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Preadaptation toward parasitism in order Rhabditida (Nematoda)

Abstract approved:

Rhabditid nematodes include free-living, phoretic, and parasitic species, making the order useful for investigating the evolution of parasitism in nematodes. Continuum theory proposes that adaptation to host environments occurred gradually through increasingly invasive stages of phoresis and the consequent accumulation of traits required for vertebrate parasitism. Preadaptation theory suggests that the habitats of saprobiotic rhabditids provide immediate selective pressures, including elevated temperature, high osmotic potential, low pH and oxygen levels, and the presence of proteolytic enzymes, that preadapted saprobiotic nematodes for parasitism of vertebrate hosts. To assess their ability to survive in a host environment, free-living rhabditid nematode species utilizing non-invasive, minimally invasive, and maximally invasive phoretic strategies, were collected, cultured, and exposed to fully-crossed levels of temperature, acidity, and oxygen concentration in the presence of proteolytic enzymes. Significant differences in relative survivorship were observed among the species; however, increased survivorship did not follow phylogenetic or ecological patterns. Species of nematodes fed to frogs via feeding needles had varying survivorship, with the maximally invasive species demonstrated highest survivorship. However, when these species were fed to frogs via invertebrate vectors, the minimally invasive species demonstrated greatest survivorship. These data demonstrate that infectiousness is not associated with phoretic association, suggesting preadaptation as a possible mechanism for the evolution of parasitism in the Rhabditida.

PREADAPTATION TOWARD PARASITISM IN ORDER RHABDITIDA (NEMATODA)

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PREFACE

This thesis follows the guidelines for publication in the *Journal of Parasitology* because this research will ultimately be submitted to that journal for publication. Please refer to the journal's instructions to authors for any stylistic inquiries.

TABLE OF CONTENTS

CHAPTER 1

INTRODUCTION

METHODS AND MATERIALS

RESULTS

DISCUSSION

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1

Introduction

Preadaptation, continuity, and functional shifts

After Darwin first proposed the theory of natural selection, a critic of his theory, George Mivart, posed the problem of "incipient stages" (Mivart, 1871 *in* Gould, 2002). Mivart argued that although a certain character might be adaptive in its finished state, no adaptive value existed in a character's intermediate state. Gould (2002) commonly termed this the "five percent of a wing principle", in that a whole wing has adaptive value for flight; however, five percent of that wing would not have adaptive value and would not be selected. To address the problem of continuity in evolution, Darwin (1872) utilized the concepts of redundancy and functional shifts. Today this concept of functional shifts in characters relate to the phenomenon of preadaptation. In preadaptation, a character previously shaped by natural selection for one particular function can be co-opted for a new use for which it was not previously selected, causing a functional shift in the character (Bock, 1959; Gould and Vrba, 1982; Futuyma, 1998).

The term "preadaptation" differs from the "exaptation" of Gould and Vrba (1982) in that they are on two different points of a theoretical timeline; "exaptation" can only be determined *ex post facto*. In exaptation the characters already have been co-opted for a new use, while preadaptation, however, refers to characters that arise under the ancestral selective regime and that have the potential for co-option under future selective pressures. For instance, coelurosaur dinosaurs, ancestors of *Archaeopteryx*, had hollow bones and a fused clavicle, both of which were vital to the evolution of flight in *Archaeopteryx*

(Futuyma, 1998). *Archaeopteryx* also possessed proto-feathers, originally selected for thermoregulation, which were then co-opted for flight (Gould and Vrba, 1982; Gould 2002). Insect wings also appear to have been selected for thermoregulation as well, with flight being a co-opted function (Kingsolver and Koehl, 1985), indicating that preadaptation can account for some convergent evolutionary events.

According to Osche (1962), preadaptive phenomena require a platform of structural and physiological characteristics that promote survival in two environmentally similar but disperate habitats, and a bridge of ecological characteristics that are appropriate for both habitats and create the potential for dispersal from one habitat to the other. Preadaptation can account for the possession of necessary traits to permit a shift into a new niche or habitat (Bock, 1959; Futuyma, 1998; Gould, 2002), thereby facilitating reproductive isolation. Bock (1959) even went so far as to suggest that many of the unsolved problems of macroevolution would be elucidated with a better understanding of preadaptation.

Preadaptation and parasitism

Parasitism, in which an organism uses a host as habitat, has evolved numerous times in various taxa (Blaxter, 2001; Bush et al., 2001). Read (1972) described three distinct properties of parasitism: infectiousness, establishment, and transmission. Infectiousness occurs first and requires immediate tolerance to the environment. Establishment is the major hurdle for parasites because it requires evading the immune system, making it the least likely character to be acquired through preadaptation. Transmission entails colonization of new hosts. Preadaptation might be involved in the transition to parasitism in nematodes based on the conditions that saprobiotic nematodes, namely the rhabditid nematodes, tolerate in their habitat. Conditions that these nematodes face in saprobiotic substrate are very similar to those in the vertebrate gut and might preadapt them toward Read's (1972) principle of infectiousness (Osche, 1952, 1962, 1965).

The order Rhabditida (Nematoda: Secernentea) includes soil-dwelling bacteriovores, fungivores, and predators, as well as parasites of both vertebrates and invertebrates, making it useful for studying the origins of vertebrate parasitism. Features that allow terrestrial nematodes to exploit spatially and temporally discontinuous habitats such as decomposing organic matter, are considered to have been keystone adaptations in saprobiotic Secernentea that became parasites of animals and plants (Clark, 1994). Many of the free-living rhabditids, such as *Cheilobus quadrilabiatus*, *Diplogaster* spp., and *Rhabditis* sp., associate phoretically with arthropods as a means of dispersing among patches of saprobiotic substrate (Bovien, 1937). Rhabditids such as *Strongyloides* spp. are heterogonic vertebrate parasites that alternate between free-living and parasitic cycles (Fisher and Viney, 1998). Phylogenetic analyses suggest that the parasitic habit in the order Strongylidia (obligate nematode parasites of vertebrates) originated in Rhabditia (Blaxter, 2001). These characteristics of rhabditid nematodes have prompted a number of authors to use the variation in life history patterns to speculate about the origins of the parasitic habit.

