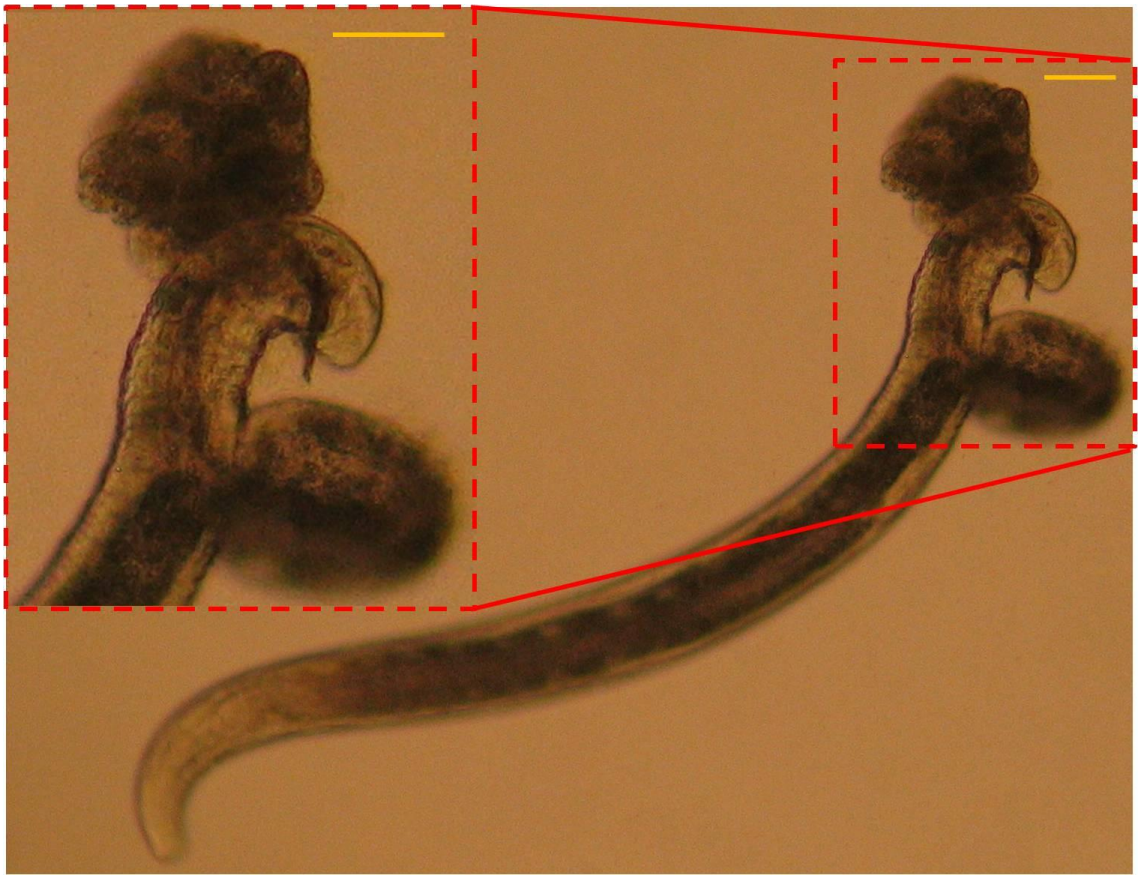


Figure 5.12 ***S. longicaudum*** **male suffering a crush-ruptured body wall and protrusion of its entrails through this wound immediately after the application of local pressure with a Platinum wire 0.20 mm diameter.The male had twisted a little under the pressure which might have added to the severity of the wound. The inset is a blowup (1.6x) from the red square on the original image.**

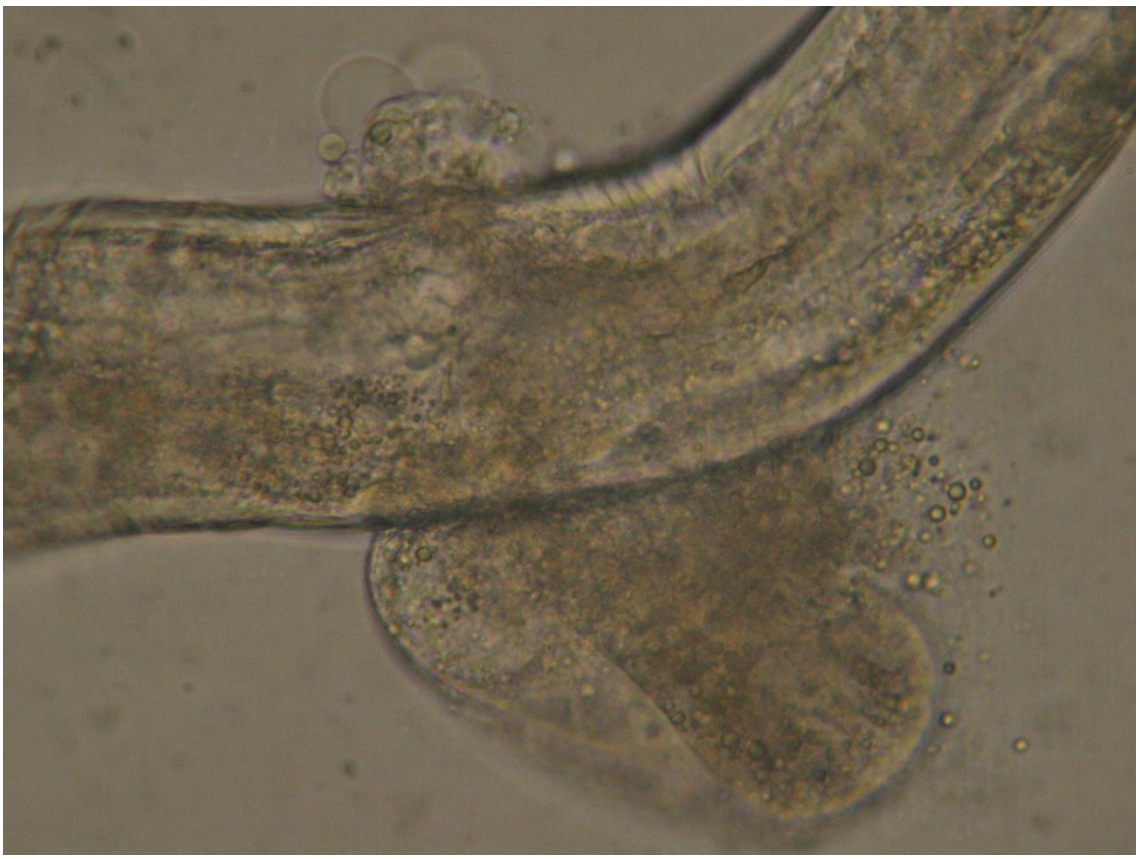


Figure 5.13 ***S. longicaudum*** **male suffering a crush-ruptured body wall and protrusion of its entrails through this wound immediately after the application of local pressure with a dissecting needle.**

Figure 5.14 ***S. longicaudum*** **male showing a constriction and even a twist of the gonad and the intestine 24 h after the application of local pressure with a 0.20 mm diameter platinum wire.**

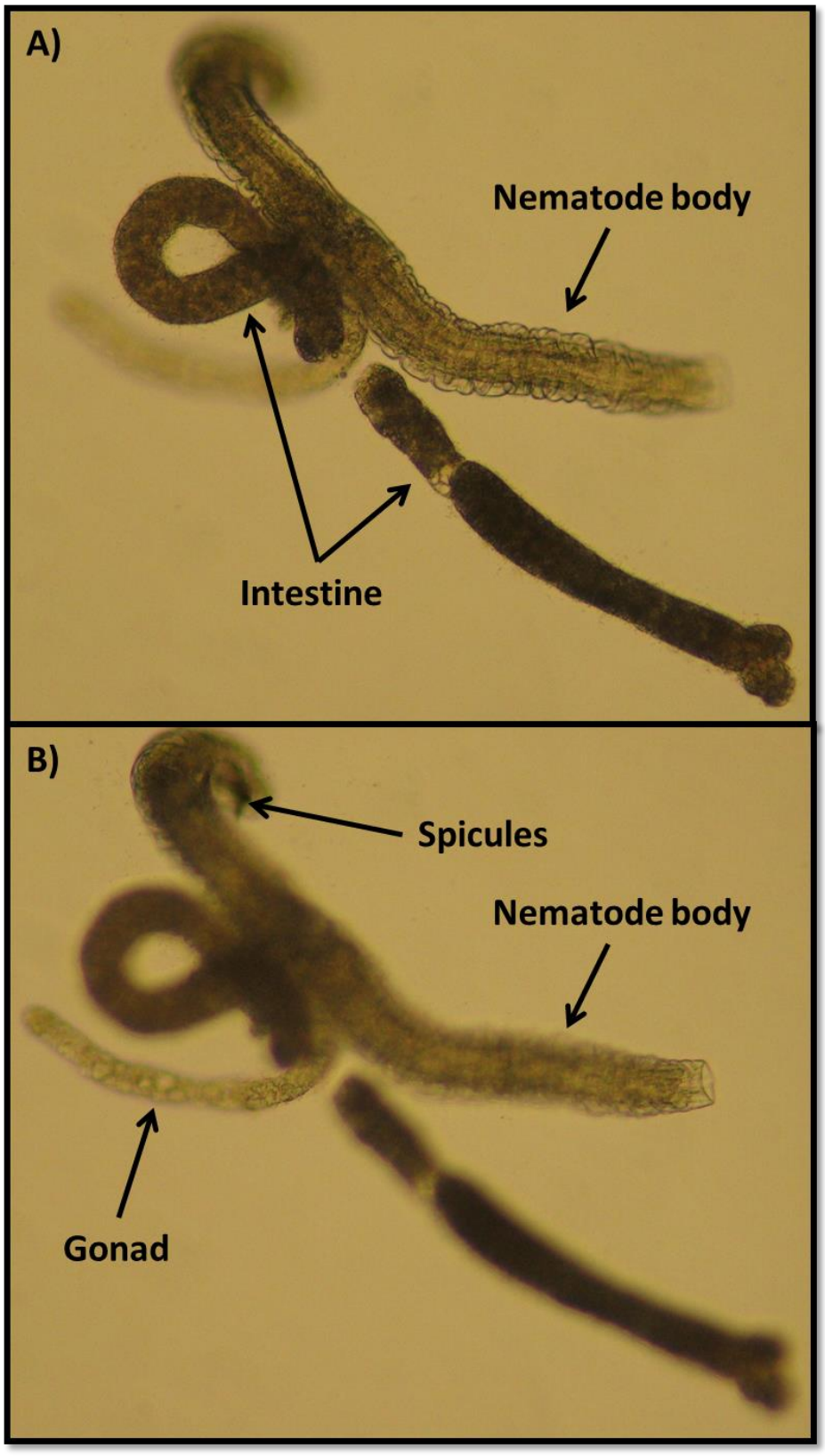


Figure 5.15 Picture of a *S. longicaudum* male 24 h after the application of pressure with a 0.20 mm diameter platinum wire during wich the male's body wall had crush-ruptured (Figure 5.12). A) is focussed on the intestine that has completely come out of the male's body. B) is focussed on the gonad that has also completely come out of the male's body.

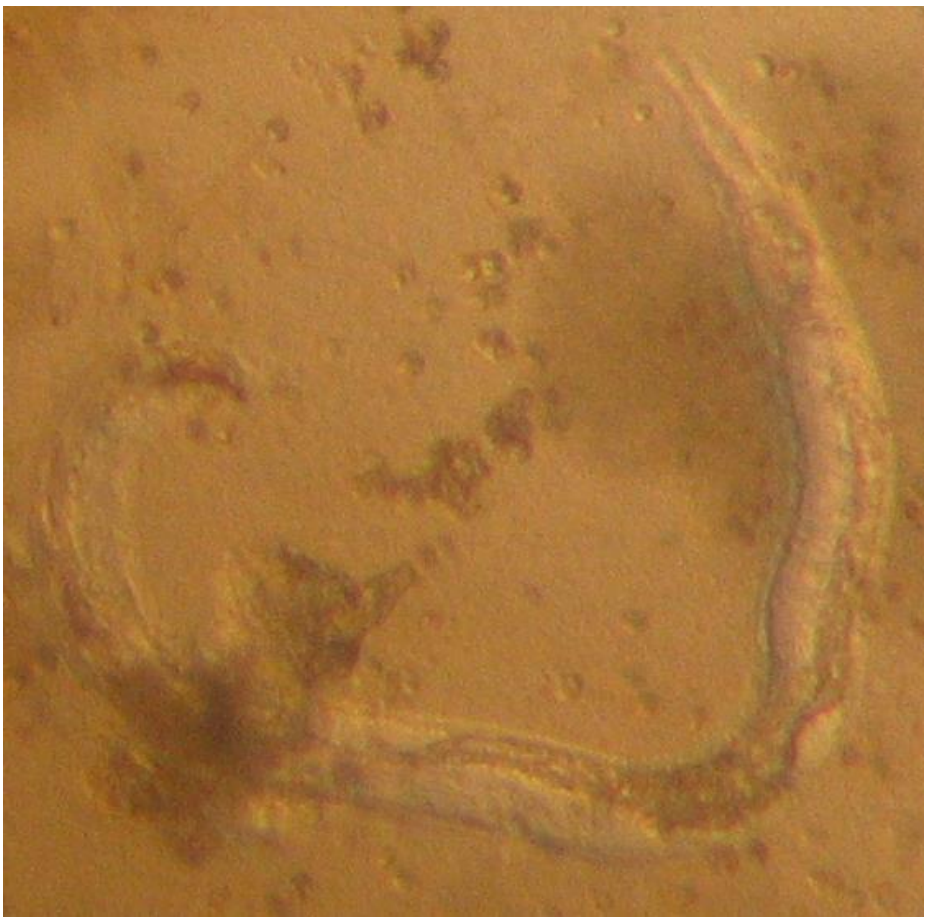


Figure 5.16 Picture of a *S. longicaudum* male 24 h after the application of pressure with a 0.200 mm diameter platinum wire during wich the male's body wall had crush-ruptured (Figure 5.12). The male is in very bad shape, but is still moving as can be seen in the recording "*S. longicaudum* Crush injuries.AVI" on the attached CD.

5.4.4. Summary & conclusions

S. longicaudum males that had been subjected to local pressure in an attempt to mimic the wounding and paralysis occurring in *Steinernema* male-fights, tended to show either severe injuries or no visible evidence of injuries. Four out of 13 males had suffered a crush-ruptured body wall and ruptured intestines. Although a lot of attention was paid to keeping the duration of application of pressure and the applied pressure low, it is still likely that the pressure applied was higher than the pressure another male would apply during a fighting wrap. The fact that 7 out of the 13 treated males didn't show injuries or paralysis can be explained by the single, short (mere seconds long) exposure to pressure, whereas the males in male-male fights, including those of the experiments in section 5.3, were possibly subjected to repeated attacks from the other male during more than 24 h.

Seven out of 13 males died within 72 h of the application of localised pressure, but only 1 of these dead males had showed disruption of the content of internal organs immediately after the application of pressure. Localised pressure on the body of a male didn't lead to disruption of the contents of internal organs in the majority of males. Nor did the application of pressure lead to impeded movement of the majority of males within 24 h after the pressure was applied. The majority of males died within 72 h after pressure was applied, however, this could not be linked to injuries like disruption of content of internal organs, only to more severe injuries which are only occasionally seen after normal male-male *S. longicaudum* fights.

The occurrence of injuries combined with the lack of proper paralysis indicates either that pressure alone doesn't explain the paralysis and the deadly consequences of male-male *S. longicaudum* fighting, or that the type of pressure and/or the region of the male to which it was applied did not adequately mimic the pressure applied by a male *S. longicaudum* wrapping around the body of another male.

Since the cuticle was not deliberately exposed to a sharp edge in this experiment, the crush-rupture of the body wall observed in 4 worms may be associated with internal pressure changes. This further suggests that a ruptured body wall observed after a male-male fight is not necessarily caused by puncturing of the body wall by the spicules.

In *S. longicaudum*, ruptures of the body wall after a normal male-male fight are not as intense as the crush-ruptures recorded in this crushing experiment. However in *S. feltiae* and *S. kraussei* this kind of severely ruptured body wall whether or not accompanied by ruptured internal organs protruding through the wound, was more often observed after male-male fights (see section 5.3.3.4). This could very well indicate that the techniques of fighting differ between species, with some species being more dependent on mechanical facets of fighting (ruptured body wall and internal organs for *S. kraussei* for example).

5.5. Mimicking injuries and paralysis: Stabbing

5.5.1. Introduction

To mimic the effect of physical insertion of the spicule into the victim's body, female *S. longicaudum* nematodes were stabbed. Stabbing a nematode asks for a lot of precision and care if the nematode is not to be accidentally cut in half. Because females are a lot bigger than males and expecting a stabbing wound to yield the same effect in females as in males, these experiments were carried out on female nematodes.

If stabbing a nematode in a bacteria- infected haemolymph drop causes paralysis, the diffusion of a toxin from this medium may be the cause of the observed paralysis. In this section, females are stabbed in their own drops which should contain their symbiont and associated excreted toxins, but not any toxins produced by males. In a second experiment, females are stabbed in medium that had contained either one male or a pair of fighting males, to allow for entry of any male produced as well as bacteria produced toxins.

Hypotheses

- In case a paralysing toxin is secreted into the medium by the symbiotic bacteria, stabbing females in their own drop, in medium in which 1 male had been present or in medium in which 2 males had fought, will all result in a significantly larger proportion of paralysed or dead females in comparison to the control females.
- In case a paralysing toxin is secreted into the medium by males, stabbing females in their own drop will not result in a significantly larger proportion of paralysed or dead females in comparison to the control females. But, stabbing females in medium in which 1 male had been present or in medium in which 2 males had fought, will result in a significantly larger proportion of paralysed or dead females in comparison to the control females.
- In case a paralysing toxin is secreted into the medium by fighting males, stabbing females in their own drop or in medium in which 1 male had been present, will not result in a significantly larger proportion of paralysed or dead females in comparison to the control females. But, stabbing females in medium in which 2 males had fought, will result in a significantly larger proportion of paralysed or dead females in comparison to the control females.
- In case stabbing females in their own drop, in medium in which 1 male had been present or in medium in which 2 males had fought, doesn't result in a significantly

larger proportion of paralysed or dead females in comparison to the control females, either no paralysing toxin is secreted into the medium (but the possibility of injection into the victim by the attacking male is still plausible), females are not as sensitive to the toxin as males or no toxin is involved in paralysis.

