Nematology, 2011, Vol. 13(8), 985-993

The effect of temperature on hatch and activity of second-stage juveniles of the root-knot nematode, *Meloidogyne minor*, an emerging pest in north-west Europe

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Received: 31 January 2011; revised: 29 March 2011
Accepted for publication: 2 April 2011; available online: 3 June 2011

Summary – *Meloidogyne minor* is a serious threat to turfgrass in north-west Europe, and has a broad host range that includes other economically important plants. The species was described only recently and little is known about its biology. This study examines the effect of temperature on hatch and motility of second-stage juveniles (J2), and records hatching from egg masses collected from golf greens in different seasons. Eggs were present throughout the year and a high percentage hatch (46-88%) was recorded when they were incubated at 20°C. When egg masses were incubated at constant temperatures, J2 hatched between 15 and 25°C, with limited hatch (<1%) at 10 and 30°C. The percentage hatch was lower at 15°C (43%) than at 20-25°C (63-76%). J2 hatched fastest at 23°C, with an average duration to hatching of 7 days compared to 17 days at 15°C. The range of temperatures at which J2 was active was broader than that at which they hatched. J2 were active from 4-30°C, with greatest activity between 15 and 25°C. The addition of grass root extract temporarily increased J2 activity at 10-20°C, but not at lower temperatures.

Keywords – *Agrostis stolonifera*, creeping bentgrass, diapause, egg mass, golf green, stimulation, turfgrass.

Since 1997, golf greens in the United Kingdom and Ireland have displayed unusual yellow patches caused by a previously unidentified root-knot nematode (Entwistle, 2003). In 2004, the nematode was described as a new species, *Meloidogyne minor* (Karssen *et al*., 2004). This species favours sandy soils such as those at golf greens constructed according to United States Golf Association (USGA) guidelines. *Meloidogyne minor* has also been recorded in coastal dunes, pastures and sports grounds (Karssen *et al.*, 2004; Turner & Fleming, 2005; Lammers *et al.*, 2006). In 2000, an outbreak of *M. minor* was recorded on potatoes in The Netherlands; the species has a wide host range that includes tomato and barley (Lammers *et al*., 2006). Prediction of the risk posed by the species, currently or in the context of future climate change, is hampered by the scarcity of fundamental knowledge of its biology, including its response to ambient temperatures.

Temperature influences all aspects of nematode life cycles and behaviour, including hatching, motility, invasion and development (Wallace, 1963; Davide & Triantaphyllou, 1968; Bird, 1972; Evans & Perry, 2009). There have been many studies showing the effects of temperature on *Meloidogyne* embryogenesis and hatch (Wallace, 1971; Ogunfowora & Evans, 1977; Vrain & Barker, 1978; Goodell & Ferris, 1989; Ploeg & Maris, 1999; Tzortzakakis & Trudgill, 2005). *Meloidogyne* species vary in the temperature range over which hatching occurs; the optimal temperature for hatching is generally indicative of the geographic region or seasonal preference of their plant hosts (Lee & Atkinson, 1976). Eggs produced in autumn may play an important role in the winter survival of *Meloidogyne* species (Jeger *et al.*, 1993; Starr, 1993). In some species, such as *M. naasi* (Franklin, 1965), there is an obligate diapause requiring a period of chilling before second-stage juveniles (J2) will hatch (Ogunofowura & Evans, 1977). Motility of the J2 is important both for

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DOI:10.1163/138855411X571902

hatching and for penetration into the host and is affected by temperature (Prot & Van Gundy, 1981; Roberts, 1987; Jeffers & Roberts, 1993; Ploeg & Maris, 1999).

In this study, we investigate the effects of constant temperatures on hatching and J2 motility of *M. minor*. We also assess the numbers and hatch rate of *M. minor* in egg masses collected throughout the year from a golf green sown with creeping bentgrass (*Agrostis stolonifera* var. *stolonifera* L.).