The continuum theory of the origin of parasitism in rhabditid nematodes proposes that adaptation to the host environment occurred gradually by progression through increasingly invasive phoretic associations with invertebrates, and the subsequent accumulation of traits required for vertebrate parasitism (Osche, 1952). In fact, Rühm

(1956) suggested that only by first being symbiotic can nematodes achieve vertebrate host utilization. Blaxter (2001) also suggested insect parasitism might have been a repeated prelude to vertebrate parasitism based on data showing that vertebrate parasites in several monophyletic clades are closely related to insect parasites. However, this close relationship between insects and nematodes also would be expected under preadaptive theory because of the association between saprobiotic nematodes and insects. The continuum begins as a phoretic habit of attaching to arthropods' exoskeletons, then proceeds to a minimally invasive state, such as phoretic attachment under the elytra, followed by progression to an internally invasive state whereby the nematodes invade the host body cavity. Finally, the invasive state exerts selective pressures resulting in the evolution of parasitism in these nematode species (Osche, 1952).

In contrast to the continuum theory, Osche (1952, 1962, 1965) suggested that the environment of free-living saprobiotic rhabditids provides immediate selective pressures that preadapt them for parasitism in vertebrate hosts. Infectiousness would be comparable to rhabditids encountering the vertebrate gut and surviving passage through the alimentary canal. Conditions that saprobiotic nematodes endure are mediated by the activity of bacteria, fungi, and other microflora and include elevated temperatures and osmotic potential, lowered pH and oxygen levels, and the presence of proteolytic enzymes (Osche, 1962). These conditions are similar to those in the gut of a vertebrate host, therefore, surviving passage through a vertebrate would be likely because the physiological pressures of saprobiotic substrate requires a suite of characteristics that are readily co-optable for survival inside a host. Therefore, the key question focuses on if invasive species have any advantage in infectiousness over non-invasive species.

Transmission, the third requirement for parasitism, is dependent on successful establishment and entails passing a new generation of parasites to a new set of hosts (Read, 1972). In rhabditids, the 3rd juvenile (J_3) or "Dauerlarva" stage possesses a characteristic oily, water-repellant covering originating from the second molt called a "Schutzhülle" that provides resistance to chemicals, heat, and desiccation (Bovien, 1937; Anderson, 1978; Charwat et al., 2002). Dauerlarvae also exhibit questing behavior similar to that of strongylid juveniles (Osche, 1965; Viney, 1999), seeking out the highest point in the vicinity and attaching to visiting insects (Voelk, 1950). In free-living nematodes, questing is found only in conjunction with juveniles that exhibit substrate specialization and occurs when the substrate desiccates (Osche, 1952; Riddle et al., 1981; Viney, 1999). Transmission from one habitat patch to another by phoretic associates is vital to rhabditids because compost is an ephemeral habitat, becoming uninhabitable as the substrate decomposes and dessicates (Osche, 1962).

Phoretic association with insects (Bovien, 1937) readily satisfies Osche's bridge criteria, solving the problem of transmission, and one would expect no advantage for invasive or non-invasive nematodes in terms of the potential for transmission. The use of insect vectors by phoretic rhabditids represents both a proximal and an ultimate solution to the problem of spatially and temporally discontinuous habitats and creates the potential for ingestion of dispersing nematodes by insectivorous vertebrates (Osche, 1962).

Passage through the gut also has some ecological advantages. Excretion with the feces provides the surviving juveniles with an immediate saprobiotic habitat: dung provides bacteria for nutrition, creates conditions favorable for reproduction, and attracts feeding invertebrates that provide a phoretic route to other resource patches. The ability to survive passage, reproduce and then find a phoretic associate relate to Read's (1972) concept of transmission.

Examination of the selective pressures faced by saprobiotic nematodes suggest that it is unlikely that a more invasive phoretic habit provides an advantage to rhabditids in terms of infectiousness and, possibly, establishment. The purpose of the present investigation was to determine if the saprobiotic habit preadapts rhabditids for infectiousness. Preadaptation would be supported if invasive and non-invasive juveniles were equally able to survive in the host environment. Phoretic invasiveness should not confer higher survivorship to juvenile nematodes under *in vitro* conditions, while survival under *in vivo* conditions should be similar to those *in vitro*. Therefore, a good predictor of survivorship in a vertebrate host should be tolerance in *in vitro* conditions that mimic host conditions; invasiveness should not be a good predictor.

Materials and Methods

Collection and culturing of nematodes

During 2003 and 2004, 50 ml samples of compost and dung were collected from decomposing vegetation in Emporia (Lyon County, Kansas) and 50 ml samples of dung were collected from 800-acres of grazed pasture surrounding the Ross Natural History Reservation (Lyon County, Kansas). Dung beetles were collected manually from dung and from baited pitfall traps. Compost was collected in 50 ml samples from garden compost heaps in Emporia, Kansas. Earthworms (*Lumbricus terrestris*) were purchased from a local merchant to obtain invasive juvenile nematodes.

Dung beetles were examined and dissected with the aid of a dissecting scope. Superficially attaching nematodes, nematodes under the elytra, and nematodes in the sutures were collected. Compost samples, dung samples, and dissected, captured insects were macerated in 0.06% saline solution. Nematodes were collected from these solutions by placing compost, dung, or macertated insects on the screen of a Baermann apparatus (MacInnis and Voge, 1970) with the modification of adding light and heat via a 40 watt bulb and the use of a fine wire mesh without gauze. Resulting filtrate was collected after one hour and examined using an inverted compound microscope. Individual gravid females from compost and dung extractions were each transferred to a single well of a 6-well polystyrene tissue culture plate containing cornmeal agar. Third-stage (J_3) juveniles isolated from insects were transferred to similar wells containing cornmeal agar. Earthworms were washed in distilled water, cut into small sections, and each section was placed in a well of a 6-well plate containing cornmeal agar. A total of seven species of rhabditid nematodes, representing three levels of phoretic association were obtained in this manner. Three species (*Cruzema tripartitum*, *Rhabditis longicaudata*, *Protorhabditis oxyuroides*) utilize superficial attachment, three species (*Diploscapter coronatus*, *Diploscapter lycostoma, Stomachorhabditis fastidosa*) attach under the wing case of dung beetles, and one species (*Rhabditis maupasi*), obtained from *L. terrestris*, invades the body cavity of earthworms.