5.5.2. Methods

The *S. longicaudum* CB2B females were reared in separate drops as described in section 2.3. Single females of 2 days old were immobilised so that they could be stabbed with either an insect dissecting needle or a micro-injection needle.

Different methods were used for immobilisation of the nematode:

1. The nematode was pulled out of its drop using a platinum wire. Outside of its drop, it was subjected to mild desiccation which slowed down its movement. This made it possible to more precisely stab the nematode with a sharp insect dissecting needle mounted on a sharpened long toothpick. About 20-40 µl of Ringer's solution was placed on top of the nematode. By pulling this Ringer's solution drop into the haemolymph drop the nematode was moved back into its hanging drop.

2. Using a mouth suction pipette, the nematode was transferred from its drop onto an agarose pad. By taking off as much as possible of the co-transferred liquid, the nematode was immobilized by sticking to the agarose pad. This made it possible to more precisely stab the nematode with an Eppendorf Femtotip II needle (cat No: 5242957000= sterile glass injection capillary, 0.5 µm inner and 0.7 µm outer diameter) using a micro-injection setup (see section 2.5.2). Before pulling the needle out of the nematode a drop of 50-70 µl of the medium to be tested was added. After pulling the needle out, the nematode was left 5 - 30 min to come apart from the agarose pad in a humid chamber, before being put back into a hanging drop of fresh haemolymph. The testing media were: haemolymph in which two males had fought resulting in paralysis; haemolymph in which one nematode had resided, or haemolymph from the female's own drop. Additional females were treated as above but were not stabbed and were left as controls.

5.5.3. Results

5.5.3.1. Stabbing females with an insect dissecting needle

Already 3 h after the females were stabbed a significant portion of them (36.4 %) showed impeded movement. After 4 h, this had risen to 50 % (Figure 5.17) and a significant portion of the stabbed females (38.9 %) were no longer able to move (Figure 5.18). After 24 h, more than 65 % showed impeded movement and more than 50 % were no longer able to move. These females showed no sign of recovery over a further 2 days and were presumed dead.

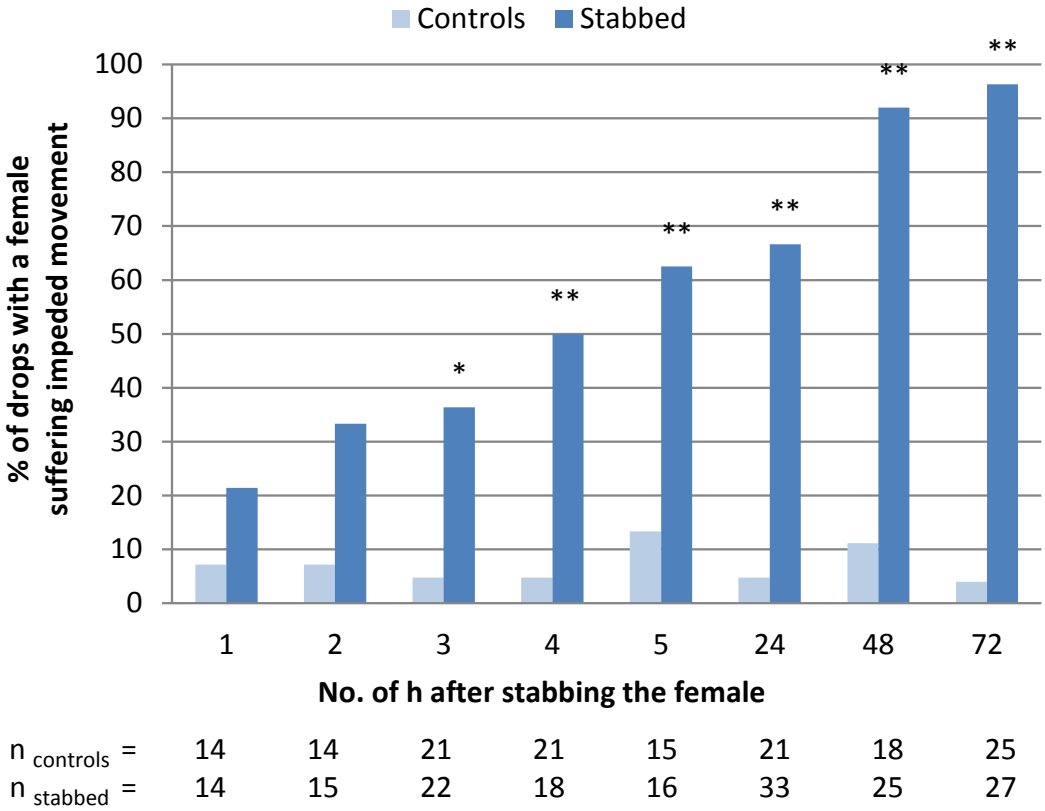


Figure 5.17 The percentage of drops containing a female that suffered impeded movement at the specified timepoint after stabbing. Two by two Chi-Square tests were carried out on the stabbed females and controls at each timepoint. Those bars marked with an asterix (*) show the time points where the difference between controls and stabbed females was significant with 0.05 > p ≥ 0.01. Those bars marked with a double asterix (**) show the time points were the difference between controls and stabbed females was significant with 0.01 > p > 0.001. Raw data and statistics in Appendix table 8.

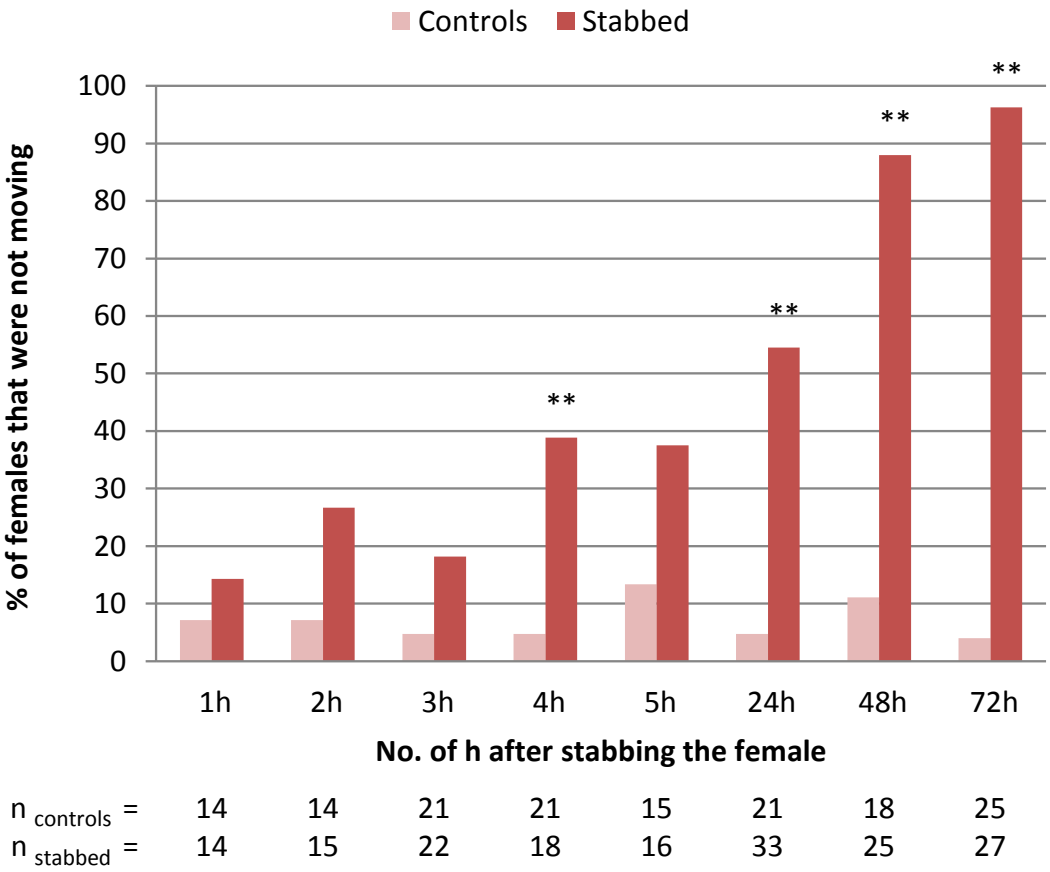


Figure 5.18 The percentage of drops containing a female that was no longer moving at the specified timepoint after stabbing. Two by two Chi-Square tests were carried out on the stabbed females and controls at each timepoint. Those bars marked with an asterix (*) show the time points where the difference between controls and stabbed females was significant with 0.05 > p ≥ 0.01. Those bars marked with a double asterix (**) show the time points were the difference between controls and stabbed females was significant with 0.01 > p. Raw data and statistics in Appendix table 9.

5.5.3.2. Stabbing females using a micro-injection setup

When females were stabbed with a micro-injection needle in a drop of haemolymph, only few females suffered impeded movement or worse (maximum 29 % after 48 h, Table 5.5). There was no significant difference between all 4 treatments, including the control females that had not been stabbed (Table 5.5). In addition, there was no difference between the 3 stabbed treatments, nor were there differences between any of the stabbed treatment and the unstabbed controls (Chi-square tests, $p > 0.05$).

Table 5.5 The effect of stabbing a female with a micro-injection needle in the specified medium.

	No. of females after 24 h that		No. of females after 48 h that	
The stabbing medium is haemolymph in which	**were moving normal**	**suffered impeded movement or worse**	**were moving normal**	**suffered impeded movement or worse**
• 2 males had fought and paralysed	7	2	7	2
• A single male had resided	12	2	10	4
• A female had resided	26	2	25	3
• The female was not stabbed	10	1	9	2
Statistics:	χ^2 (3, n = 62) = 1.773, p = 0.630		χ^2 (3, n = 62) = 2.198, p = 0.532	

5.5.4. Summary & conclusions

Four hours after being stabbed with an insect dissecting needle, about 50 % of the females showed restricted movement. More than 50 % of the females that were stabbed with an insect dissecting needle died within 24 h of being stabbed. This could have indicated the involvement of toxins and/or bacteria present in the hanging drop in the paralysis and/or killing process. However, it needs to be noted that the insect dissecting needle was fairly blunt, even in comparison to the dimensions of the female nematodes and most definitely in comparison to a *Steinernema* spicule. It is thus more likely that the massive amount of damage caused by stabbing the nematode with an insect dissecting needle is responsible for the impeded movement and following deaths. Moreover, using the much finer micro-injection needle

showed a significant reduction in the amount of deaths and movement restriction (maximum 29 % after 48 h). The medium in which a female was stabbed had no significant influence on the occurrence of movement restriction or death, but we need to keep in mind that the total numbers were low. The results of the micro-injection needle stabbing experiment do not support the hypothesis that a toxin from the medium causes paralysis and death following entry through the damaged cuticle. Nor does it suggest that a small stab-wound causes paralysis and death of a *S. longicaudum* female; however, the effect may be different for a male.

5.6. Mimicking injuries and paralysis: Injecting

5.6.1. Introduction

Merely stabbing a nematode might not allow diffusion of the putative toxin or sufficient amounts of the toxin into the body of the nematode. Injecting a male or female *S. longicaudum* with fractions of bacteria- infected haemolymph or with fractions of the seminal fluid of male *S. longicaudum* nematodes might reveal the origin of the toxin. Attempts to extract seminal fluid from *S. longicaudum* males were unfortunately unsuccessful. It also proved to be very difficult to obtain fractions of haemolymph that were injectable (i.e. did not clog up the micro-injection needle). Due to these difficulties an artificial medium inoculated with the symbiotic bacteria that supported fighting and lent itself to injection through a micro injection setup was used.

Hypotheses

- In case a paralysing toxin is secreted into the medium by the symbiotic bacteria, injecting males with medium in which 1 male had been present or with medium in which 2 males had fought, will result in a significantly larger proportion of paralysed or dead males in comparison to the control males that were injected with sterile medium.
- In case a paralysing toxin is secreted into the medium by males, injecting males with medium in which 1 male had been present or with medium in which 2 males had fought, will result in a significantly larger proportion of paralysed or dead males in comparison to the control males that were injected with sterile medium.
- In case a paralysing toxin is secreted into the medium by fighting males, injecting males with medium in which 1 male had been present, will not result in a

significantly larger proportion of paralysed or dead males in comparison to the control males that were injected with sterile medium. But, injecting males with medium in which 2 males had fought, will result in a significantly larger proportion of paralysed or dead males in comparison to the control males that were injected with sterile medium.

5.6.2. Materials and Methods

Media for injection were prepared as follows: on day 1 of the experiment, infective juveniles of *S. longicaudum* were put in fresh haemolymph drops (1 per drop) and stored for development in a 27 °C incubator. On day 2, 50 ml vials with 15 ml Brain Heart Infusion Broth with extra chemicals (BHIB extra; see section 2.4.4) were each inoculated with 6 loops of medium from haemolymph drops that had contained developing juveniles (from drops inoculated on day 1) and therefore the symbiotic bacteria. These vials were then incubated on a shaker-rotator at 27 °C for about 24 h. On day 3 the adult nematodes were sexed and placed into hanging drops of incubated BHIB extra. In 14 drops, the males were left single, in another 14 drops, 2 males were placed together in a drop. In 6 of the 14 drops with 2 males, one of the males was highly likely to be matured because the drop it had developed in had also contained at least 1 female (in one drop 2 females had been present and mating was observed).

After 18-21 h at 27 °C (day 4 of the experiment), the drops were checked for survival. All the single males were alive and moving normally whereas of the coupled males only 3 of the 14 drops now contained males that were both moving normally. In the other 11 drops, at least 1 male suffered impeded movement or worse. These 11 drops were collected in an Eppendorf filter and spun for 10-15 min at 14000 rpm. Ten of the single male drops went through the same procedure as did an equal volume of sterile BHIB extra. There were thus 3 media: BHIB in which 2 males had fought, BHIB in which 1 male had resided, and sterile BHIB.

Using the micro-injection setup (see section 2.5.2) healthy looking *S. longicaudum* males, 2 -3 days post infection of live *Galleria mellonella* larvae, were injected with the 3 different media. The protocol used was adapted from micro-injections in *C. elegans* (Evans, 2006). *S. longicaudum* nematodes do not survive desiccation as well as *C. elegans*, the worms had thus to be injected quickly which is why the flow of the needle was checked before putting the nematode on the agarose pad. The injection was stopped when the pseudocoel seemed to be filled with liquid (a few nl). The worms were returned to the dissecting microscope where a drop (~20 µl) of M9 -buffer was added so that it completely surrounded the worms and they could release off of the pad and recover. Once the worm began swimming briskly, it was

transferred to a hanging drop of fresh haemolymph. Nematodes that were too desiccated or were damaged by the injection were discarded so that only males where the injection went well are included in the analyses.

Some control males were left uninjected but went through all of the other handling steps (move to another drop, slight desiccation on the agarose pad) so that the effect of desiccation could be established.

5.6.3. Results

More than 80 % of the males in each treatment were still moving a day after injection, and most (66-75 %) were moving normally (Figure 5.19). The 3 injection-media did not have any significantly different (Appendix table 11) effect on the survival or on the movement capabilities of male *S. longicaudum*. Impeded movement recorded 2-3 h after injection had the smallest p-value ($\chi^2(2, n = 81) = 4.802$, $p = 0.091$, 1 cell with expected count < 5), all other treatments and time points were $p > 0.5$. Lacking male controls over a longer period of time, the male injection data were compared with the data of females that were not stabbed in the injection needle stabbing experiment in 5.5.3.2: $p = 0.171$ with 1 cell with an expected count less than 5, $\chi^2(1, n = 72) = 1.872$.

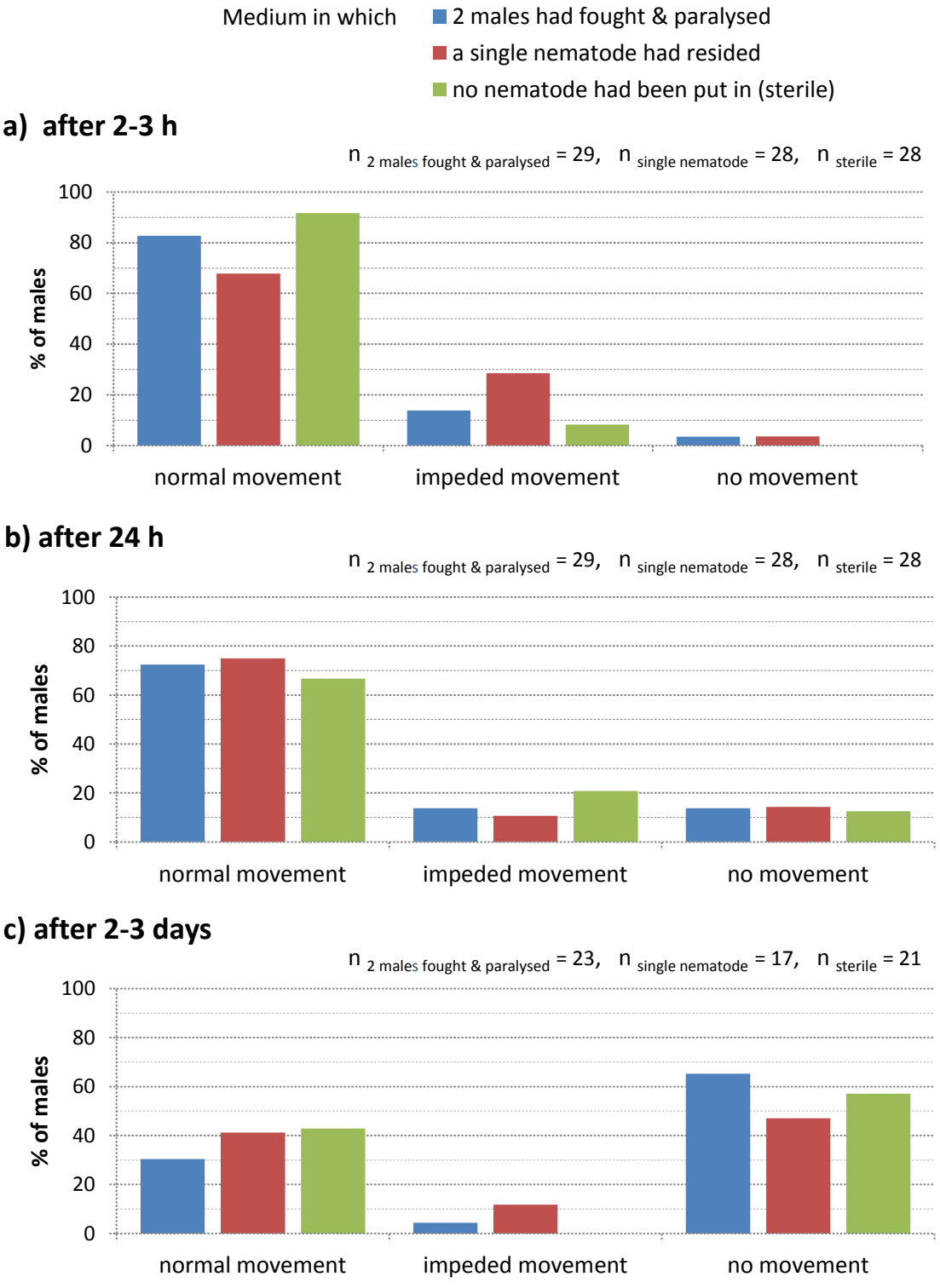


Figure 5.19 The percentage of males that were moving normally, suffered only impeded movement or were dead at the specified time point after injection. At none of the different observation time points were there significant differences observed (all $p > 0.1$). Raw data and statistics in Appendix tables 10 and 11.