Established cultures were extracted at two-week intervals to isolate individual gravid females to create subcultures. Mature nematodes from each culture were stained using an acetic-acid/lactophenol series and identified (Andrassy, 1983).

In vitro **survivorship tests**

For each species, groups of 30-70 third-stage juvenile rhabditids were transferred to each well of a 6-well tissue culture plate. The isolated J_3 rhabditids were exposed to a solution of 0.5% trypsin and 0.5% pepsin in a fully-crossed design with three levels of pH, three different temperatures, and two oxygen levels, resulting in a total of 18 combinations of environmental conditions, each represented by one 6-well tissue culture plate per condition.

Enzyme solutions were brought to pH 2, 4, or 6, by addition of hydrocholoric acid or potassium hydroxide. After adjusting pH, three milliliters of solution were pipetted into each well containing J_3 rhabditids. Inoculated plates were exposed to one of three different temperatures by placing replicate tissue culture plates in a refrigerator (7 C), leaving them at room temperature (20 C ± 1 C), or by placing them in a 30 C incubator. For normoxic trials nematodes were left at ambient oxygen levels. For hypoxic trials, nitrogen gas was bubbled through the solution for one minute. This hypoxic solution was pipetted into the wells of tissue culture plates containing J_3 rhabditids, the wells were sealed with Parafilm®, and plate lids secured over the Parafilm®. Juvenile survivorship was quantified by motility assays, in which numbers of moving nematodes were counted. Assays were performed every 2 hours for 12 hours, and once every 12 hours following the initial 12 hour observation period. This procedure was repeated for a total of three trials per *in vitro* condition. *In vitro* survivorship data were analyzed using the accelerated time failure model as reviewed by Fox (1998) via SAS 9.0 statistical software (The SAS Institute, 2002) to evaluate test conditions' effect over time on the survivorship of each species and to compare time until failure data for each species' with χ^2 denoting weighted differences between the observed and expected number of events.

***In vivo* survivorship tests**

After determining *in vitro* survivorship for nematode species, one species representing each level of phoretic association was selected for *in vivo* tests based on which species had highest survivorship in the most adverse conditions (pH 2, 30 C, normoxia). *C. tripartitum* represented superficial association, *S. fastidosa* represented minimal invasiveness, and *R. maupasi* represented maximum invasiveness. Fifty third-stage juveniles per species were fed to eight female leopard frogs (*Rana pipiens*) via a feeding needle for a total of 24 exposed frogs.

In addition, laboratory-reared grain beetles (*Tenebrio molitor*) were placed within cultures of *C. tripartitum* and *S. fastidosa* for 24 hours to allow for attachment of the third-stage juveniles to the insect host. One exposed beetle from each culture was fed to each of eight frogs by placing the insects in the mouth of the frogs, for a total of 16 frogs fed nematode-attached insects. Two exposed grain beetles were dissected in saline and examined after exposure to the culture to ensure that nematodes had attached to the beetles. To feed *R. maupasi* to frogs, two earthworms were washed in distilled water, and each cut into five sections of similar size. Eight of these sections were selected haphazardly and fed to eight frogs. The two remaining sections were placed in culture and 24 hours later the number of juveniles that escaped the body cavity of the earthworm was counted to estimate the infective dose delivered to each frog.

For each species of nematode and for each method of feeding, two frogs were killed by double-pithing, and their intestines dissected in saline solution and examined with the aid of a stereo microscope after 1, 12, 24, and 72 hours of exposure to determine survivorship of nematode juveniles in frog hosts. Survivorship was determined by motility assay. The order in which nematode species were assayed for survival in the frog host was randomized via a random number generator (Urbaniak and Plous, 1997) to ensure that differences in the timing of the assays did not affect interpretation of the results. Exposed frogs were housed separately and their feces examined until they were killed to determine if any juvenile rhabditids survived passage through the gut. All *in vivo* data were analyzed using logistic regression of categorical data as reviewed by Floyd (1998) via SAS 9.0 statistical software (The SAS Institute, 2002) to evaluate the effects of passage time and feeding method of exposure on survivorship.

Results

In vitro tests

Combined analysis of overall *in vitro* survivorship for the seven species (Fig. 1) in all experimental conditions determined that survivorship varied significantly among species (χ^2=4224.89, p<0.0001), temperatures (χ^2=1436.47, p<0.0001), pH levels (χ^2=2018.42, p<0.0001), and oxygen levels (χ^2=45.90, p<0.0001). Interactions between pH and oxygen concentration (χ^2=902.37, p<0.0001), temperature and pH (χ^2=2039.38, p<0.0001) as well as temperature, pH, and oxygen concentration (χ^2=1028.15, p<0.0001) also occurred. As denoted by their χ^2 values, temperature and pH had the strongest effect, with the effect of pH increasing as temperature increased *in vitro*. Evaluation of 95% confidence intervals for survivorship estimates in Fig. 1 produced two distinct survivorship groups. The group with the highest survivorship consists of *P. oxyuroides*, *R. maupasi*, and *C. tripartitum* while the group with the lowest survivorship consists of *S. fastidiosa, R. longicaudata, D. coronatus*, and *D. lycostoma. In vitro* survivorship rankings for each nematode species for all 18 test combinations, based on the intercepts of each survivorship curve, are illustrated in Table 1.

In vivo tests

Species that had the highest *in vitro* survivorship ranking of their respective phoretic group at pH 2, 30 C, normoxia (Table 1) were fed to frogs. Assessment of delivery via a feeding needle revealed that *R. maupasi* had significantly higher survivorship (t = 0.11; p<0.05) than *C. tripartitum* and *S. fastidosa*, which did not differ significantly from each other ($t = 0.01$; $p>0.05$) based on multiple comparisons of slopes generated by logistic regression (Fig. 2). With invertebrate delivery, *C. tripartitum* had significantly higher survivorship ($t = 0.12$; $p<0.05$) than *R. maupasi* and *S. fastidosa* which did not differ significantly from each other ($t = 0.01$; $p>0.05$) (Fig. 3).

Discussion

Preadaptation, with habitat switching via functional shifts of characters, and continuum theory, with the gradual acquisition of characters through increasing invasiveness, both have been proposed as possible mechanisms for the evolution of parasitism in rhabditid nematodes. If preadpatation for the host environment does occur in saprobiotic habitats, no difference in the ability to survive host-like conditions *in vitro* should occur between invasive and non-invasive juveniles and survivorship under these conditions should be the best predictor of survivorship in a vertebrate host. In the present investigation, non-invasive species were just as likely, or more likely, to survive the *in vitro* conditions.

In vitro tests

The *in vitro* experiments were designed to evaluate whether saprobiotic rhabditids could tolerate host-like conditions, and determine whether phoretic habit contributes to survivorship in these host-like conditions. The three varying treatments (pH, temperature, and oxygen concentration) all factored significantly into J_3 survivorship *in vitro*. Optimum pH is vital for proper enzyme function and deviations of one pH unit or more disrupt the biochemistry of organisms by slowing or halting critical metabolic pathways (French et al., 2001). Overall nematode survivorship was greater at higher pH levels. In low pH conditions, non-invasive species, such as *C. tripartitum*, were just as likely, or more likely to survive, as the mid-invasive and invasive species (Table 1). These observations demonstrate that non-invasive species, particularly *C. tripartitum*, possess tolerance to a low pH, a trait vital for survival in a vertebrate gut.

Temperature also affects a variety of physiological processes. High temperatures denature proteins by disrupting the conformation of polypeptide chains. Enzymes have slower reaction rates when temperatures are low due to low kinetic energy of substrate molecules; however, denaturation at high temperatures decreases reaction rates in enzyme-catalyzed reactions (French et al., 2001). If an ectotherm, like a nematode, is subjected to temperatures above their critical thermal maximum, a breakdown in critical physiological processes occurs (French et al., 2001).

Even though some species survived at all three temperatures for at least 144 hours, overall nematode survivorship was higher at 7 C and 20 C than at 30 C. Higher survivorship at lower temperatures could be due to several factors. Trypsin and pepsin, the proteolytic enzymes in the *in vitro* tests, might not be fully functional at 7 C. Depressed development and metabolic rate of the J_3's in lower temperatures (Salih and Grainger, 1981; Klekoski and Wasilewska, 1982) could slow depletion of internal food stores, such as glycogen, of non-feeding J_3's. In addition, 7 C and 20 C might be closer to the nematodes' optimal physiological reaction rate because this temperature range is similar to what a free-living ectotherm might experience. Survivorship patterns did not correspond to phoretic associations. In lower temperatures of 7 C and 20 C, *P. oxyuroides*, a non-invasive nematode, and *R. maupasi*, an invasive nematode, both had high survivorship (Table 1). On the other hand, *C. tripartitum*, a non-invasive species exhibited higher survivorship at 30 C than all other species (Table 1).

Hypoxia also is a characteristic of the host habitat and rhabditids that survive well in hypoxia have the potential to survive passage through a vertebrate's alimentary canal (Osche, 1965). Oxygen concentrations can vary widely in saprobiotic habitats due to the amount of microbial action taking place, therefore, saprobiotic rhabditids should be able to tolerate both normoxia and hypoxia (Osche, 1965). Hypoxia did have a significant effect on overall survivorship rate with higher overall survivorship occurring in hypoxic than in normoxic conditions. *Protorhabditis oxyuroides* had the highest survivorship and *C. tripartitum* had the second highest survivorship under hypoxic conditions. Under normoxic conditions overall survivorship was lower and *R. maupasi* had highest survivorship. These results support the idea that invasiveness has no physiological advantage in surviving this characteristic of the host habitat. Dauerlarvae are adept at surviving oxygen deprivation (Anderson, 1978) and all species in the study had higher survivorship in hypoxic conditions. Thus, hypoxic conditions might not be a meaningful barrier for the transition to parasitism in the rhabditid nematodes.

According to J_3 survivorship data, pH most strongly influenced survival, followed by temperature. In addition, significant parallel interactions of pH and oxygen concentration, temperature and pH, as well as temperature, pH, and oxygen concentration were observed. However, no pattern of survival corresponding to phoretic associations occurred under any test conditions. Low pH and high temperature cause a rapid and dramatic decrease in the survivorship of most of the species examined and are the conditions a J_3-stage rhabditid would encounter immediately after ingestion, therefore, J_3-stage rhabditids that can survive these conditions should be more likely to survive in the host environment.

***In vivo* tests**

Based on the hypothesis that saprobiotic substrate preadapted rhabditids for survival in a host environment, one would expect rhabditids that had high survivorship in extreme *in vitro* conditions that might be found in a vertebrate host (e.g., pH 2, 30 C) to have high survivorship *in vivo*. Given that all frogs were housed at room temperature, and the tolerance of all species to hypoxia, high *in vitro* survival under conditions of low pH should be the best predictor of *in vivo* survivorship.

The present investigation tentatively supports the prediction that species with greater survivorship at low pH levels *in vitro*, have greater *in vivo* survivorship than species with relatively low survivorship at low pH. However, utilizing two different *in vivo* feeding methods created two different results. *Rhabditis maupasi* had highest survivorship when fed to frogs with a feeding needle (Fig. 2) while *C. tripartitum* had highest survivorship when fed to frogs via a phoretic associate. Generally, these *in vivo* results correspond with *in vitro* data comparing species survivorship in different pH levels, with the two species with the greatest *in vitro* survivorship having the highest survivorship under the two *in vivo* conditions. Invasiveness level does not seem to predict free-living rhabditids' experimental survivorship and preadaptation may be the mechanism for this phenomenon.

Cruznema tripartitum had highest survivorship when fed via invertebrate phoresis (Fig. 3). Utilizing two different feeding methods (feeding needle and invertebrate association) creates two different *in vivo* test conditions. Amphibians require the presence of food in their stomachs to stimulate proliferation of mucus, pepsinogen, and hydrochloric acid (Reeder, 1964). Therefore, trials using a feeding needle would not be expected to stimulate a gustatory response. Trials that employed invertebrate association, however, would elicit acid secretion and enzymatic activity. Consequently, invertebrate phoresis trials would be analogous to *in vitro* tests with low pH, whereas feeding needle trials would be more like pH 4 because they do not elicit a gustatory response. In addition, amphibians would most likely ingest a rhabditid when it is attached to a phoretic associate; therefore, invertebrate phoresis trials most accurately mimic potential ingestion in nature.