5.6.4. Summary & conclusions

There was no difference in paralysis or death after injecting a male *S. longicaudum* with artificial media differing in whether 2 males had fought and paralysed in it or not. There was even no difference in the effect an inoculated medium and a sterile medium had on the survival and movement capabilities of the males. This injection experiment further suggests that the main cause of paralysis and/or death of a fighting victim is not a chemical in the medium that enters through a wound in the victim. As the production of metabolites by *Xenorhabdus* depends on the growth medium, the artificial medium used here may not support the production of toxins in the same way that haemolymph does (Maxwell *et al*, 1994;Wang *et al*, 2010).

5.6.5. Comparison of different methods of mimicking injuries and paralysis

Comparing the results of the stabbing and injection experiments (Figure 5.20), it is clear that stabbing a female with an insect dissecting needle resulted in significantly more impeded movement than stabbing a female with a micro-injection needle or injecting a male with various BHIB media (24 h: $\chi^2(2, n = 165) = 28.751$, $p < 0.001$; Pairwise comparisons: comparing the 2 experiments with stabbed females but with different utensils: $\chi^2(1, n = 84) = 27.176$, $p < 0.001$; comparing the injection and the stabbing with micro-injection needles: $\chi^2(1, n = 132) = 5.049$, $p < 0.05$, DF = 1; comparing the stabbing with a dissecting needle to injection: $\chi^2(1, n = 114) = 14.375$, $p < 0.001$; 48 h: the 2 experiments with stabbed females but different utensils: $\chi^2(1, n = 75) = 38.047$, $p < 0.001$. Raw data in Appendix table 12). This is presumably due to the blunt nature of the dissecting needles used relative to the micro-capillary injection tip.

Injecting males with different media resulted in significantly more paralysis and death after 24 h than for females that were only stabbed with a micro-injection needle. Maybe males are more fragile than female nematodes or the size dimorphism between males and females caused males to be more prone to suffer from stabbing, even with an injection needle. Or the significant difference in the level of paralysis and death between micro-injected males and micro-injection needle stabbed females is due to the media getting into the body of the nematode. Further experiments using the same sex in both stabbing and injecting experiments could clarify this.

Mimicking injuries and paralysis with the methods used above didn't bring insight into the mechanisms behind paralysis and death after male-male *S. longicaudum* fighting. Even the

involvement of a toxin in the medium or the involvement of the symbiotic bacteria could be clarified, however, it does seem unlikely that a medium-born toxin is involved or that the bacteria are necessary for paralysis and/or death.

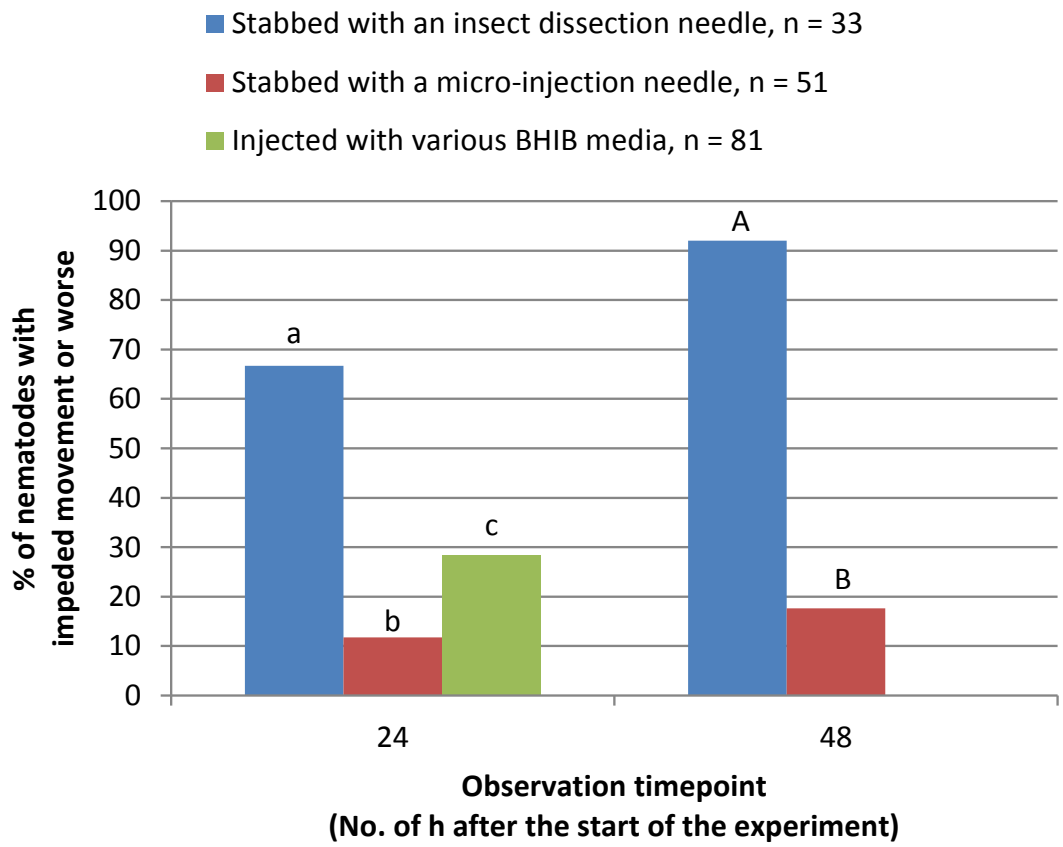


Figure 5.20 The effects of stabbing a female with an insect dissecting needle (blue bars), stabbing a female with a micro-injection needle (red bars) and injecting a male with different BHIB-media (green bar) on the movement capabilities of *S.longicaudum*. Bars marked with a different letter are significantly different from each other. No observations were carried out for the 48 h observation timepoint of the injected males. Raw data in Appendix table 12.

5.7. Media and bacteria

5.7.1. Introduction

Steinernematidae are symbiotic with *Xenorhabdus* bacteria which are released upon entry into the insect haemocoel and proliferate in this medium. The bacteria are known to influence their symbiotic nematode's behaviour: e.g. IJ-recovery (Aumann & Ehlers, 2001;Hirao *et al*, 2010) and cadaver attractiveness to late arriving nematodes (Grewal *et al*, 1997). These bacteria might thus also influence the fighting, paralysis and killing behaviour of the worms in a number of ways. This was investigated by also pairing males in drops that had only been inoculated with *Xenorhabdus* at the time point of pairing or in drops that contained no *Xenorhabdus*, through the following 2 experiments:

1. Normal, separately reared *S. longicaudum* males were set up for fighting in haemolymph drops at different time points since inoculation with the symbiotic bacteria.

Hypotheses

- Drops of haemolymph in which males were setup for fighting and that were inoculated by the insertion of one of the males of the pair as an infective juvenile (normal culture), will show evidence of fighting in a normal time-pattern (see section 3.3.3.2.), i.e. the occurrence of paralysis and death will be significantly different between couples of males and single males.
- Drops of haemolymph in which males were setup for fighting and that were inoculated by the insertion of both males, so at the moment the 2 males were put together (starting culture), might show fighting but will show no or significantly less fighting related paralysis and death than in the drops with a normal culture of the symbiotic bacteria. In essence, the number of drops with at least 1 male paralysed or dead will be significantly less for the starting culture 2-males drops than for the normal culture 2-males drops. There will also be no or almost no difference in paralysis or death between couples of males and single males in drops with a starting culture of the bacterial symbiont, especially at the earlier observation time points.
- Because the bacteria in the starting culture will grow and multiply, the differences in the results of fighting between the 2 types of cultures will diminish over time as

the starting culture develops into a normal culture and the effects of fighting also become observable in these drops.

- Single male death, injury or paralysis will be more likely to occur in the drops with a bacterial starting culture than in normal culture drops because of the activity of the hosts' immunity system or contaminating bacteria.

2. *Xenorhabdus*-free infective juveniles were separately reared in haemolymph drops. The resulting aXenic males were then staged for fighting in these *Xenorhabdus*-free haemolymph drops. *Xenorhabdus*-containing infective juveniles were separately reared in haemolymph drops. The resulting Xenic males were then staged for fighting in these *Xenorhabdus*-containing haemolymph drops. The *Xenorhabdus*-free infective juveniles should not have brought in any *Xenorhabdus* bacteria into the drop in which they had developed, however, the presence and development of other bacteria could not be prohibited.

Hypotheses

- Drops of haemolymph in which males were setup for fighting and that were inoculated with *Xenorhabdus*-containing IJs will show evidence of fighting in a normal time-pattern, see section 3.3.3.2.
- Drops of haemolymph in which males were setup for fighting and that were inoculated by the insertion of *Xenorhabdus*-free IJs will probably show fighting, but significantly less paralysis or death related to this fighting, i.e. there will be no or almost no difference in paralysis or death between aXenic groups of males and single males and the occurrence of paralysis and death of at least 1 male in a 2- or 5-males drop will differ significantly in Xenic and aXenic drops.
- Single male death, injury or paralysis will be more likely to occur in the drops inoculated with *Xenorhabdus*-free IJs than in drops inoculated with *Xenorhabdus*-containing IJs due to contaminating bacteria or remnants of the insect's immune system.
- The differences in results of fighting between the *Xenorhabdus*-containing drops and the *Xenorhabdus*-free drops will not diminish over time.

5.7.2. Materials and Methods

5.7.2.1. Haemolymph drops with differing incubation periods of *Xenorhabdus*

Infective juveniles were let to develop into adults in hanging drops of fresh haemolymph as described in section 2.3. After 4-5 days in the 27 °C incubator, the resulting males were divided over the following treatments:

- 2 males of the same age paired in the drop of one of the males (normal culture);
- single male in its original drop (controls in normal culture);
- 2 males of the same age were paired in a fresh drop of haemolymph (starting culture);
- single male in a fresh drop of haemolymph (control in starting culture).

The age of males at observations varied, but 2 males in a drop were always of the same age.

Drops were examined every hour after insertion of the males for up to 7 h. They were also examined about 24, 48 and 72 h after the start of the experiment.

5.7.2.2. Haemolymph drops with and without *Xenorhabdus* (Xenic and aXenic)

Fresh haemolymph drops were infected with 1 *Xenorhabdus*-free infective juvenile (see section 2.4.5.) (resulting drops should not have contained *Xenorhabdus* and are therefore termed aXenic, although there may have been other bacteria present) or 1 *Xenorhabdus* -containing infective juvenile (should have contained *Xenorhabdus* and are therefore termed Xenic).

The *Xenorhabdus*-free developed males were placed together with 1 or 4 other *Xenorhabdus*-free males or left singly in an aXenic drop. The Xenic males were placed together with 1 or 4 other Xenic males or left singly in a Xenic drop.

The age of males at grouping varied from 2 to 8 days, but 2 males in a drop were always of the same age.

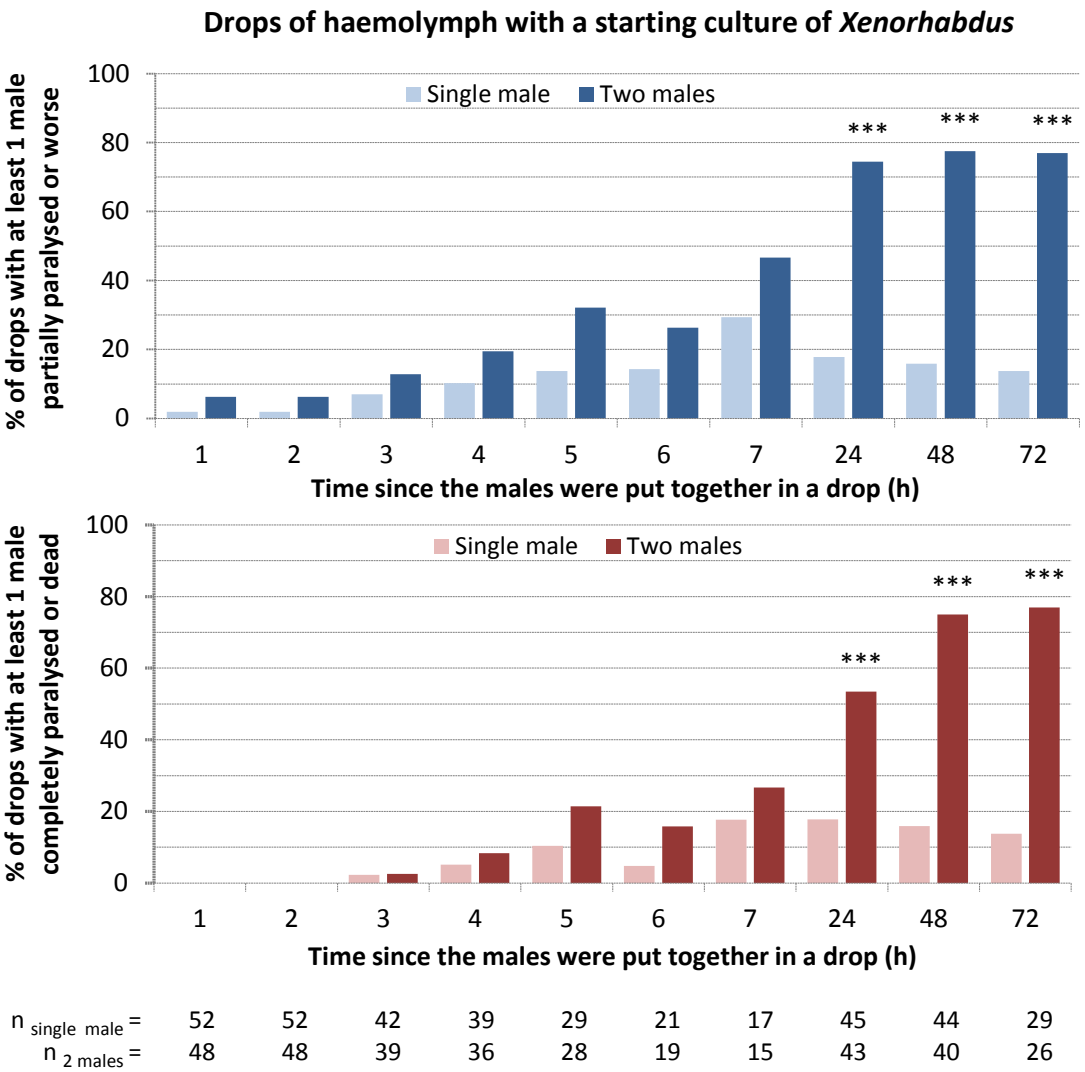
The drops were examined about 24 and 48 h after the start of the experiment.