Non-invasive species were just as likely to survive host-like conditions, *in vitro* and *in vivo*, as the more invasive species. Thus, infectiousness does not appear to differ among species. In fact, some non-invasive species actually had higher survivorship in some key test conditions, such as low pH and high temperature. A non-invasive nematode, *P. oxyuriodes*, had the highest overall survivorship in *in vitro* tests. Even though *C. tripartitum* was not the best survivor over all levels of treatment, it survived in low pH values and high temperatures, and *C. triparitum* survived passage through a frog via invertebrate phoresis. These data indicate that a non-invasive free-living rhabditid might be just as likely to infect a host as an invasive rhabditid, supporting Osche's (1952, 1962, 1965) contention that preadaptation is sufficient to explain infectiousness.

One possible explanation for the observed differences in *in vitro* and *in vivo* survival rates could be that high or low survivorship under the experimental conditions is a phylogenetically conserved trait, and not a product of immediate selective pressures. Examination of hypothesized relationships within Nematoda (Fitch 2000; Dolinski et al.,1998; Baldwin et al., 1997; Blaxter, 2001) reveals no apparent phylogenetic pattern that explains survival differences among the species examined.

Implications for the evolution of parasitism in Nematoda

Even though *C. tripartitum*, *R. maupasi*, and *S. fastidiosa* demonstrated differing survivorship values in the varying *in vitro* conditions, both *C. tripartitum* and *R. maupasi* survived passage through the frogs' digestive systems when fed via invertebrate phoresis (Fig. 3). These data indicate that like transmission, infectiousness might not be related to levels of invasiveness in phoretic associations. Infectiousness is vital because it is the necessary first step toward parasitism (Read, 1972). Without infectiousness, establishment and transmission cannot occur. Given that infectiousness requires tolerance to the environment, akin to Osche's (1962) platform, many species of the rhabditids examined could meet the criteria for infectiousness. All of these species are associated with invertebrates that could provide a "bridge" (Osche, 1962) between the saprobiotic substrate and the host; therefore, all species could meet the criteria for transmission.

Invasiveness does not seem to affect infectiousness; however, it might influence establishment, where invasive species might have an advantage. Cases involving establishment of saprobiotic rhabditids in a vertebrate have been anecdotally reported (Chandler, 1938). These findings suggest that infectiousness, rather than establishment might be more of an issue in the gut because these free-living nematodes have been found in humans; however, a nematode's degree of invasiveness could provide it with adaptations, such as immune evasion, vital for establishment. *In vivo* data do not reflect any establishment advantage for invasive species; however, only one species from each phoretic group was tested. Establishment, which involves evasion of the host immune response (Zelmer, 1998), is key for a switch to parasitism. Moreover, establishment might be a phenomenon that develops from selective pressure exerted by the vertebrate host, with no advantage to species occupying internal habitats in invertebrate hosts. In this case, initial functional shifts in infectiousness would result in "secondary adaptation" (Gould and Vrba, 1982) toward establishment, with transmission already accounted for by preadaptation. Therefore, preadaptive and adaptive phenomena would work in conjunction to produce prerequisites toward parasitism. Thus, evaluation of potential parasitic habits in the Rhabditida should focus on mechanisms of establishment and the likelihood of such "secondary adaptations" occurring in this group.

Literature Cited

Anderson, G.L. 1978. Responses of dauerlarvae of *Caenorhabditis elegans* (Nematoda: Rhabditidae) to thermal stress and oxygen deprivation. *Canadian Journal of Zoology* **56**: 1786-1791.

Andrassy, I. 1983. A taxonomic review of the suborder Rhabditina (Nematoda: Secernentea).ORSTOM, Paris, France. 241 p.

Baldwin, J.G., Frisse, L.M., Vida, J.T., Eddleman, C.D., and W.K. Thomas. 1997. An evolutionary framework for the study of developmental evolution in a set of nematodes related to *Caenorhabditis elegans*. *Molecular phylogenetics and Evolution* **8**: 249-259.

Bock, W.J. 1959. Preadaptation and multiple evolutionary pathways. *Evolution* **13**: 194-211

Bovien, P. 1937. Some types of association between nematodes and insects. *Dansk Natur-Historisk Forening Copenhagen Videnskabelige Meddelelser* **101**: 1-114.

Blaxter, M.L. 2001. Molecular analysis of nematode evolution. *In* Parasitic Nematodes. M. Kennedy and W. Harnett (eds.). CABI Publishing, Wallingford, UK. 486 p.

Bush, A.O., Fernandez, J.C., Esch, G.W., and J.R. Seed. 2001. Parasitism: The diversity and ecology of animal parasites. Cambridge University Press, New York, NY. 566 p.

Chandler, A.C. 1938. *Diploscapter coronata* as a facultative parasite of man, with a general review of vertebrate parasitism by rhabditid worms. *Parasitology* **30**: 44-52.

Charwat, S.M., Fisher, J.M., and U. Wyss. 2002. The effect of osmotic stress on desiccation survival and water content of four nematode species. *Nematology* **4**: 89-97.

Clark, W.C. 1994. Origins of the parasitic habit in the nematoda. *International Journal for Parasitology* **24**: 1117-1129.

Darwin, C. 1872. The Origin of Species, 6th ed. J. Murray, London, UK. 544 p.

Dolinski, C., Borgonie, G., Schnabel, R., and J.G. Baldwin. 1998. Buccal capsule development as a consideration for Phylogenetic analysis of Rhabditida (Nemata). *Development genes and Evolution* **208**: 495-503.

Fitch, D.H.A. 2000. Evolution of "Rhabditiae" and the male tail. *Journal of Nematology* **32**: 235-244.

Fisher, M.C. and M.E. Viney. 1998. The population genetic structure of the facultatively sexual parasitic nematode *Strongyloides ratti* in wild rats. *Proceedings of the Royal Society of London Series B* **265**: 703-709.

Floyd, T. 1998. *In* S.M. Scheiner and J. Gurevitch (eds). Design and Analysis of Ecological Experiments. Chapman and Hall, New York, NY. 415 p.