5.7.3. Results

5.7.3.1. Haemolymph drops with differing incubation periods of *Xenorhabdus*

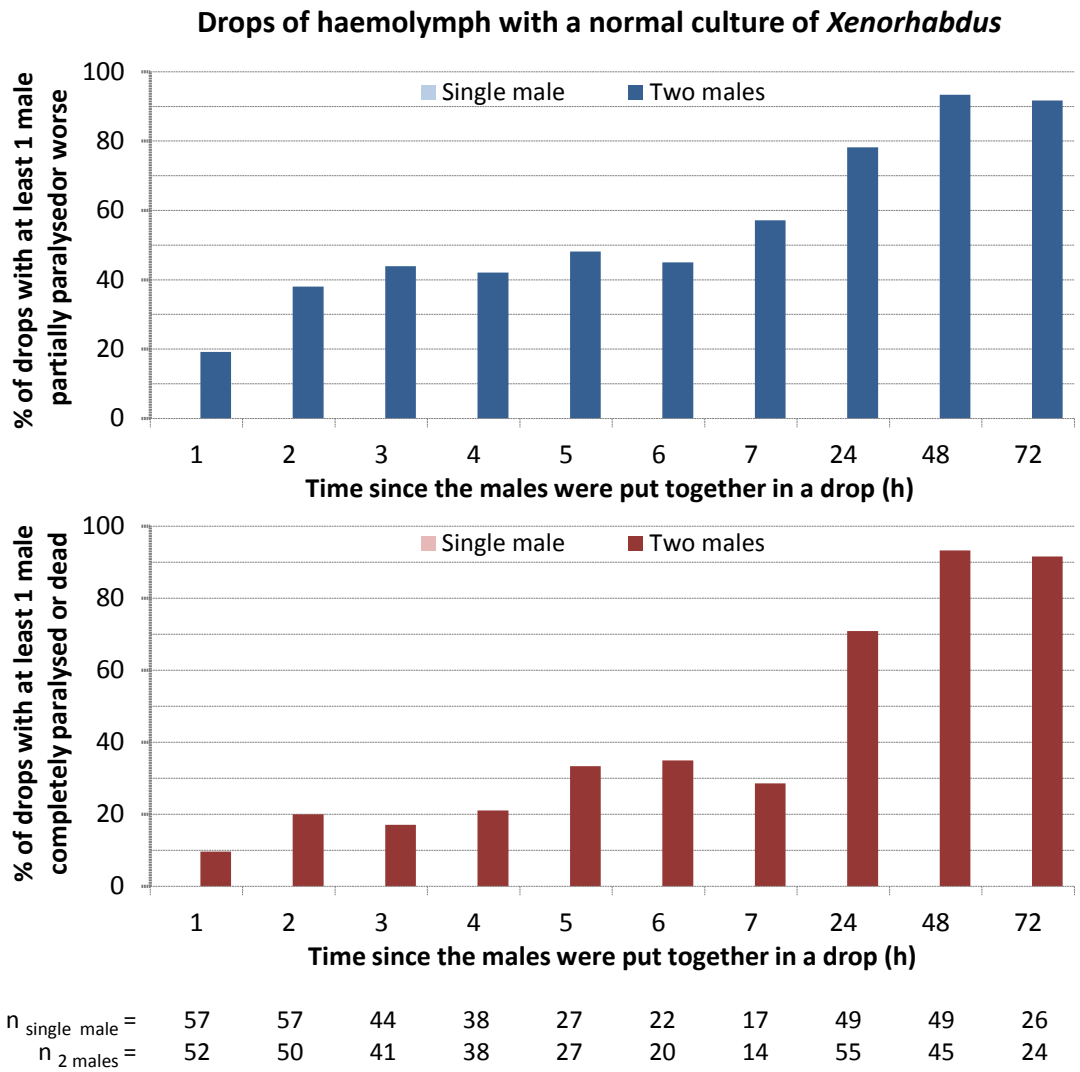
There was relatively high mortality for single males placed in haemolymph drops that had not previously contained a nematode and its *Xenorhabdus* symbiont ("starting culture"; Figure 5.21) compared to drops with a normal culture of bacteria (Figure 5.22). When males were put together for fighting in a fresh drop of haemolymph (starting culture), there was significantly more paralysis and death in the paired males than the single males, but only 24-72 h after the males were paired in the drop (24 h: paralysis: $\chi^2(1, n = 88) = 28.453$, $p < 0.001$; death: $\chi^2(1, n = 88) = 12.289$, $p < 0.001$; 48 h: paralysis: $\chi^2(1, n = 84) = 32.084$, $p < 0.001$; death: $\chi^2(1, n = 84) = 29.685$, $p < 0.001$; 72 h: paralysis and death: $\chi^2(1, n = 55) = 22.214$, $p < 0.001$; Figure 5.21). Whereas when the bacteria had already colonised the drop over a couple of days (normal culture), the difference in paralysis and death in the paired males compared to the single males was already significant 1 h after the males were paired in the drop (paralysis: Fisher's exact: n = 109, $p < 0.001$; death: Fisher's exact: n = 109, $p = 0.0222$; Figure 5.22).

Comparing the occurrence of paralysis and death in drops with 2 males, the occurrence of paralysis started earlier in the drops with a normal culture of the symbiotic bacteria than in drops with a starting *Xenorhabdus* culture (2 h ($\chi^2(1, n = 98) = 14.180$, $p < 0.001$), 3 h ($\chi^2(1, n = 80) = 9.427$, $p < 0.005$) and 4 h ($\chi^2(1, n = 74) = 4.432$, $p = 0.035$) (Figure 5.23). The number of drops with at least 1 dead male differed significantly between the drops with different incubation times at the 2 h observation time point only (Fisher's exact: n = 98, $p = 0.0012$ Figure 5.23). There was also significantly more paralysis and death in the drops with a normal culture of the symbiotic bacteria than in the drops with a starting culture 48 h after the males were placed together.



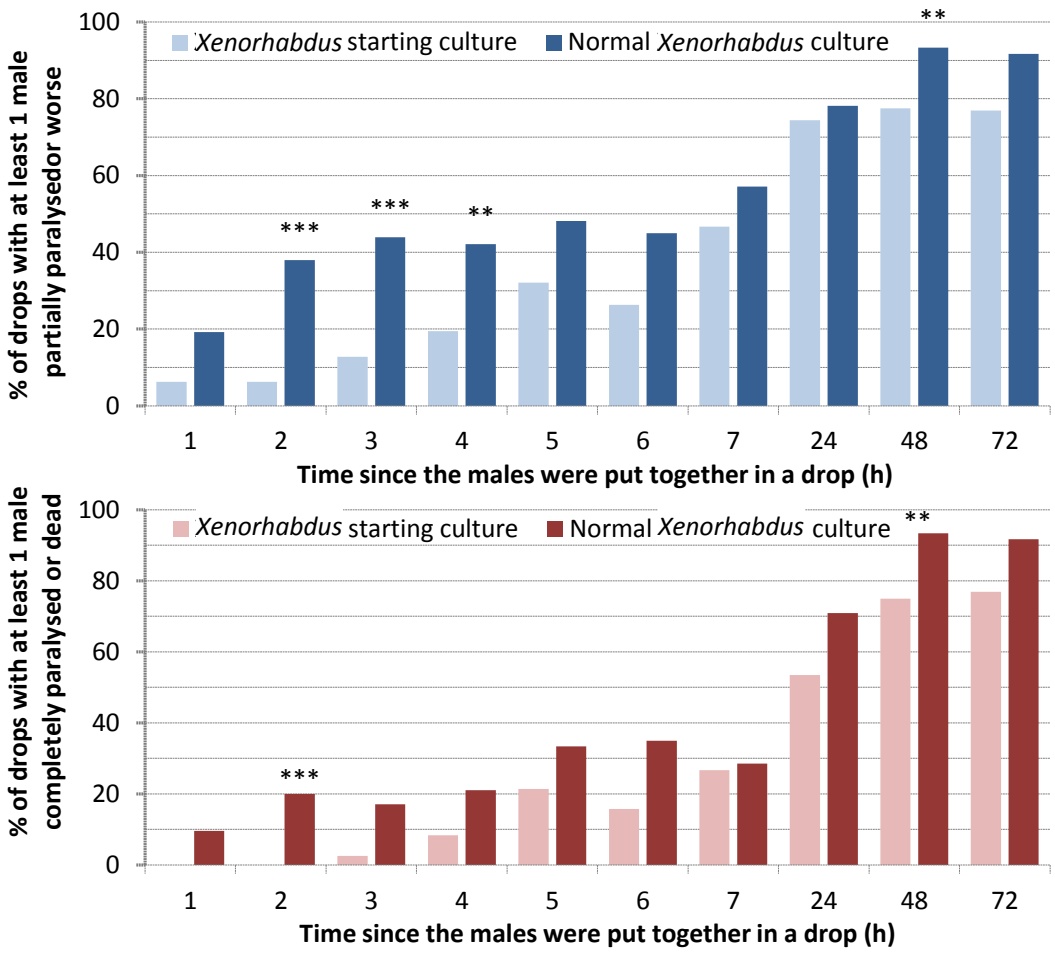
$n_{single\ male}$ =	52	52	42	39	29	21	17	45	44	29
$n_{2\ males}$ =	48	48	39	36	28	19	15	43	40	26

Figure 5.21 The occurrence of paralysis and death in drops with 2 males and a starting culture of symbiotic bacteria. Those bars marked with a triple asterix (***) show the time points were the difference between single controls and 2-males drops was significant with 0.01 > p.



$n_{single\ male}$ =	57	57	44	38	27	22	17	49	49	26
$n_{2\ males}$ =	52	50	41	38	27	20	14	55	45	24

Figure 5.22 The occurrence of paralysis and death in drops with 2 males and a normal culture of symbiotic bacteria.
At all observation time points, the number of drops with at least 1 male partially paralysed and the number of drops with at least 1 male completely paralysed or dead, is significantly higher in the drops with two males than in the drops with the single male ($p < 0.05$).



$n_{\text{starting culture}}$ =	48	48	39	36	28	19	15	43	40	26
$n_{\text{normal culture}}$ =	52	50	41	38	27	20	14	55	45	24

Figure 5.23 The occurrence of paralysis and death in drops with 2 males compared between drops with a starting culture of symbiotic bacteria and drops with a well established culture of symbiotic bacteria. Those bars marked with a double asterix (**) show the time points where the difference between drops with a starting culture and drops with a normal bacterial culture was significant with 0.05 > p ≥ 0.01. Those bars marked with a triple asterix (***) show the time points were the difference between controls and stabbed females was significant with 0.01 > p.

5.7.3.2. Haemolymph with and without *Xenorhabdus* (Xenic and aXenic)

Less than 3 % of the single males showed impeded movement (Figure 5.24) and less than 2 % of single males died in each of the treatments, even after 48 h. Whether the infective juveniles started out Xenic or aXenic did not influence the condition of the single males in their own drop at either of the observation time points.

The different treatments affected the occurrence of **paralysis** of at least 1 male at both 24 and 48 h after the males were put together in 1 drop. Drops with pairs of males of Xenic or of aXenic origin showed more drops with paralysis than their single controls (Figure 5.24; 24 h Xenic: Fisher's exact n = 214, $p < 0.001$; 24 h aXenic: Fisher's exact n = 178, p = 0.001; 48 h Xenic: χ^2 (1, n = 210) = 122,627, $p < 0.001$; 48 h aXenic: Fisher's exact n = 138, p = 0.001). The occurrence of paralysis of at least 1 male in Xenic 5-males groups was significantly higher than the occurrence of paralysis of at least 1 male in the Xenic 2-males drops (Figure 5.24; 24 h: χ^2 (1, n = 70) = 31.023, $p < 0.001$; 48 h: χ^2 (1, n = 71) = 8.208, p = 0.004). The occurrence of paralysis of at least 1 male in Xenic 5-males groups was also significantly higher than the occurrence of paralysis of at least 1 male in the aXenic 2- and 5- males drops, the latter only at 24 h (Figure 5.24; 2 aXenic – 5 Xenic males: 24 h: χ^2 (1, n = 42) = 27.300, $p < 0.001$; 48 h: χ^2(1, n = 37) = 16.306, $p < 0.001$; 5 aXenic– 5 Xenic males: 24 h: χ^2(1, n = 52) = 21.081, $p < 0.001$; 48 h: Fisher's exact n = 47, p = 0.158). Only 48 h after the males were put together, more aXenic 5-males drops showed at least 1 paralysed male than aXenic 2 males-drops, but this difference was no longer significant when the significance levels were adjusted according to the sequential Bonferroni rule (Figure 5.24; 24 h: χ^2 (1, n = 42) = 1.292, p = 0.256; 48 h: Fisher's exact n = 32, p = 0.020, sequential Bonferroni adjusted $\alpha = 0.0125$).

The different treatments affected the occurrence of at least 1 **dead** male at both 24 and 48 h after the males were put together in a similar matter. Pairs of males of Xenic origin showed more drops with at least 1 dead male than their single controls (Figure 5.24; 24 h: Fisher's exact n = 214, $p < 0.001$; 48 h: Fisher's exact n = 210, $p < 0.001$). Even significantly more Xenic drops with 5-males than with 2 males showed at least 1 dead male (Figure 5.24; 24 h: χ^2(1, n = 196) = 10.432, p = 0.001) ; 48 h: χ^2(1, n = 191) = 12.498, $p < 0.001$). Significantly more drops with 5 aXenic males showed at least 1 male dead than the single controls (Figure 5.24; 24 h: Fisher's exact n = 188, $p < 0.001$; 48 h: Fisher's exact n = 148, $p < 0.001$), but these numbers were not different from the numbers of aXenic 2-male drops with at least 1 male dead (Figure 5.24; 24 h: Fisher's exact n = 42, p = 0.270; 48 h: Fisher's exact n = 32, p = 0.061). More aXenic couple drops showed at least 1 male dead than their single controls, but this difference was no longer significant when the significance levels were adjusted according to the sequential Bonferroni rule (Figure 5.24; 24 h: Fisher's exact n =178 ,p = 0.022, sequential Bonferroni adjusted $\alpha = 0.0125$; 48 h: Fisher's exact n = 138, p = 0.032, sequential Bonferroni adjusted $\alpha = 0.0125$).

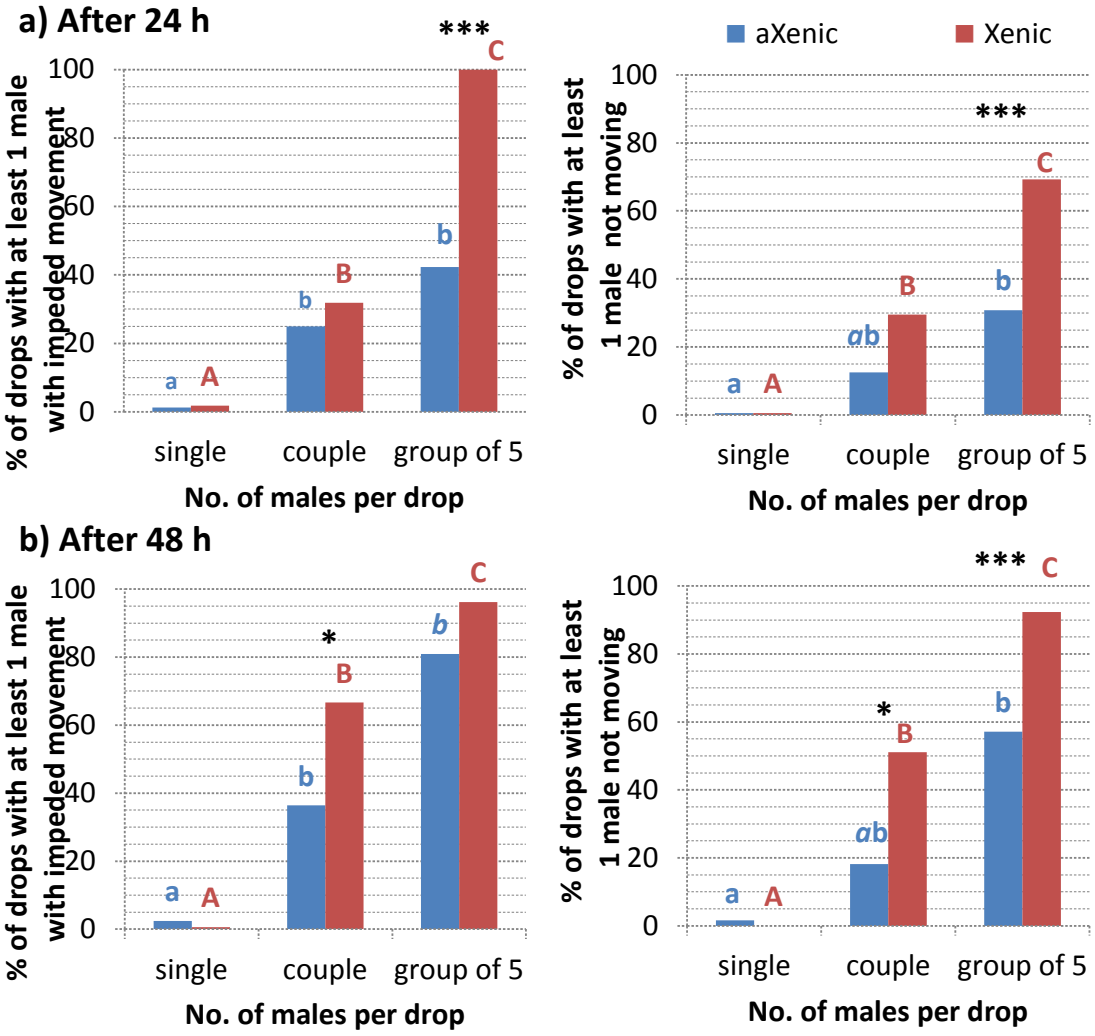


Figure 5.24 The occurrence of paralysis and death in drops with 1, 2 or 5 aXenic or Xenic males in respectively aXenic and Xenic drops. Bars marked with a different letter indicate that the number of males in a drop had a significant influence on paralysis or death within the Xenic or aXenic treatments. Italic letters indiciate p-values that are significant according to α=0.05 but not according to sequential Bonferroni adjusted significance level. Asterixes (*) on top of the bars mark the treatments for which Xenicity had a significant influence on paralysis or death.
***: 0.05 > p ≥ 0.01; **: 0.01>p>0.001; ***: 0.001>p.**
Raw data and statistics in Appendix tables 13, 14, 15, 16, 17 and 18.

5.7.4. Summary & conclusions

5.7.4.1. Haemolymph drops with differing incubation periods of *Xenorhabdus*

The importance of a well-established culture of the symbiotic bacteria in the fighting, paralysing and killing behaviour of the males of *S. longicaudum* CB2B was indicated by the

delay in fighting-related paralysis and death in haemolymph drops that were only inoculated with the symbiotic bacteria when the males were put in the drop together. This suggests that the bacteria are involved in the paralysing and killing processes, but as seen in the other experiments (stabbing (see sections 5.5, 5.5.4), injection (see sections 5.6, 5.6.4, 5.6.5)), the bacteria alone are not sufficient to elicit paralysis and death in the nematodes. In the symbiosis between *Steinernema* nematodes and *Xenorhabdus* bacteria, the nematode's infective juvenile stage is a safe vector for the bacteria ensuring colonisation of new hosts (see also sections 1.2.1, 1.2.2 and 1.2.3)(Snyder *et al*, 2007;Goodrich-Blair, 2007). The relationship is beneficial for the nematode because the *Xenorhabdus* bacteria ensure rapid death of the insect host and provide the nematode with a suitable medium for development and reproduction (Sicard *et al*, 2003;Goodrich-Blair, 2007). This is mediated by toxins and antibiotics produced by the *Xenorhabdus* bacteria (Fodor *et al*, 2010), mainly in its stationary phase of growth. Dunphy (1997) even showed that a critical concentration of *Xenorhabdus* bacteria necessary before the onset of insect mortality (Dunphy *et al*, 1997). The relatively high mortality of single males in drops with only a starting culture of the symbiotic bacteria is very likely due to the lack of *Xenorhabdus* produced toxins and antibiotics making it possible for remnants of the insect's immune system or for contaminating bacteria in the haemolymph drop to weaken the adult nematodes (Walsh & Webster, 2003;Ehlers *et al*, 1990). Such weakened nematodes might be less fit for fighting or the nematodes might lack a fighting stimulus produced by the bacteria. However, no or less fighting is not the only explanation for a delay in fighting related paralysis and death, these might also be caused by non-successful fighting. The abnormally low concentrations of *Xenorhabdus* metabolites in the drops with a starting culture might cause the absence of the paralysing and killing toxin. Recording the incidence of fighting would enable finding out if paired males in new haemolymph drops perform less fighting or are less able to fight successfully.