Fox, G.A. 1998. *In* S.M. Scheiner and J. Gurevitch (eds). Design and Analysis of Ecological Experiments. Chapman and Hall, New York, NY. 415 p.

French, K., Randall, D. and W. Burggren. 2001. Eckert Animal Physiology. W.H. Freeman and Company, New York, NY. 752 p.

Futuyma, D.J. 1998. Evolutionary Biology. Sinauer Associates, Sunderland, MA. 763pp.

Gould, S.J., and E.S. Vrba. 1982. Exaptation: A missing term in the science of form. *Paleobiology* **8**: 4-15.

Gould, S.J. 2002. The Structure of Evolutionary Theory. Belknap Press, Cambridge, MA. 1433 p.

Kingsolver, J.G. and M.A.R. Koehl. 1985. Aerodynamics, thermoregulation, and the evolution of insect wings: Differential scaling and evolutionary change. *Evolution* **39**: 488-504.

Klekowski, R.Z. and L. Wasilewska. 1982. Oxygen consumption by a typical saprobiotic nematode *Peldera* (*Cruznema*) *monhysteroides*. *Ekologia Polska* **30**: 205-219.

MacInnis, A.J. and M. Voge. 1970 Experiments and Techniques in Parasitiology. W.H. Freeman and Company, San Francisco, CA. 232 p.

Osche, G. 1952. Die Bedeutung der Osmoregulation und des Winkverhaltens für freilebende Nematoden. *Zeitschrift für Morphologie und Oekologie der Tiere* **41**: 54-77.

Osche, G. 1962. Das Praädaptationsphänomen und seine Bedeutung für die Evolution. *Zoologischer Anzeiger* **169**:14-49.

Osche, G. 1965. Parasitismus und Evolution. *Naturwissenschaftliche Rundschau* **18**:189-196.

Reeder, W.G. 1964. *In* J.A. Moore (ed). Physiology of the Amphibia, Academic Press, New York, NY. 654 p.

Read, C.P. 1972. Animal Parasitism. Prentice-Hall, Englewood Cliffs, NJ. 182 p.

Riddle, D.L., Swanson, M.M. and P.S. Albert. 1981. Interacting genes in nematode dauer larva formation. *Nature* **290**: 668-671.

Rühm, W. 1956. Die Nematoden der Ipiden *In* W. Eichler, C.E. Sprehn, and H.J. Stammer (eds). Parasitologische Schriftenreihe, Gustav Fischer Verlag, Jena, Germany. 93 p.

Salih, N.E. and J.N.R. Grainger. 1981. Effect of changing temperatures on the rate of development of the eggs of *Rhabditis longicaudata*. *Journal of Life Sciences* **2**: 181-185.

SAS Institute. 2002. SAS 9.0. Cary, NC.

Urbaniak, G.C. and S. Plous. 1997. Research Randomizer 3.0. http://www.randomizer.org/form.htm

Viney, M.E. 1999. Exploiting the life cycle of *Strongyloides ratti*. *Parasitology Today* **15**: 231-235.

Voelk, J. 1950. Die Nematoden der Regenwürmer und aasbesuchenden Käfer. *Zoologischer Jahrbücher* **79**: 1-70.

Zelmer, D.A. 1998. An evolutionary definition of parasitism. *International Journal for Parasitology* **28**: 531-533.

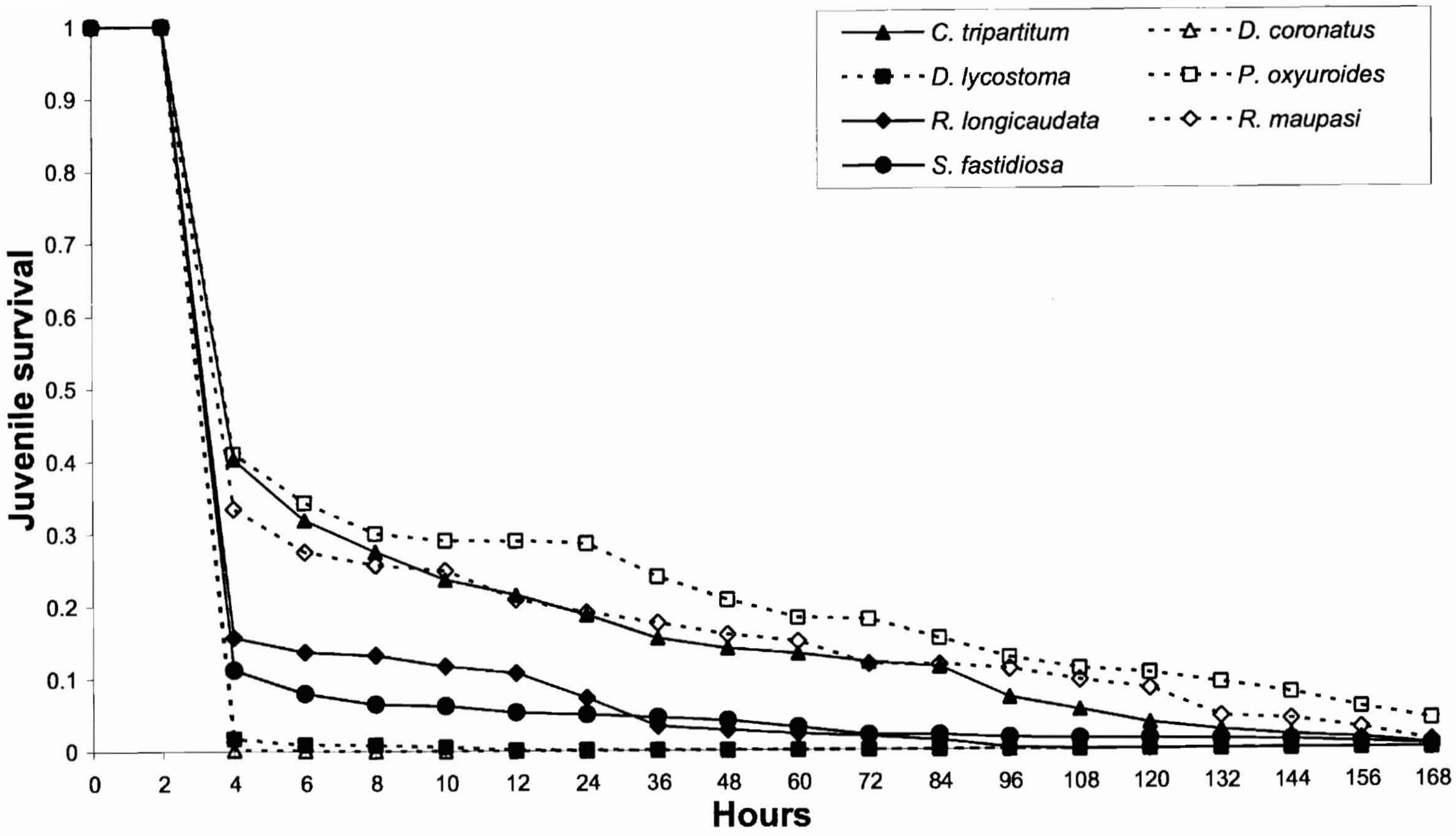
Table 1. Ranked survivorship of all juvenile nematode species in all experimental combinations of pH, temperature, and oxygen levels *in vitro*. One represents the species with highest survivorship in each condition while seven represents the species with lowest survivorship. Tied ranks were averaged.

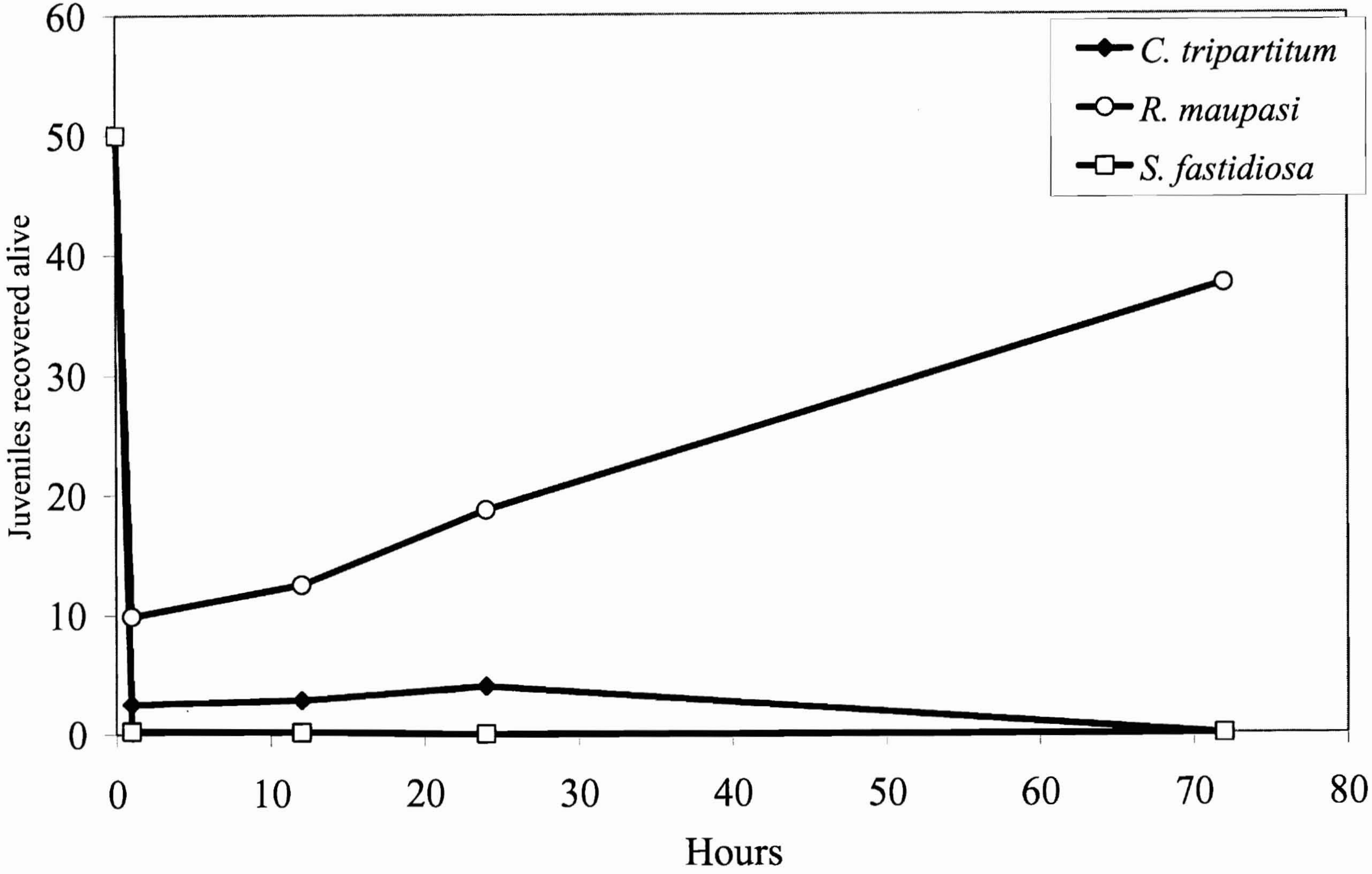
pH	Temperature (C)	O_2	Ranked juvenile survivorship						
			Superficial attachment			Attachment under elytra			Invasive
			C.tripartitum	*R.longicaudata*	*P.oxyuroides*	*D.coronatus*	*D.lycostoma*	*S.fastodiosa*	*R.maupasi*
2	10	Norm	1	5.5	3	5.5	5.5	5.5	2
2	20	Norm	1	5.5	2	5.5	5.5	5.5	3
2	30	Norm	1	6	2	6	6	4	3
2	10	Hypo	1	5	3	7	6	4	2
2	20	Hypo	2	7	1	6	5	4	3
2	30	Hypo	1	5.5	2	5.5	5.5	5.5	3
4	10	Norm	3	4	2	6	5	7	1
4	20	Norm	3	4	1	6	6	6	2
4	30	Norm	1	6	2	6	6	4	3
4	10	Hypo	3	6	2	5	7	4	1
4	20	Hypo	2	6	1	5	7	4	3
4	30	Hypo	1	6	2	6	6	4	3
6	10	Norm	3	5	1	7	6	4	2
6	20	Norm	2	6	1	7	5	4	3
6	30	Norm	1	6.5	2	6.5	4.5	4.5	3
6	10	Hypo	3	5	1	6.5	6.5	4	2
6	20	Hypo	2	5	1	6.5	6.5	4	3
6	30	Hypo	1	6	2	6	6	3.5	3.5