5.7.4.2. Haemolymph with and without *Xenorhabdus*

Males developed from aXenic infective juveniles fought, paralysed and killed in a *Xenorhabdus* free medium, but did so at a lower rate than Xenic males in *Xenorhabdus* containing drops. The presence of *Xenorhabdus* for fighting, paralysis and death is thus not essential, but it did enhance these events. In contrast to the experiment with different incubation times for *Xenorhabdus* in the haemolymph drop, single males suffered very little mortality (less than 2 % after 48 h) where there was no *Xenorhabdus.* This is probably due to the elimination of the insect's immune system by contaminating bacteria before the IJ's had developed into the more vulnerable adults. As the IJ is the only *Steinerema* stage that can

persist outside of an infected insect and is the stage that invades the living hosts, it is only logical that adult nematodes are more vulnerable to the insect's immune system that is still active in a fresh drop of haemolymph.

Males without *Xenorhabdus* may have been in poorer condition than those with their symbiont (Ehlers *et al*, 1990;Sicard *et al*, 2003), and thus might have fought less, as was also suggested in the previous experiment. Looking into the intensities and number of fights between drops with 2 aXenic or 2 Xenic males would clarify whether the aXenic males are less fit to fight or less inclined to fight resulting in a lower paralysis and death rate. As the bacteria are known to influence their symbiotic nematode's behaviour (e.g. IJ-recovery (Aumann & Ehlers, 2001;Hirao *et al*, 2010) and cadaver attractiveness to late arriving nematodes (Grewal *et al*, 1997)), the symbiotic bacteria could produce a chemical enhancing the males' aggressiveness. Less paralysis and death could then be explained by the absence of this chemical in aXenic cultures.

5.8. Conclusions

Examining the victims of stimulated fights (5.3), the injuries ranged from separation of the content of internal organs owing to the effects of physical constriction (most common), tissue damage to loss of content from internal organs protruding through a wound in the body wall. Immediate paralysis following a fight did not occur frequently in the stimulated fights experiment (only 2 out of 9 injured males) and was not related to visible puncture wounds. Stimulated fights are probably quite different from normal fighting in *S. longicaudum* paired males: they resulted in 5 times more injuries within the first 20 min of pairing the males (section 3.7).

Pressure from the tight grip from the attacker might be important in male-male *S. longicaudum* fighting, but the local pressure in the crushing experiment did not result in paralysis and death as seen in normal male-male fighting. These incongruities might be caused by differences in the type, amount and/or the area of the application of pressure.

Perforation of the body wall didn't necessarily result in immediate paralysis, irrespective of how the lesion had been inflicted: by another male in a stimulated fight (see section 5.3), by crushing (see section 5.4), by stabbing (see section 5.5) or by injecting (see section 5.6).

Comparing Figure 5.17 and Figure 5.18 to Figure 5.22, the stabbing of females with an insect dissecting needle produced paralysis and death levels comparable to those normally seen for male-male *S. longicaudum* fighting. Due to the size of the female and the size and nature of the needle, stabbing a female with an insect dissecting needle looked like a combination of applying a crushing pressure and puncturing. Immediate paralysis was, however, not observed and the restricted movement of the females was very likely due to the ruptured body wall and internal organs.

Stabbing males with a micro-injection needle resulted in death after 24 h in 10 % of the drops. Injecting a male with a medium resulted in more death (28 % after 24 h) and death rate was independent of the nature of the artificial medium. Normal male-male fighting (see section 3.3.3.2) resulted in about 70% of drops with at least 1 dead male after 24 h. Stabbing and injecting with a micro-injection needle resulted in lower levels of death after 24 h and therefore don't reflect the normal mechanism of paralysis and death after fighting. The micro-injection needles used in experiment 5.5.3.2 and 5.6 are thinner (0.7 µm outer diameter) than a spicule (median of average width of 1st generation males of several *Steinernema* species: 12 µm (Nguyen & Duncan, 2002;Phan *et al*, 2005;Nguyen, 2010)). As the difference in width is

more than 10-fold, wounds made with the micro-injection needle of 0.7 µm could be too small to allow a sufficient amount of medium to enter the nematode's pseudocoel. These micro-injection stabbing and injecting experiments (see sections 5.5.3.2 and 5.6) should be repeated with a micro-injection needle with a larger outside diameter, preferably the same width as the particular species' spicule width. In this manner the wounds caused by the injection or stabbing would better mimic the wounds made by the insertion of a male's spicule.

On the basis of the experiments recapitulated above, it is not clear how paralysis and death are inflicted during male-male *S. longicaudum* fighting. It is possible that there are multiple causes of both paralysis and death. Paralysis without damage to the cuticle seems to be more likely the result of pressure, and it is plausible to suggest that paralysis ensues when there is damage to the nervous system. An alternative hypothesis is that toxins released into the pseudocoel from damaged organs interfere with neuromuscular activity. Death might ensue from these injuries, or from the major disturbance to the internal environment resulting from tearing of the body wall and/or rupture of internal organs such as the intestine.

The presence and quantity of the symbiotic *Xenorhabdus* bacteria was not essential for fighting, paralysis and death, but enhanced these (see section 5.7). This might be due to an increase in the stimulus or motivation to fight, an increase in the health and physical condition of the males or the bacteria might contribute to the production of a toxin by the nematode.

The techniques of fighting probably differ between species, with some species more dependent on rupturing effects (e.g. *S. kraussei*, see sections 5.3.3.4 and 4.6) and others more on more subtle, not necessarily visible effects of fighting (e.g. *S. longicaudum*).

6. Discussion

6.1. Fatal fighting of *Steinernema* nematodes

Various explanations have been proposed for same-sex sexual-like behaviour, including weak sex recognition, social bonding, and intrasexual competition (Abele and Gilchrist, 1977; Connor and Mann, 2006; Dukas, 2010; Field and Waite, 2004; Preston-Mafham, 2006b; Ryne, 2009; Vervaecke and Roden, 2006b). Even with direct access to females, male *S. longicaudum* wrap and inflict injury on or even kill other males present in a drop (Section 3.5) or in an insect cadaver (O' Callaghan, 2006). Because the presence of a female did not result in reduction of fighting behaviour (Section 3.5.3), it is very unlikely that males wrapped around another male due to weak sex recognition, i.e. because they were mistaken the other male for a female. The wrapping behaviour itself also differs: males rapidly attack and wrap around another male irrespective of the place on the body of the victim and remain where the wrap was initiated, whereas the males wrap around the females more gently and perform obvious searching behaviour for the female's vulva (own observations). Not unexpectedly, females of the male's own species are not at all common receivers of injurious fighting behaviour, but a small number of females (less than 5 over the course of the whole study) were observed with puncture wounds or other fight-like injuries when they were physically abnormal (own observations) like dumpy females (Rahimi et al., 1993).

Due to the reduction in reproductive output of the victims of fighting, social bonding is not at all a very likely explanation for the occurrence and subsistence of same-sex sexual like behaviour and intrasexual competition (Abele and Gilchrist, 1977; Preston-Mafham, 2006) is a more likely explanation than practice for intersexual mating (Dukas, 2010; Vervaecke and Roden, 2006).

Fatal fighting is rare in the animal kingdom because most animals have developed strategies to avoid costly escalation of fighting (Innocent et al., 2011; Pereira and Do Prado, 2005). Assessment, display and defensive behaviour might explain the survival of *Steinernema* males in multiple male drops or insect cadavers (O' Callaghan, 2006). Prior to wrapping, any particular behaviour related to fighting was not observed. Coiling in on itself as a defensive behaviour or the scraping of its own body with its spicule and then "flicking" it occurred in many different situations (with or without presence of a male or female) and taking into

account that these behaviours can occur at very high numbers in a short amount of time, or not at all, no further attempt was made to quantify these behaviours. Also, situations where the reproductive value of the future is near zero don't favour the development of defensive behaviours or injury-avoiding assessment (Enquist and Leimar, 1990).

The lack of obvious pre-fight assessment behaviours may partly reflect the unnatural conditions of the test protocol, where utilised males were reared in isolation and then placed together. In nature, adult male *Steinernema* are unlikely to encounter each other unless they have developed together in the same insect cadaver. Being reared in isolation affects the development of neuronal connectivity of *C. elegans*, with consequent effects on behaviour (Rose et al., 2005).

O'Callaghan (2006) found differences between the intraspecific killing behaviour of group-reared or *in vivo* reared males and of singly-reared males. She found that singly reared males were not deterred from combat with increasing numbers of male competitors whereas group-reared males' tendency to fight was modified by the number of males present. Group-rearing might allow some sort of behaviour conditioning that lessens the tendency to fight to death. Due to the high number of male competitors in the natal drop, males might assess their chances of winning a fight as not being greater than their chances of losing it which would deter these males from attacking each other (Reinhold, 2003). When the cadaver contained more than 50 males, in vivo reared males showed a decreased speed of killing (O' Callaghan, 2006). Experiments aimed for assessment behaviour should thus be performed using group reared males. O'Callaghan also noted the possibility that mixed-sex rearing might be the cause of a lower fighting tendency of group-reared males. The presence of a female had no effect on the fighting behaviour of singly-reared males, but the effect of female presence might play its influence at the developmental stages which was not investigated in this work.

When taking into account the above rationale that group-rearing of *Steinernema* males might allow for some sort of assessment, the closed system of the insect cadaver and that *Steinernema* males fight before mating, all assumptions for Reinhold's theoretical model on conflict over mating partners in a closed system are met (Reinhold, 2003). An important conclusion from this model is the expectation that the frequency of fatal fighting will decrease with increasing male numbers, just like it did in O'Callaghan's (2006) *in vitro* and *in vivo* group-reared males experiments.

The observation that solitary *S. longicaudum* males attacked each of the male-like objects -a dead or incapacitated male, or an artificial male- with which they were presented in a drop of

haemolymph equally (Section 3.6.) may point to a lack of discrimination in these animals. However, the fact that two normal males when together did not attack all male-sized objects equally, but attacked real males (incapacitated, dead or alive) more than the suture indicates that they can at least distinguish between worms and inanimate objects. Attacking an incapacitated male may be adaptive, as a temporarily incapacitated worm might recover; attacking all incapacitated worms, rather than attempting to distinguish between those that would and those that would not recover is a reasonable strategy, as attacks on an incapacitated opponent are unlikely to be costly except in time. Similarly, recently killed males may be difficult to distinguish from temporarily incapacitated ones. What was recorded as attacks may have included assessments of viability. It is reasonable to assume that attacks on dead or incapacitated worms were shorter than those on live ones, but these data have not yet been analysed. The fact remains that *S. longicaudum* males do "attack" (or wrap around) an inanimate object, a suture, which might suggest that wrapping behaviour is a thigmotactic response to any approximately worm-sized object, followed by assessment of contact chemical cues which would lead them to modulate their subsequent behaviour (search for vulva; squeeze and pierce, or let go). Male nematodes have chemoreceptors located at the tail (Bird and Bird, 1991; Emmons and Sternberg, 1997).

Due to the use of haemolymph drops as the test arena, males in the experiments in this study did not have any possibility to flee and hide in insect tissue. This particular setup might have caused less aggressive and more defensive behaviours to be overlooked in this thesis.

6.2. Factors influencing the fighting behaviour of males of *Steinernema longicaudum*.

Resource holding potential (RHP) and resource value (RV) have been identified as important determinants of the escalation of intraspecific competition. The resource holding potential of a contestant reflects this contestant's ability to win a fight when it occurs (Connor and Mann, 2006; Jennings et al., 2004).

Residency and previous fighting experience are common factors influencing perceived RHP. Typically, residents are more likely to win mainly because they value the resource more than an intruder (Bentley et al., 2009; Haley, 1994). For *Steinernema longicaudum*, residency might have an effect, but lacking a good way to identify single males, this could not be independently

studied in this thesis. Previous fighting experience determines fight outcomes in many animal contests: a winner is more likely to win again in a new contest, even when it is against a different opponent (Hsu and Wolf, 1999; Hsu and Wolf, 2001; Jennings et al., 2004; Rutte et al., 2006). In *S. longicaudum* the winner of a fight was identified by the injuries or paralysis of the loser. This made it impossible to stage new fights with the loser. However, new fights with a previous winner did result in more aggression (measured in drops with a paralysed male) in the first hour the males were put together (Section 3.8). Even though it was not possible to identify the individual males in this thesis, it is more likely that the male who had fought previously was the more aggressive opponent in the new fight. The practical fighting experience it had acquired might make the winner more skilled in fighting, but it could also have boosted the winner's perception of its RHP and thus given him an edge. An accurate way to identify males would shed more light on the factors influencing the likelihood of winning or losing a fight. During the course of this thesis, undergraduate student research projects experimented with natural dyes and fluorescent micro-beads for the identification of individual males but these methods proved ineffective for staining live nematodes for more than 24 hours after contact with the dye (Brennan, 2009).

Maturity had a very substantial effect on the fighting of *S. longicaudum*: the speed of fighting, paralysis and kill were higher when one of the contestants was able to mature before the males were paired (Section 3.4). Forty-eight hours after the pairing of the competitors, these differences were no longer detected. Immature males were also more likely to be the victim of a fight. This might be due to biological effects like size: mature males are wider (Ebssa et al., 2008). Size or weight of an animal or the size of certain body parts are often related to resource holding power (Batchelor and Briffa, 2010; Brown et al., 2006; Jennings et al., 2004; Jenssen et al., 2005). A mature male had also already mated which would have given him experience in wrapping and might thus have prepared him better for other behaviours involving wrapping. Maturity might also give a male a chemical advantage for winning a fight. In case there is a paralysing toxin associated with the production of sperm and/or seminal fluid (discussed below), matured males might be physiologically better equipped for fighting, paralysing and killing by having more of the toxin. In the experiments in this thesis, males were given complete access to females in order to mature. Males can however also mature without physical contact with a female (Ebssa *et al.*, 2008) so that any possible wrapping experience a male gains by mating or attempting to mate with a female can be one variable less. Further experiments could aim to untangle how maturity can physically benefit a male for fighting.

Maturity of a male also changes the subjective value of available females. A *S. longicaudum* male needs the presence of a female to mature (Ebssa *et al.*, 2008), so his experience indicates that a female is present somewhere in "its" cadaver (even if it is no longer detected in the drop used as a fighting arena). This may make the value of the resource (a drop presumed to house a female) higher for a mature male than for an immature male and will increase the former's willingness to fight. Moreover, the matured male has already invested in the production of spermwhich is comparable to the higher value of a host to a female parasitoid *Eupelmus vuilleti* with a higher egg-load (Mohamad et al., 2010). Speed is of importance here to the matured male, because in case the competition is not eliminated as soon as possible, an opponent may have had the opportunity to mate with the female, or an immature male could have become mature. An already mated male could also be expected to be more aggressive to prevent a female he has mated with from being mated again. However, in section 3.5, the results indicated that whether a female had already been mated or was big or small did not influence the fighting or mating behaviour of the mature males, this argues against the hypothesis that a male's tendency to fight is affected by the value of the current resource (when taking the female present as the current resource). If the current resource value has no effect, it is also unlikely that prior experience of resource value affects fighting tendency.

The above paragraph also brings up the issue of whether a male can distinguish male juveniles from female juveniles. For an adult male *Steinernema* nematode, attacking a juvenile male implies a higher chance of winning and a much lower probability of sustaining injuries than fighting another adult male. Further experiments could be done with males that are not matched for age.

6.3. Sequential male polymorphism and kin recognition in fighting behaviour of S. longicaudum

This thesis suggests that *Steinernema longicaudum* exhibits a strategy with 2 behaviourally different male varieties that are sequential in time and arise from conditional strategies where the environmental conditions of the parental generation determine the strategy followed (Cook, 2005).

First generation males were more aggressive than males of subsequent generations (Section 3.9.3.1). This difference between the founder generation and subsequent generations

translates into sequential male polymorphism with "fighter" 1st generation males versus "peaceful" subsequent generation males. First generation males develop from infective juveniles and have thus followed a different developmental pathway compared to adults from subsequent generations, analogous to development through the dauer juvenile stage in *C. elegans* (Hall et al., 2010). This differing developmental pathway of founder adults may entail differences in the gene expression leading to phenotypic differences of these 1st generation adults compared to subsequent generations developing within the host, which do not pass through the IJ stage. In *C. elegans*, expression of thousands of genes was affected by developmental pathway, including genes that alter direct fitness such as the number of progeny and mean adult life span (Hall *et al.*, 2010).

The second generation adults in this thesis were separated very early in their development, but the initial group-rearing (discussed in Section 6.1) might still have had an effect. Males that developed from IJs did not experience this group rearing in my experiments, though would have developed to the IJ stage surrounded by conspecifics within the cadaver, some weeks earlier. O'Callaghan's (2006) results suggest that speed of fighting rather than overall tendency to fight was affected. Still, future research with group rearing of males would be needed to take the group-rearing effect out of possible explanations for the differences seen between founder and subsequent generations.

A second difference between generations was that relatedness of the contestants did not influence the occurrence of fighting in the post-dauer males, but it did influence the occurrence of fighting between males in the subsequent generation (Section 3.9.3.2). The latter were less aggressive towards siblings than towards less closely related males which also means non-founder generation males are able to discern kin from unrelated males. These results show both a reduction of detrimental competitive behaviour when relatedness is high (second generation) and the absence of an effect of relatedness on fighting (first generation) within the same species. It would be very interesting to find out whether male *Steinernema* nematodes of the 1st generation will also attack their own male offspring. If they don't fight their male offspring, 1st generation males possess some kind of mechanism to distinguish own offspring from related and unrelated males from the second generation.

A noteworthy difference between the generations is that males of the first generation develop from infective juveniles that have dispersed from the natal cadaver and so are much less likely to encounter siblings than males of the second generation that develop within the cadaver.

The latter are much more likely to experience competition for mating opportunities between relatives in a restricted environment (Local mate competition, LMC). LMC has received a lot of attention and many (though often interrelated) factors accommodating the development of fighting between relatives or favouring brother (or sister) competition-avoidance behaviour have been identified, including number of valuable females (Anderson et al., 2003), dispersal possibilities (Nelson and Greeff, 2009; West et al., 2001), kin recognition (Reinhold, 2003), level of relatedness (Cronin and Monnin, 2010), and male variance in fighting strength (Reinhold, 2003).

Highly female biased sex ratios have been put forward as an avoidance mechanism for brother competition (Abe et al., 2005; Hamilton, 1963; Hamilton, 1967; Hamilton, 1979), but the different effects of number of females and males, the operational sex ratio and the relatedness between the males and between the males and the females are hard to disentangle (Nelson and Greeff, 2009). Many brother competition studies used fig wasps to untangle the confounding effects of competition and relatedness which are both increased in closed environments. *Steinernema* spp. are characterised by slightly female-biased sex ratios (Alsaiyah et al., 2009; Hirao et al., 2010). The operational sex ratio (OSR) on the other hand, where only the sexually reproductive adults are taken into account, has not yet been investigated for *Steinernema* spp. More research on the OSR of *Steinernema* spp. could give more insight into the competition pressure the male nematodes experience and explain why *Steinernema* species show both slightly female biased sex ratios and high levels of competitive behaviour, even between related males.

Dispersal is another means of avoiding local mate competition between relatives. In fig wasps, at least 1 of the sexes is capable of dispersing from the natal fig. As noted above, in entomopathogenic nematodes infective juveniles destined to become either of both sexes disperse; adults, however, are not able to survive outside the host cadaver. Thus several generations of adults and their progeny are restrained to the same host cadaver. The life cycle of dicœcious *Steinernema* species thus poses different opportunities and constraints to the different generations. Non-founder generations of dicœcious *Steinernema* spp. have more potential mating partners, but also have a much higher likelihood to compete with related males. A male from such a generation will have the highest reproductive outcome if all its female siblings reproduce; the male fertilises all of the available females (both related and unrelated) to the extent of its capacity, and the remaining females are fertilised by its siblings. A 1st generation male however might only get 1 mating opportunity (depending on the number of co-infecting conspecific females). However, each first generation female can produce a higher number of progeny than those of subsequent generations (Baliadi et al., 2001). A first

generation male that succeeds in being the only male reproducing in that insect has a high potential number of offspring; for example, Ebssa et al. (2008) showed that *S. longicaudum* can fertilise multiple females in just a few hours, and a single first generation female can produce several hundred juveniles (Baliadi *et al.*, 2001). Moreover, all of this progeny are direct descendants which have a much higher impact on the male's reproductive success than indirect progeny from related males. In summary, if 1 *Steinernema* founder generation male is able to inseminate all the first generation females in "its" cadaver, his direct descendants (from all generations) would amount to hundreds of thousands of directly related infective juveniles. Competition to be this male could be intense and explain the evolution of fatal fighting in *Steinernema* spp.

6.4. Fighting behaviour in the *Steinernema* genus

Most of the other *Steinernema* species tested showed at least some evidence of fighting: fighting was seen in clades II, III and V, but not IV (Table 6-1). According to the phylogenetic tree shown in Section 1.2.3, Clade IV is not ancestral and so it would appear likely that fighting evolved early in the genus and was lost in *S. bicornutum* (and possibly other Clade IV species).

The other *Steinernema* species displayed varying levels of fighting, but not one was more aggressive than *S. longicaudum* CB2B. Table 6-1 depicts differences between the studied *Steinernema* species that might be associated with competition including fighting in these species.

Steinernema species are classified as ambush, cruise or intermediate foragers (Section 1.2.1). Foraging behaviour of infective juveniles is often used as a phylogenetic characteristic (Campbell et al., 2003; Lewis et al., 2006; Nadler et al., 2006) and has been linked to the patchiness of populations of species (Campbell et al., 1995; Puža and Mrácek, 2010; Stuart and Gaugler, 1994) and to the sex ratios in infected insects (Lewis and Gaugler, 1994). The results of this thesis combined with those of O'Callaghan (2006) do not indicate a correlation between IJ foraging behaviour and the level of intraspecific male-male competition (Table 6-1). After all, the ambusher species *S. carpocapsae* shows higher levels of aggression than *S. kraussei*, but lower levels than *S. longicaudum* which are both cruisers (Table 6-1).

S. longicaudum is very closely related to *S. hermaphroditum* (Nadler *et al.*, 2006). Hermaphroditism in this latter species might have arisen under conditions in which IJs disperse widely and find themselves in hosts with few other conspecifics (Griffin et al., 2001). In case these same conditions apply to *S. longicaudum*, the frequent occurrence of a low number of IJs

infecting an insect would favour a high fighting tendency due to a lower number of males one male might have to compete with (Reinhold, 2003). The opposite, higher population densities, could eventually lead to situations where males would benefit more from mating with as many as possible of the available females than trying to defeat the large number of competitors (Emlen and Oring, 1977; Reinhold, 2003). Ecological factors leading to high natural infection rates could thus promote lower levels of fatal fighting.

Sperm competition is another possible means of competition for reproduction within Steinernematidae and could for some species have triumphed over fighting as the main mode of intrasexual competition. Insemination plugs have not been observed for Steinernematidae, but this is a sperm competitive strategy often used in other Nematoda (Barker, 1994; Cutter, 2008). Studies on different *Caenorhabditis* species have shown that larger sperm cells are likely to outcompete smaller spermatozoa (Geldziler et al., 2006; Lamunyon and Ward, 1998; Lamunyon and Ward, 1999; Lamunyon and Ward, 2002). Four of the species tested here (*S. longicaudum*, *S. glaseri*, *S. bicornutum* and *S. carpocapsae*) show sperm dimorphism (see Table VI-1) where macrospermatozoa are giant (20-60µm) amoeboid cells (ACs) that transport microspermatozoa (±2µm) into the female's seminal receptacle where the latter can fertilise the oocytes (Spiridonov et al., 1999; Yushin et al., 2007). The significance of this sperm dimorphism in *Steinernema* is unclear, but such dimorphism is typically associated with sperm competition in other taxa (Gomendio and Roldan, 2008; Lamunyon and Ward, 1999; Lamunyon and Ward, 2002; Murray et al., 2011). *S. kraussei* and *S. feltiae* are 2 of the *Steinernema* species that don't show this sperm polymorphology, instead, the males produce spermatozoa of medium size (5-16µm) that can form chains inside the female's uterus (Spiridonov et al., 1999; Yushin et al., 2007). Killing cannot be easily correlated one way or another with sperm size as had been suggested by O'Callaghan (2006), since both the most and the least aggressive species (*S. longicaudum* and *S. bicornutum*) show dimorphic sperm. However, *S. bicornutum* males that had not been in contact with a conspecific female or another conspecific worm, were observed to contain large spermatozoa in their seminal vesicle (own observation, data not shown). This is in sharp contrast with S*. longicaudum* which is a very aggressive species in which the males only show large spermatozoa when they had been in contact with a female for at least 6 hours (Ebssa *et al.*, 2008). The delay in production of sperm in *S. longicaudum* males until a female is present is likely to be associated to the costs of the formation of competitive macrospermatozoa. Earlier maturation would risk the decay of valuable sperm cells in the case no females arrive soon after. The occurrence of giant spermatozoa in *S. bicornutum* males that have not been in contact with a conspecific female is

thus quite puzzling but might give an indication of the explanation of the very sharp contrast in aggressiveness between these 2 species.

Female reproductive structure also differs between species (see Table 6-1) with *S. bicornutum* showing the most aberrant female gonoduct system (Zograf et al., 2008). The female's sexual structures may influence sperm competition (not addressed by Zograf et al., 2008) and might thus also be related to the male's fighting behaviour. The low level of aggression observed in *S. bicornutum* might thus in some way be related to the female gonoduct structure and the male's giant megaspermatozoa. However, it should be noted that Zograf et al. (2008) described sperm of 6-7 µm diameter in the uterus of *S. bicornutum*, instead of the giant cells described by Spiridonov et al. (1999) for this species, throwing doubt on the identity of the species with the unusual gonoduct.

Spicules and gubernaculum morphology are phenotypic characteristics often used in phylogenetic analyses of Steinernematidae and might also be of great importance in the evolution of fighting behaviour of Steinernematidae. In case the spicule is used during fighting for stabbing and maybe also for injection of a toxin, the shape of the spicules and the gubernaculum might be of great importance during a fight. Differences in these *Steinernema* males' weapons could then be translated into fighting advantages or disadvantages. Which characteristics might have an important influence on paralysis and kill, is of course dependent on the mechanism behind paralysing and killing, i.e. injection of a toxin, diffusion of a toxin or no toxin involved in paralysis and kill. Illustrations of the spicules in Nguyen (2007) show that *Steinernema* spicules vary considerably in both sharpness and angle. The spicules of *S. longicaudum*, *S. glaseri* and *S. carpocapsae* are relatively sharp compared to those of *S. kraussei* and *S. feltiae*, corresponding to the greater frequency of injury in the first three species. The spicules of *S. bicornutum* are intermediate in sharpness, indicating inferior weaponry is not the reason why this species does not cause injuries.

Table 6-1 Summary of aggressiveness of the *Steinernema* species studied in this thesis and summaries of phenotypic characteristics possibly influencing fighting behaviour.

Species	*S. bicornutum*	*S. feltiae*	*S. kraussei*	*S. carpocapsae*	*S. glaseri*	*S. longicaudum*
Clade	IV	III	III	II	V	V
Level of Aggression	Very low[1]	Moderate	Moderate	High	High	Very high[4]
	Bacterial symbiont: *Xenorhabdus* spp. (Tailliez et al., 2006)					
	X. budapestensis	*X. bovienii*	*X. bovienii*	*X. nematophila*	*X. poinarii*	*X. ehlersii*
	IJ foraging strategy (Campbell *et al.*, 2003)					
	Intermediate	Intermediate	Cruise	Ambush	Cruise	Cruise
	Sperm morphology in the ♀'s uterus (Spiridonov *et al.*, 1999)					
Number of amoeboid cells	5-6	Chains of up to 16	Chains of 3-8	Up to 24	Few, elliptical	Few
Length (µm)	50	7-14	8-16	40-50	30	50-60
Ø (µm)	20-40	5-8	6-9	30-35	20	30
	♀ gonoduct structure (Zograf *et al.*, 2008)					
Ovarial sac	Single	Several	-	Several	Single	-
Oviduct length	Extremely short (25-30 µm)	Very long (>180 µm)		Short (<70 µm)	Medium (100-150 µm)	-
Uterus	2 unequal parts	Spermatheca-uterus complex – no constriction – rest of uterus	-	No uterine sac	Spermatheca-uterus complex – sphincter-like constriction – rest of uterus	-

[1] Very low level of aggression: <20% of drops with at least 1 paralysed or dead male after 24-48hrs; [2] Moderate level: 40-60% of drops with at least 1 paralysed or dead male after 24-48hrs;[3] High level: 60-80% of drops with at least 1 paralysed or worse male after 24-48hrs; [4] Very high level: ≥80% of drops with at least 1 paralysed or worse male after 24-48hrs. [5] ACs = amoeboid cells; [6] More than 1000 ACs per branch observed; [7] Completely sealing the gonad lumen.

6.5. Mechanism of paralysis and kill

Victims of fights frequently showed massive physical trauma including lacerations of the cuticle and damage to internal organs. The scale of damage is such as might account entirely for paralysis (e.g. nerve damage) or death. The possible additional involvement of toxins, produced either by the bacteria or the nematode, is less clear. Even though some worms that had fought showed paralysis independent of visible injection wounds (Section 5.3), which would appear to show that paralysis is not always (if ever) due to entry of a toxin, small punctures may have been overlooked, and other experiments point to possible involvement of a toxin. Already fought and thus possibly injured aXenic males transferred singly to Xenic drops did not show higher degrees of incapacitation (own observations, data not shown). This suggests that a possible toxin does not enter the nematode from the medium through an open wound, but in case any paralysing toxins are involved, they would need to be injected by the male victor.

Although there is no evidence that the bacteria are directly responsible for paralysis or death by production of a toxin into the medium that enters wounded males, the nematodes do appear to need the bacteria for normal fighting behaviour. AXenic nematodes performed fighting behaviour, but did not show the same amount of paralysis or incapacitation as normal Xenic males (Section 5.7). AXenic males transferred to Xenic drops for fighting (own observations, data not presented) showed paralysis and killing comparable to the level of paralysis and kill that Xenic males achieve in a Xenic drop, which is significantly higher than aXenic males achieve in an aXenic drop over the same time period. The symbiotic bacteria provide the most efficient development of their associated nematodes (Han and Ehlers, 2000; Sicard et al., 2004). Thus the presence of the symbiotic bacteria implies better physical condition of the worms which could be the main cause for differences in fighting behaviour and fighting outcome. In addition, they or their products may influence the motivation of the nematodes to fight, just as they can stimulate recovery of juveniles from the arrested IJ stage to resume development (Aumann and Ehlers, 2001; Strauch and Ehlers, 1998). A nutritionally more suitable environment would be a more valuable resource worth fighting for.

Haemolymph drops and insects successfully infected with *S. longicaudum* CB2B often show a blue-green colouration (O' Callaghan, 2006). Haemolymph drops infected with any of the other *Steinernema* species used in this thesis did not show a specific coloration. When culturing the symbiotic bacteria of *S. longicaudum* CB2B on Nutrient agar, a blue-green colour originating from the bacterial colonies diffused through the agar (own observations, data not shown). Literature doesn't report on coloration of Nutrient agar when cultivating other Xenorhabdidae

(Akhurst, 1983; Akhurst, 1986; Boemare, 2002b; Lengyel et al., 2005; Lengyel et al., 2007). All this indicates that *X. ehlersii* produces a blue-green metabolite when cultured on Nutrient agar or in haemolymph. Even though the experiments looking into the involvement of the bacteria didn't show that *Xenorhabdus ehlersii* or its secondary metabolites are necessary for fighting behaviour or for paralysis and killing, the presence of the bacteria did enhance the effects of fighting (paralysis and death). No investigation into a possible link of paralysis, death and fighting behaviour to the coloration of the drop was done in this thesis.

Pyocyanin, a blue-green phenazine-derived secondary metabolite produced by some *Pseudomonas aeruginosa* strains, was linked to rapid paralysis of *C. elegans* nematodes (Darby et al., 1999; Mahajan-Miklos et al., 1999). This neuromuscular paralysis involved spasmodic twitching of the nematode followed by a kinked body posture indicating aberrant hypercontraction of body wall muscles (Darby et al., 1999; Liu and Nizet, 2009). This is very similar to the rapid paralysis that can occur in a *Steinernema longicaudum* male-male fight (described in 3.3.3.1). During such a fight the victim's initial frantic movements slowed down, until the nematode kept a kinked posture and was only capable of slight head movements (also see the attached CD for the recording "*S. longicaudum* Fight.mpg"). Even though at present there is no evidence that *X. ehlersii* is responsible for producing a diffusible toxin in the media causing the rapid paralysis during a fight, further research into the occurrence of rapid paralysis during a fight with aXenic males and the circumstances that allow for this to happen, might reveal the necessity of *X. ehlersii* in that specific type of rapid paralysis and it might even be linked to the production of the blue-green colorant.

The effects of the bacteria on fighting were also examined through pairing *S. longicaudum* males in haemolymph drops that differed in the time since inoculation with symbiotic bacteria (Section 5.7.2.1, 5.7.3.1 and 5.7.4.1). The time of inoculation influences more than only the amount of bacteria in a drop. It also determines the growth phase of the bacteria and it might also influence which phenotypic phase variant of *Xenorhabdus* will occur or will be dominant. Different growth phases in the bacteria's growth cycle allow for the production of different chemicals (Maxwell et al., 1994; Webster et al., 2002). Phenotypic phase I and phase II cells produce different substances and it has also been shown that *Steinernema* nematodes develop best on phase I bacteria (Boemare, 2002a). Growth phase and phase variant are thus 2 factors which might influence fighting behaviour or its outcome and which could also contribute to the differences between the founder and subsequent generations.

The time since inoculation influences the metabolites excreted by the bacteria and the number of bacterial cells available as a food source to the nematode. The bacteria also play a vital role in overcoming the insect's immune system (Fodor et al., 2010; Goodrich-Blair, 2007; Sicard et al., 2003). Information on the timing of the bacteria's growth cycle and the occurrence of the 2 intraspecific phenotypic variants within an insect cadaver or within a haemolymph drop is scarce and what is available, shows that the stationary phase is probably the only growth phase of the bacteria that the adult nematodes come in contact with (Maxwell et al., 1994; Webster et al., 2002). More research could shed light on whether the condition of the bacterial culture (e.g. the metabolites produced and phase variation) affects nematode behaviour such as fighting.

As the hypothesis of a toxin present in the media diffusing into an open wound is not supported by the results in 5.5 and 5.6, rapid paralysis might be caused by a toxin injected by the male. Since the spicule is very likely used for wounding during fighting, glands associated with the spicule and thus with sperm or seminal fluid might produce a toxin responsible for rapid paralysis. *Drosophila melanogaster* females' lifespan is shortened by mating owing to a toxin in the seminal fluid (Chapman et al., 1995; Wigby and Chapman, 2005).