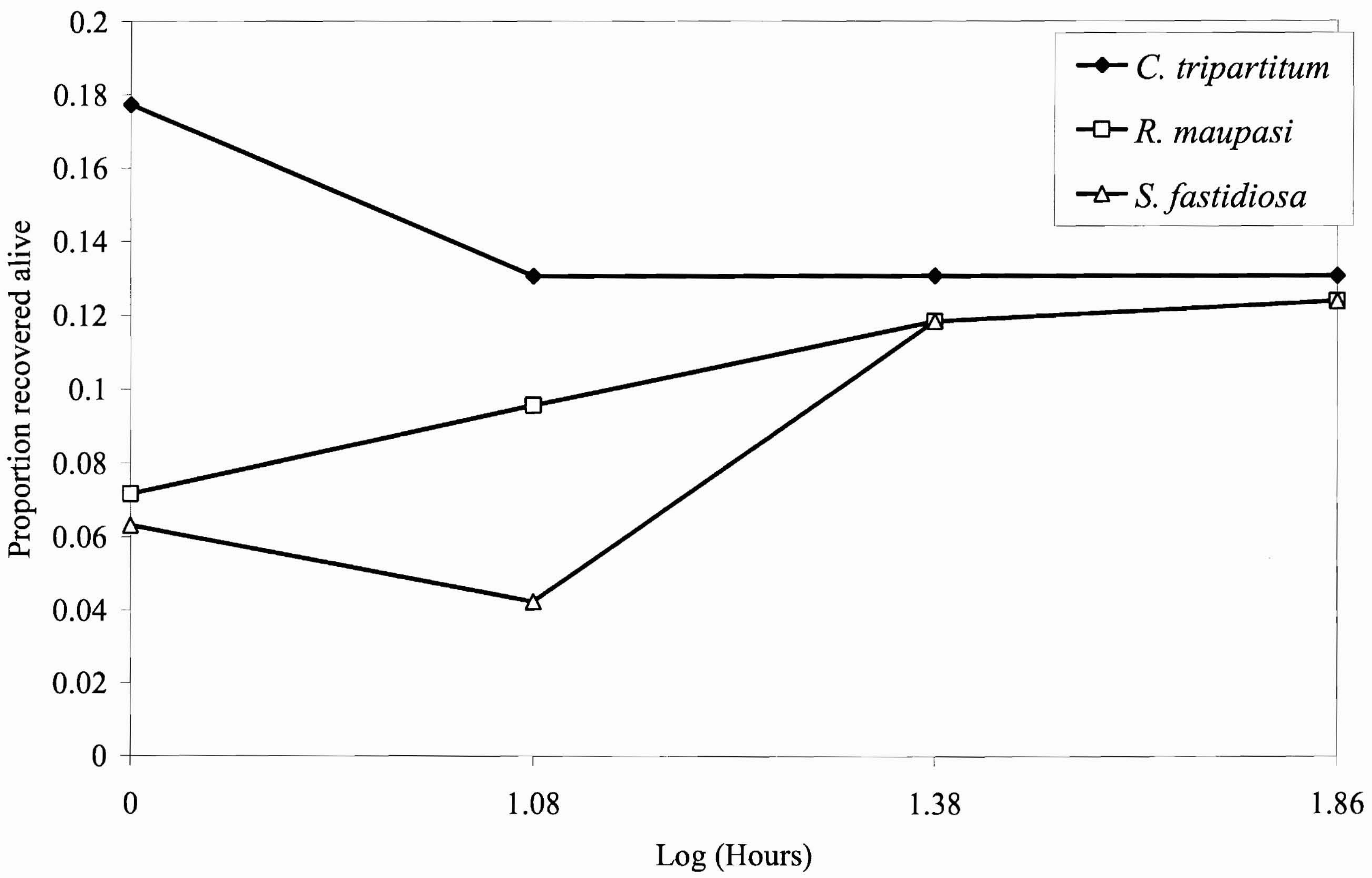
Fig. 1. Overall *in vitro* survivorship of J_3 rhabditids as determined by the accelerated time failure model under varying conditions of pH, temperature, and oxygen level.

Fig. 2. Survivorship of juvenile nematode species (*C. tripartitum*, *R. maupasi*, and *S. fastidiosa*) representing each phoretic group when fed to *R. pipiens* via feeding needle and analyzed via logistic regression. *Rhabditis maupasi* (invasive) differs significantly ($p<0.05$) from *C. tripartitum* (non-invasive) and *S. fastidiosa* (mid-invasive), which did not differ significantly ($p>0.05$) from each other.

Fig. 3. Juvenile nematode survival of selected species representing each phoretic group when fed to *R. pipiens* by invertebrate phoresy on *T. molitor* (*C. tripartitum*, *S. fastidosa*) or in *L. terrestris* (*Rhabditis maupasi*) and analyzed via logistic regression. *Cruznema tripartitum* (non-invasive) differs significantly ($p<0.05$) from *R. maupasi* (invasive) and *S. fastidiosa* (mid-invasive), which did not differ significantly ($p>0.05$) from each other.


C. tripartitum
D. lycostoma
R. longicaudata
S. fastidiosa
D. coronatus
P. oxyuroides
R. maupasi
Juvenile survival
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Hours
0
2
4
6
8
10
12
24
36
48
60
72
84
96
108
120
132
144
156
168


60
50
40
30
20
10
0
Juveniles recovered alive
0
10
20
30
40
50
60
70
80
Hours
C. tripartitum
R. maupasi
S. fastidiosa


0.2
0.18
0.16
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
Proportion recovered alive
0
1.08
1.38
1.86
Log (Hours)
C. tripartitum
R. maupasi
S. fastidiosa

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Elizabeth Warburton
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July 22, 2005
Date

Preadaptation toward Parasitism in Order Rhabditida (Nematoda)
Title of Thesis

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