Laser ablation of specific cells or groups of cells (Garcia et al., 2007), could help elucidate the involvement of male glands or structures like spicules in the mechanism of fighting.

This thesis confirmed fatal fighting that was first noted by O'Callaghan (2006). More species than only those observed in O'Callaghan (2006) perform fatal fighting behaviour and variation in the level of lethal fights within the genus has been probed.

Knowledge of the effect of factors influencing fighting, paralysis and killing has been extended: generation, maturity, previous victory and the symbiotic bacteria have definite effects, but no obvious impact of the value of the resource present and residency have been found. *Steinernema longicaudum* CB2B males perform different fighting behaviour depending on their development. Males that had passed through the infective juvenile stage showed a higher level of fighting behaviour than subsequent generations and did not alter their behaviour depending on their relatedness with the opponent whereas following generation males fought, paralysed and killed related males less than unrelated males.

The physical injuries sustained in fighting were described and their effect on reproductive success was evaluated. The prevalence of types of injuries differed between species.

Many questions remain unanswered, including the involvement of a toxin and the mechanism behind rapid paralysis. Technical difficulties regarding the production of a medium that is both suitable for development and normal behaviour but that does not contain the *Xenorhabdus* symbiont hampered progress. Injecting males also revealed difficulties regarding fluids that are viscous or containing particles and clogged up the micro-injection needle.

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Appendix

1. Intraspecific fighting by *S. longicaudum* males (Chapter 3)

1.1. Characteristics of male sized opponents that elicit fighting behaviour

Appendix table 1 The statistical details of the effect of non-target factors (temperature, age, set and scent) on the no. of attacks per drop during the 1st 15 min time period.

Treatment		Temperature	Age	Set	Scent
2 normal males	p =	0.128	0.860	**0.035**	-
	DF =	12	3	**9**	
	F =	1.77	0.25	**2.18**	
	no. of drops with fight /total n	38/74	38/74	**38/74**	
A post hoc Tukey's test showed no significant differences in no. of attacks per drop between the different sets.					
1 normal male plus an incapacitated male	p =	-	0.205	*0.071*	-
	DF =		2	1	
	F =		1.81	3.87	
	no. of drops with fight / total n		2/15	2/15	
1 normal male plus a dead male	p =	-	0.642	0.150	-
	DF =		3	3	
	F =		0.57	2.27	
	no. of drops with fight / total n		10/29	10/29	
1 normal male plus a suture of male proportions	p =	-	0.144	0.305	0.252
	DF =		3	3	1
	F =		1.95	1.26	1.36
	no. of drops with fight / total n		4/34	4/34	4/34
2 normal males plus an incapacitated male	p =	0.675	*0.082*	0.502	-
	DF =	11	*3*	2	
	F =	0.76	*2.47*	0.71	
	no. of drops with fight / total n	18/29	*18/29*	18/29	
2 normal males plus a dead male	p =	0.960	0.379	0.578	-
	DF =	10	2	2	
	F =	0.33	1.01	0.56	
	no. of drops with fight / total n	20/31	20/32	20/32	
2 normal males plus a suture of male proportions	p =	0.168	0.406	0.821	-
	DF =	12	2	2	
	F =	1.62	0.93	0.20	
	no. of drops with fight / total n	13/32	13/32	13/32	

Appendix table 2 The statistical details of the effect of non-target factors (age and set) on the no. of attacks per drop during the 2nd 15 min time period.

Treatment		Age	Set
2 normal males	p =	0.727	0.593
	DF =	3	6
	F =	0.44	0.77
	no. of drops with fight / total n	25/60	25/60
1 normal male plus an incapacitated male	no. of drops with fight / total n	1/10	1/10
1 normal male plus a dead male	p =	0.409	**0.039**
	DF =	3	**3**
	F =	1.01	**3.37**
	no. of drops with fight / total n	5/24	**5/24**
A post hoc Tukey's test showed no significant differences in no. of attacks per drop between the different sets.			
1 normal male plus a suture of male proportions	no. of drops with fight / total n	0/6	0/6
2 normal males plus an incapacitated male	p =	0.725	0.475
	DF =	2	2
	F =	0.33	0.77
	no. of drops with fight / total n	12/29	12/29
2 normal males plus a dead male	p =	0.820	0.426
	DF =	3	2
	F =	0.31	0.88
	no. of drops with fight / total n	19/32	19/32
2 normal males plus a suture of male proportions	p =	0.187	0.891
	DF =	2	2
	F =	1.77	0.12
	no. of drops with fight / total n	7/32	7/32

Appendix table 3 The statistical details of the effect of non-target factors (age and set) on the no. of attacks per drop during the 3rd 15 min time period.

Treatment		Age	Set
2 normal males	p =	0.207	0.112
	DF =	2	3
	F =	1.71	2.30
	no. of drops with fight / total n	3/22	3/22
1 normal male plus an incapacitated male	no. of drops with fight / total n	1/4	1/4
1 normal male plus a dead male	p =	0.310	0.539
	DF =	3	2
	F =	1.31	0.65
	no. of drops with fight / total n	4/18	4/18

Appendix table 4 The statistical details of the effect of non-target factors (temperature, age and set) on the no. of attacks per drop during the 4th 15 min time period.

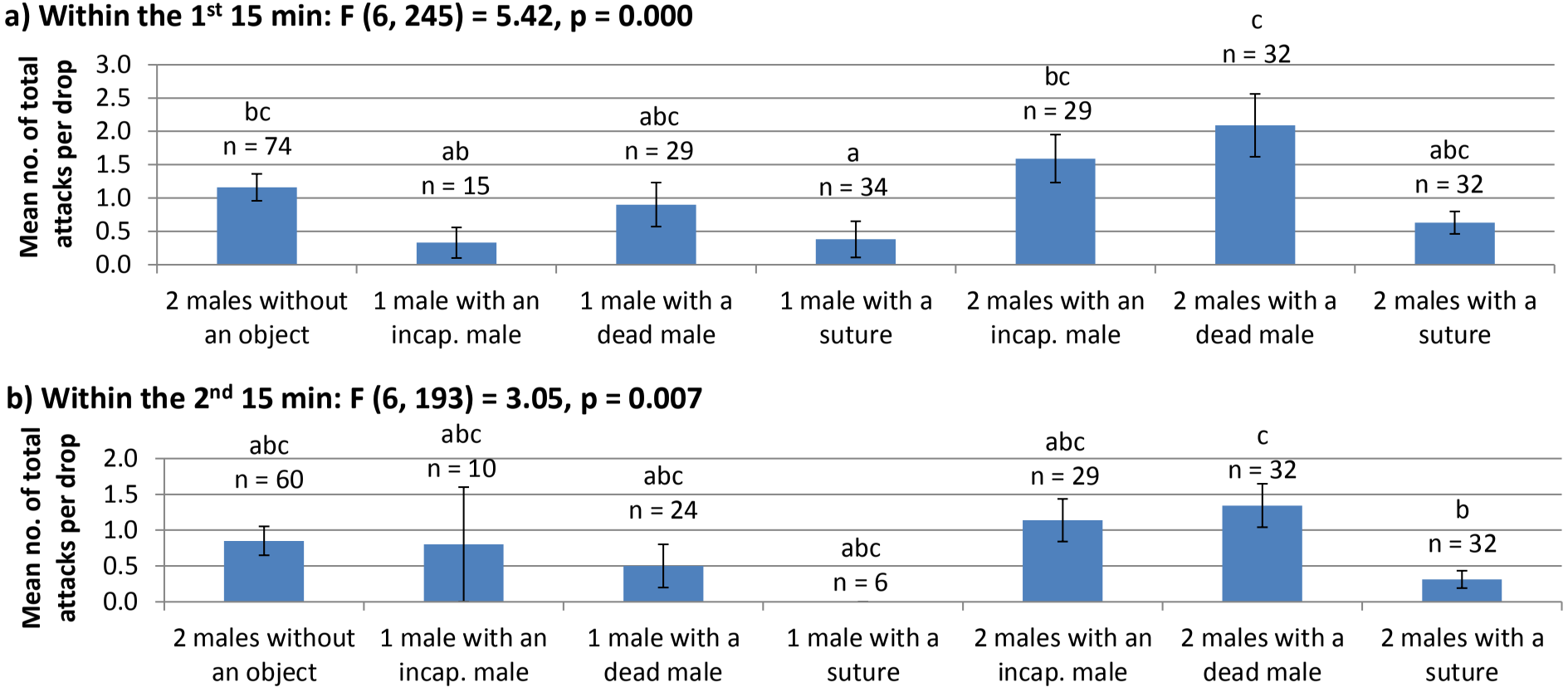
Treatment		Temperature	Age	Set
2 normal males	p =	0.836	0.606	0.904
	DF =	7	2	1
	F =	0.46	0.52	0.02
	no. of drops with fight / total n	9/14	14/21	14/21
2 normal males plus an incapacitated male	p =	0.685	0.726	0.121
	DF =	7	2	1
	F =	0.69	0.33	2.65
	no. of drops with fight / total n	7/13	10/21	10/21
2 normal males plus a dead male	p =	0.182	0.946	0.544
	DF =	9	2	1
	F =	3.23	0.06	0.38
	no. of drops with fight / total n	5/13	8/19	8/19
2 normal males plus a suture of male proportions	p =	0.136	0.549	0.811
	DF =	9	2	1
	F =	4.10	0.62	0.06
	no. of drops with fight / total n	5/13	8/19	8/19

1.2. The effect of all 7 treatments on total amount of fighting in drops with or without an object:

The 7 treatments:

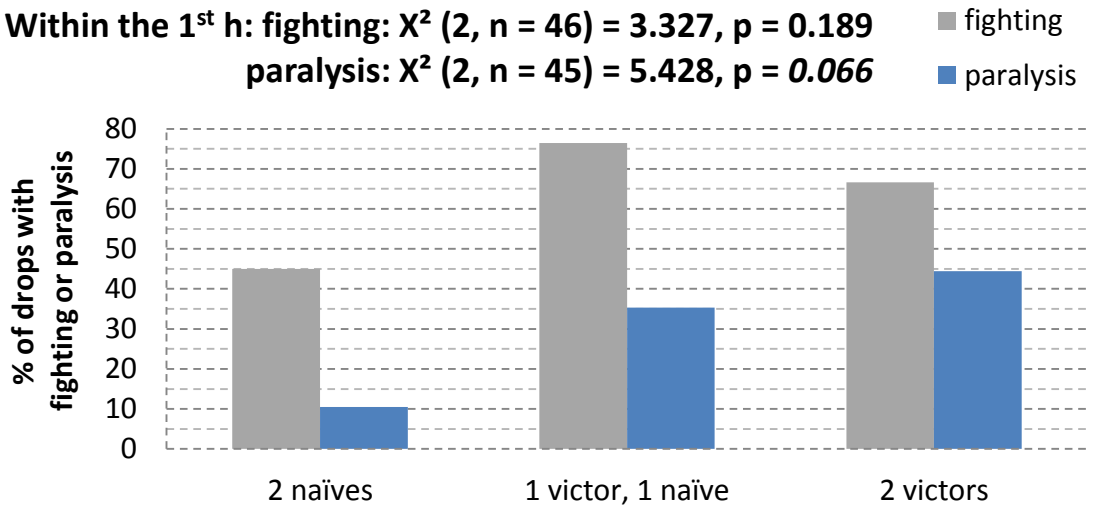
- 2 males without an object
- 1 male with an incapacitated male
- 1 male with a dead male
- 1 male with a conspecific male sized suture
- 2 males with an incapacitated male
- 2 males with a dead male
- 2 males with a conspecific male sized suture

When all seven treatments were analysed for total no. of attacks in a drop, significant differences between the no. of attacks depending on the nature of the object were found for both the 1st as the 2nd time periods (1st 15 min: p = 0.000; F= 5.42; DF=6b, 2nd 15 min: p =0.007; F=3.05; DF=6).

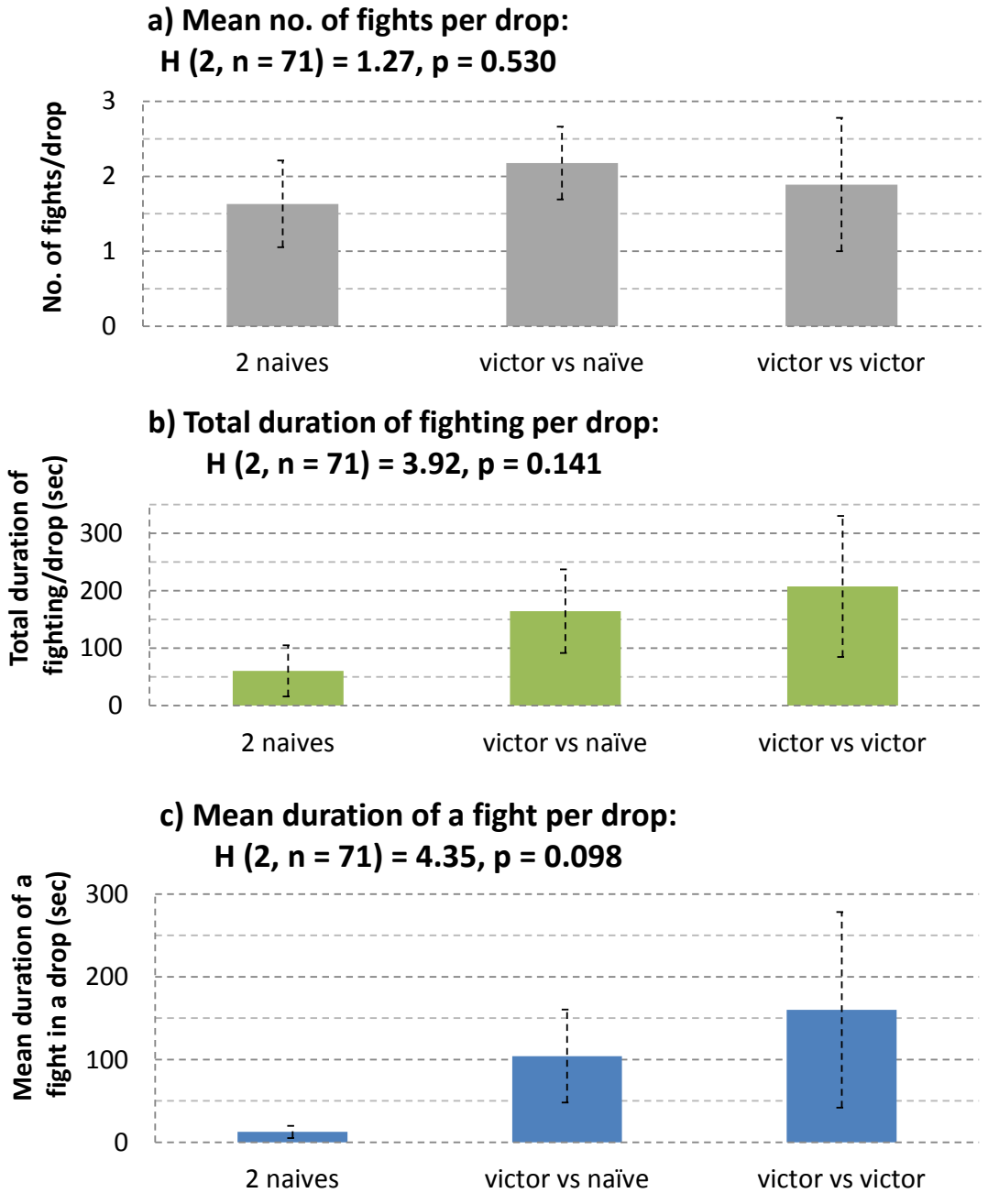


Appendix figure 1 **All attacks, regardless of receiver. Mean (+/- SE) no. of attacks made in a drop. Observations were made over 2 time periods: a) 0-15 and b) 16-30 mins after the worms and objects were added to the drop. Within a graph, bars accompanied by the same or by no letters are not significantly different.**

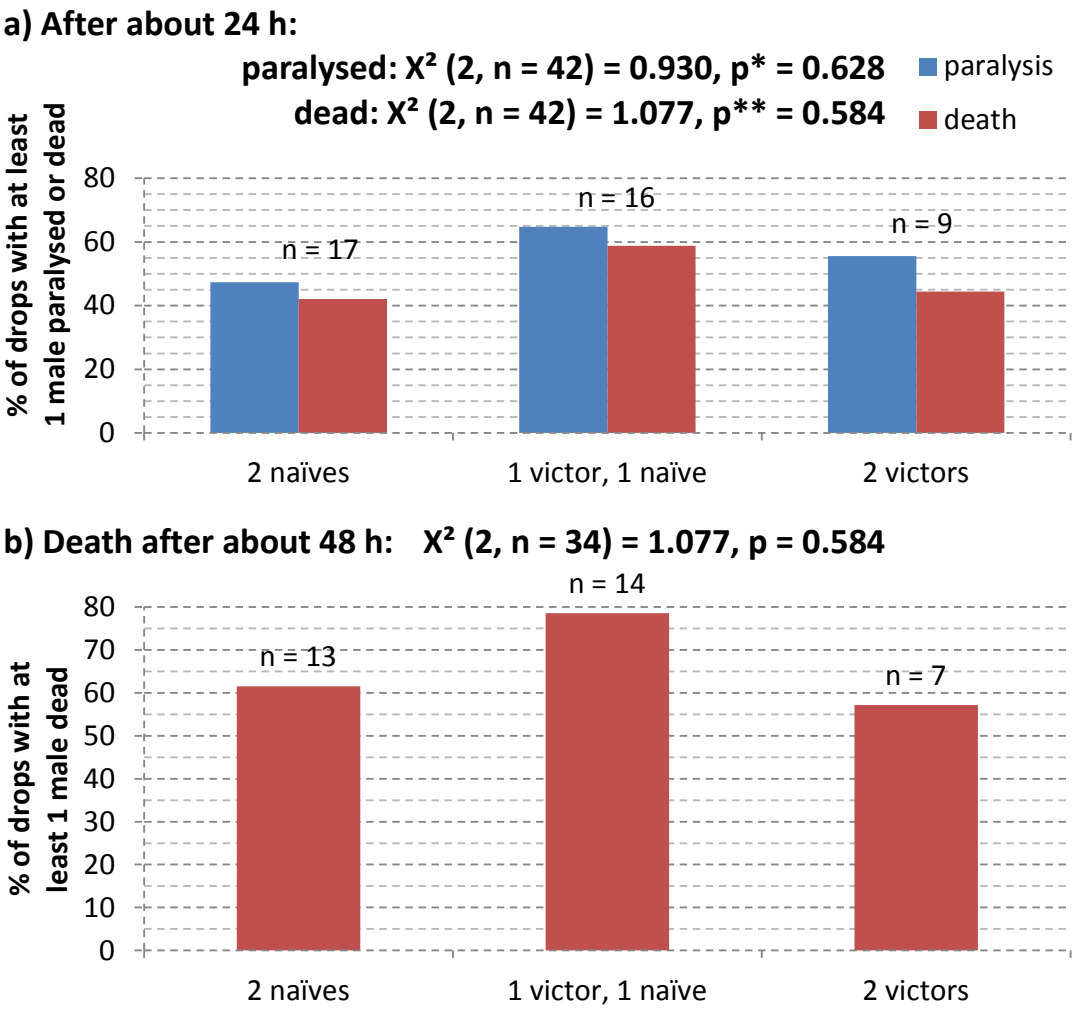
1.3. The effects of winning a previous fight:



Appendix figure 2 **The effect of winning a previous fight on the no. of drops with fighting (orange) or with paralysis (blue) during the 1st h after the 2 males were put together in a drop. Bars accompanied by the same, or by no letter are not significantly different from each other. The trend for differences in no. of drops with paralysis in the 1st h of the battle could not be translated into differences between the different treatments by a Bonferroni-Holm step down test. The no. of drops with 2 naïves =19, with 1 naïve and 1 victor =17, with 2 victors =9.**



Appendix figure 3 **The effect of winning a previous fight on a) the no. of attacks per drop; b) the total duration of attacks per drop and c) the mean duration of an attack in a drop during the 1st h after the males were put together in a drop. Bars accompanied by the same, or by no letter are not significantly different from each other. All values shown are mean+/-SE. Kruskal-Wallis, DF=2.**



Appendix figure 4 **The effect of winning a previous fight on the no. of drops with paralyis or death a) about 24 h and b) about 48h after the 2 males were put together in a drop. Bars accompanied by the same, or by no letter are not significantly different from each other. n is the no. of drops used for the treatment.**

2. Fighting behaviour in the Steinernematidae (chapter 4)

2.1. Comparison of fighting in *Steinernema* species

Appendix table 5 Differences between species in no. of 2-male drops with at least 1 male paralysed after 24 h using sequential χ^2-tests. Special attention is paid to differences in significance depending on the application of no adjustment (significance level α=0,05) or the sequential Bonferroni adjustment (significance level α').

24 h				Sequential Bonferroni adjustment			No adjustment
Species		χ^2	p	Rank	α'	p < α'	p < 0.05
S. carpocapsae	*S. longicaudum*	0.238	0.626	1	0.050	No	No
S. carpocapsae	*S. glaseri*	-	0.517	2	0.025	No	No
S. kraussei	*S. glaseri*	0.527	0.468	3	0.017	No	No
S. glaseri	*S. longicaudum*	-	0.322	4	0.013	No	No
S. feltiae	*S. kraussei*	2.205	0.138	5	0.010	No	No
S. feltiae	*S. glaseri*	2.989	0.084	6	0.008	No	No
S. kraussei	*S. carpocapsae*	3.706	0.054	7	0.007	No	No
S. kraussei	*S. longicaudum*	8.36	**0.004**	8	**0.006**	**Yes**	**Yes**
S. feltiae	*S. carpocapsae*	11.99	**0.001**	9	**0.006**	**Yes**	**Yes**
S. bicornutum	*S. feltiae*	16.07	**0.000**	10	**0.005**	**Yes**	**Yes**
S. bicornutum	*S. kraussei*	22.42	**0.000**	11	**0.005**	**Yes**	**Yes**
S. bicornutum	*S. carpocapsae*	36.26	**0.000**	12	**0.004**	**Yes**	**Yes**
S. bicornutum	*S. glaseri*	21.67	**0.000**	13	**0.004**	**Yes**	**Yes**
S. bicornutum	*S. longicaudum*	54.72	**0.000**	14	**0.004**	**Yes**	**Yes**
S. feltiae	*S. longicaudum*	24.92	**0.000**	15	**0.003**	**Yes**	**Yes**

Appendix table 6 Differences between species in no. of 2-male drops with at least 1 male paralysed after 48 h using sequential χ^2-tests. Special attention is paid to differences in significance depending on the application of no adjustment (significance level α=0.05) or the sequential Bonferroni adjustment (significance level α').

48 h				Sequential Bonferroni adjustment			No adjustment
Species		χ^2	p	Rank	α'	$p < \alpha'$	$p < 0.05$
S. carpocapsae	*S. glaseri*	-	1.000	1.00	0.050	No	No
S. feltiae	*S. kraussei*	0.441	0.441	2.00	0.025	No	No
S. glaseri	*S. longicaudum*	-	0.411	3.00	0.017	No	No
S. kraussei	*S. glaseri*	2.535	0.111	4.00	0.013	No	No
S. carpocapsae	*S. longicaudum*	3.093	0.079	5.00	0.010	No	No
S. kraussei	*S. carpocapsae*	3.484	0.062	6.00	0.008	No	No
S. feltiae	*S. glaseri*	4.536	***0.033***	7.00	***0.007***	***No***	**Yes**
S. feltiae	*S. carpocapsae*	7.431	**0.006**	8.00	**0.006**	**=**	**Yes**
S. bicornutum	*S. feltiae*	16.19	**0.000**	9.00	**0.006**	**Yes**	**Yes**
S. bicornutum	*S. kraussei*	18.99	**0.000**	10.00	**0.005**	**Yes**	**Yes**
S. bicornutum	*S. carpocapsae*	32.21	**0.000**	11.00	**0.005**	**Yes**	**Yes**
S. bicornutum	*S. glaseri*	*23.67*	**0.000**	12.00	**0.004**	**Yes**	**Yes**
S. bicornutum	*S. longicaudum*	65.02	**0.000**	13.00	**0.004**	**Yes**	**Yes**
S. feltiae	*S. longicaudum*	27.1	**0.000**	14.00	**0.004**	**Yes**	**Yes**
S. kraussei	*S. longicaudum*	16.31	**0.000**	15.00	**0.003**	**Yes**	**Yes**

3. Cause of paralysis and death (chapter 5)

3.1. Examination of *S. longicaudum* fight victims: Comparison of injuries in different species of *Steinernema*

Appendix table 7 No. of injured and dead males showing a ruptured body wall wound. Numbers are given for the observations made on the same day of, or straight after fighting and also at the timepoint of about 24 h after the fighting. Percentage of total injured males is put between brackets.

	Males with a ruptured body wall whether or not accompanied by ruptured internal organs protruding through the wound		Otherwise injured or paralysed males	
Species	**20 min - 5 h after fighting**	**about 24 h after fighting**	**20 min - 5 h after fighting**	**about 24 h after fighting**
S. feltiae	7 (77.8%)	14 (58.3%)	2 (22.2%)	10 (41.7%)
S. kraussei	10 (71.4%)	23 (74.2%)	4 (28.6%)	8 (25.8%)
S. longicaudum	2 (33.3%)	3 (33.3%)	4 (66.7%)	6 (66.7%)

20 min - 5 h after fighting: χ^2 (2, n = 29) = 3.566, p = 0.168;

4 cells with expected counts < 5.

About 24 h after fighting: χ^2 (2, n = 64) = 5.253, DF=2. p = *0.072*;

1 cell with expected counts < 5.

S. feltiae compared to *S. kraussei*:	χ^2 (1, n = 55) = 1.546, p = 0.214
S. feltiae compared to *S. kraussei*:	Fisher's exact: , DF = 1, n = 33, p = 0.259
S. kraussei compared to *S. longicaudum*:	Fisher's exact: DF = 1, n = 40, *p = 0.044*

3.2. Stabbing females with an insect dissection needle

Appendix table 8 The no. of drops containing a female that suffered impeded movement at the specified timepoint after stabbing.

	No. of control females that		No. of stabbed females that		Statistics: 2 by 2 χ^2test		
No. of h after stabbing	were moving normal	suffered impeded movement	were moving normal	suffered impeded movement	p	χ^2	No. of cells with expected counts < 5
1	13	1	11	3	0.28	1.167	2
2	13	1	10	5	0.08	3.027	2
3	20	1	14	8	*< 0.05*	6.484	2
4	20	1	9	9	*< 0.01*	10.403	1
5	13	2	6	10	*< 0.01*	7.888	0
24	20	1	11	22	*< 0.01*	20.113	0
48	16	2	2	23	*< 0.01*	18.135	0
72	24	1	1	26	*< 0.01*	44.297	0

Appendix table 9 The no. of drops containing a female that was no longer moving at the specified timepoint after stabbing.

	No. of control females that		No. of stabbed females that		Statistics: 2 by 2 χ^2test		
No. of h after stabbing	were moving	were not moving	were moving	were not moving	p	χ^2	No. of cells with expected counts < 5
1	13	1	12	2	0.54	0.373	2
2	13	1	11	4	0.16	1.934	2
3	20	1	18	4	0.17	1.883	2
4	20	1	11	7	**< 0.01**	6.923	2
5	13	2	10	6	0.12	2.362	2
24	20	1	15	18	**< 0.01**	13.947	0
48	16	2	3	22	**< 0.01**	25.087	0
72	24	1	1	26	**< 0.01**	44.297	0

3.3. Mimicking injuries and paralysis: Injecting

Appendix table 10 The effect of injecting a male with spun down media (BHIB extra) that had contained a fighting male couple or a single male or was sterile and had contained no nematodes.

Injection medium:	Time after injection	No. of injected males that were moving normal	suffered impeded movement	were moving	were not moving
2 males had	2-3 h	24	5	28	1
fought &	± 1 day	21	8	25	4
paralysed	2-3 days	7	16	8	15
a single	2-3 h	19	9	27	1
nematode	± 1 day	21	7	24	4
had resided	2-3 days	7	10	9	8
sterile, no	2-3 h	22	2	24	0
nematodes	± 1 day	16	8	21	3
	2-3 days	9	12	9	12

Appendix table 11 χ^2-statistics on the effects of injecting a male with spun down media (BHIB extra) that had contained a fighting male couple or a single male or was sterile and had contained no nematodes. DF=2.

	Time after injection	Statistics: 3 by 2 χ^2test p	χ^2	No. of cells	with expected counts less than
Movement impeded	2-3 h	0.09	4.802	1	5
	± 1 day	0.79	0.456	-	-
	2-3 days	0.66	0.842	-	-
Not moving	2-3 h	-	0.864	3	1
	± 1 day	0.98	0.037	3	5
	2-3 days	0.517	1.319	-	-

3.4. Comparison of stabbing and injection experiments

Appendix table 12 Comparing the effects of stabbing a female with an insect dissection needle. stabbing a female with a micro-injection needle and injecting a male with different BHIB-media.

Treatment	After 24h: No. of nematodes that were moving normal	After 24h: No. of nematodes that suffered impeded movement or worse	after 48h No. of nematodes that were moving normal	after 48h No. of nematodes that suffered impeded movement or worse
Stabbed with an insect dissection needle	11	22	2	23
Stabbed with a micro-injection needle	45	6	42	8
Injected with various BHIB media	58	23	-	-
Statistics	χ^2 (2, n = 165) = 28.751, **p < 0.001**		χ^2 (1, n = 76) = 38.047, **p < 0.001**	

3.5. Media and bacteria: Fighting in haemolymph drops with and without *Xenorhabdus* (Xenic and aXenic).

Appendix table 13 Raw data and statistics of observations of paralysis and impeded movement made about 24 h after aXenic or Xenic males were paired or grouped respectively with other aXenic or Xenic males.

Type of drop and male	No. of males per drop	No. of drops with all males moving normally	No. of drops with at least 1 male with impeded movement
Xenorhabdus containing	single	167	3 (2%)
	pair	30	14 (32%)
	group of 5	0	26 (100%)
Xenorhabdus-free	singles	160	2 (1%)
	pair	12	4 (25%)
	group of 5	15	11 (42%)

Appendix table 14 **Raw data and statistics of observations of death made about 24 h after aXenic or Xenic males were paired or grouped respectively with other aXenic or Xenic males.**

Type of drop and male	No. of males per drop	No. of drops in which all males alive	No. of drops in which at least 1 male dead
Xenorhabdus containing	single	169	1 (1%)
	pair	31	13 (30%)
	group of 5	8	18 (69%)
Xenorhabdus-free	singles	161	1 (1%)
	pair	14	2 (12%)
	group of 5	18	8 (31%)

Appendix table 15 **Raw data and statistics of observations of paralysis and impeded movement made about 48 h after aXenic or Xenic males were paired or grouped respectively with other aXenic or Xenic males.**

Type of drop and male	No. of males per drop	No. of drops in which all males moving normally	No. of drops in which at least 1 male with impeded movement
Xenorhabdus containing	single	164	1 (1%)
	pair	15	30 (67%)
	group of 5	1	25 (96%)
Xenorhabdus-free	singles	124	3 (2%)
	pair	7	4 (36%)
	group of 5	4	17 (81%)

Appendix table 16 Raw data and statistics of observations of death made about 48 h after aXenic or Xenic males were paired or grouped respectively with other aXenic or Xenic males.

Type of drop and male	No. of males per drop	No. of drops in which all males alive	No. of drops in which at least 1 male dead
Xenorhabdus containing	single	165	0 (0%)
	pair	22	23 (51%)
	group of 5	2	24 (92%)
Xenorhabdus-free	singles	125	2 (2%)
	pair	9	2 (18%)
	group of 5	9	12 (57%)

Appendix table 17 Differences in no. of drops with paralysis or death in *Xenorhabdus*-free and/or *Xenorhabdus* containing drops and males after 24 h.

	Type of drop and male		Statistics		Sequential Bonferroni adjustment		No adjustment
	Xenorhabdus - free	*Xenorhabdus* containing	χ^2	p	α'	p < α'	p < 0.05
Paralysis	single	single	-	1	0.050	No	No
	pair	pair	-	0.76	0.025	No	No
	5 males	5 males	21.081	**< 0.001**	**0.0056**	**Yes**	**Yes**
	single – pair	-	-	**0.001**	**0.0125**	**Yes**	**Yes**
	single – 5 males	-	-	**< 0.001**	**0.0063**	**Yes**	**Yes**
	pair – 5 males	-	1.292	0.26	0.0167	No	No
	-	single – pair	-	**< 0.001**	**0.0100**	**Yes**	**Yes**
	-	single – 5 males	-	**< 0.001**	**0.0083**	**Yes**	**Yes**
	-	pair – 5 males	31.023	**< 0.001**	**0.0071**	**Yes**	**Yes**
Death	single	single	-	1	0.0500	No	No
	pair	pair	-	0.31	0.0167	No	No
	5 males	5 males	7.692	**< 0.01**	0.0250	**Yes**	**Yes**
	single – pair	-	-	***0.022***	*0.0083*	*No*	***Yes***
	single – 5 males	-	-	**< 0.001**	0.0056	**Yes**	**Yes**
	pair – 5 males	-	-	0.27	0.0125	No	No
	-	single – pair	-	**< 0.001**	0.0071	**Yes**	**Yes**
	-	single – 5 males	-	**< 0.001**	0.0063	**Yes**	**Yes**
	-	pair – 5 males	10.432	**0.001**	0.0100	**Yes**	**Yes**

Appendix table 18 Differences in no. of drops with paralysis or death in *Xenorhabdus*-free and/or *Xenorhabdus* containing drops and males after 48 h.

	Type of drop and male		Statistics		Sequential Bonferroni adjustment		No adjustment
	Xenorhabdus-free	*Xenorhabdus* containing	χ^2	p	α'	p < α'	p < 0.05
Paralysis	single	single	-	0.321	0.0500	No	No
	pair	pair	-	0.089	0.0167	No	No
	5 males	5 males	-	0.158	0.0250	No	No
	single – pair	-	-	**0.001**	0.0083	**Yes**	**Yes**
	single – 5 males	-	-	**< 0.001**	0.0056	**Yes**	**Yes**
	pair – 5 males	-	-	***0.020***	*0.0125*	*No*	***Yes***
	-	single – pair	122.627	**< 0.001**	0.0071	**Yes**	**Yes**
	-	single – 5 males	-	**< 0.001**	0.0063	**Yes**	**Yes**
	-	pair – 5 males	8.208	**< 0.005**	0.0100	**Yes**	**Yes**
Death	single	single	-	0.188	0.0500	No	No
	pair	pair	-	0.088	0.0250	No	No
	5 males	5 males	-	**< 0.01**	0.0100	**Yes**	**Yes**
	single – pair	-	-	***0.032***	*0.0125*	*No*	***Yes***
	single – 5 males	-	-	**< 0.001**	0.0056	**Yes**	**Yes**
	pair – 5 males	-	-	0.061	0.0167	No	No
	-	single – pair	-	**< 0.001**	0.0083	**Yes**	**Yes**
	-	single – 5 males	-	**< 0.001**	0.0071	**Yes**	**Yes**
	-	pair – 5 males	12.498	**< 0.001**	0.0063	**Yes**	**Yes**

Content of the attached CD

Steinernema feltiae

- *S. feltiae* Fight.AVI
- *S. feltiae* Injured male.AVI

Steinernema glaseri

- *S. glaseri* Fight.AVI

Steinernema kraussei:

- *S. kraussei* Fight.AVI
- *S. kraussei* Injured male.AVI

Steinernema longicaudum

- *S. longicaudum* Crush injuries.AVI
- *S. longicaudum* Fight.mpg
- Fight-induced Injuries of a victim from a continuously stimulated pair:
  S. longicaudum Fight Injuries.AVI