Materials and methods

Hatching of J2 from egg masses collected at different times of the year

Soil was collected using a soil corer (1.9 cm diam.) to a depth of 10 cm on eight occasions over a 3-year period (Table 1). The soil was from infected patches on golf nursery greens in County Kildare, east Ireland, constructed to USGA guidelines and sown with *A. stolonifera*. Prior examination of infected greens, with nematode identification based on morphology, indicated that the dominant root-knot nematode present was *M. minor*, with *M. naasi* as an occasional minor component. Routine inspection of J2 in our trials did not reveal any *M. naasi*. Roots were gently rinsed with tap water to remove adhering soil. Egg masses with up to 2-3 mm of root on either side were placed in small hatching chambers and incubated at 20°C. The hatching chambers, based on Southey (1986), consisted of plastic cylinders 15 mm high × 10 mm diam. with 20 μm (pore size) mesh at the bottom, through which *M. minor* J2 could freely migrate. Each cylinder was suspended in a well of a 24-well flat-bottom tissue culture plate containing 500 μl tap water so that the mesh was wet but the knots/egg masses were not submerged. The water containing the migrated J2 was collected and replaced every 2-3 days and the number of J2 was recorded. When hatching had ceased (*ca* 60 days) the egg masses were dissected and the remaining eggs were examined and counted with the aid of a dissecting microscope (×40 magnification). There were at least three replicate hatching chambers for each collection date, with four egg masses per chamber (2-7 in October 2003 and 1-5 in December 2003).

The effect of temperature on hatching

Egg masses were incubated at each of ten temperatures: 4, 6, 8, 10, 15, 20, 23, 25, 30 and 36°C for up to 63 days in small hatching chambers as described above. Two experiments were conducted, using egg masses collected from *A. stolonifera* on 25 February and 24 March 2004, respectively. There were three replicate hatching chambers per temperature treatment in the first experiment and six in the second. Each hatching chamber contained four egg masses. There were fewer eggs/egg mass in the first (mean 34.4 ± 4.75) than in the second experiment (mean 64.2 ± 6.21) ($F_{1,88} = 17.96$, $P < 0.001$), but there was no difference in numbers of eggs assigned to the various temperature treatments within an experiment (February ($F_{9,20} = 0.62$, $P = 0.763$), March ($F_{9,50} = 1.09$, $P = 0.384$)).

Table 1. *Percentage hatch, number of hatched J2 per egg mass and total number of eggs per egg mass for egg masses of* Meloidogyne minor *collected at different times of the year and incubated at 20°C.*

Date	Number hatched J2 per egg mass (mean ± SE)	Total number of eggs per egg mass[1] (mean ± SE)	Percentage hatch (mean ± SE)
Oct 2003	58.1 ± 9.37 A	117.1 ± 8.08 AB	49.0 ± 4.99 AB
Dec 2003	40.0 ± 10.37 A	59.1 ± 15.67 BC	69.9 ± 7.39 AB
Feb 2004	45.0 ± 21.36 A	56.3 ± 24.55 BC	73.6 ± 8.05 AB
Mar 2004	18.2 ± 4.90 A	21.8 ± 4.80 C	76.5 ± 9.6 AB
Aug 2005	29.9 ± 10.39 A	40.4 ± 14.75 BC	72.4 ± 9.26 AB
Sept 2005	122.7 ± 10.06 B	139.4 ± 11.7 A	87.9 ± 1.08 A
Apr 2006	22.0 ± 1.55 A	30.4 ± 2.24 C	73.6 ± 3.22 AB
May 2006	33.1 ± 1.69 A	73.6 ± 13.02 BC	46.7 ± 5.49 B
	$F_{7,52} = 12.94$, $P < 0.001$	$F_{7,52} = 9.23$, $P < 0.001$	$F_{7,52} = 2.54$, $P = 0.025$

Numbers within a column accompanied by the same letter are not significantly different, Tukey's test, $\alpha = 0.05$.
[1] Total eggs = number hatched J2 + eggs remaining when hatching ceased.

The effect of temperature and stimulation on J2 motility

The J2 used in this experiment were extracted from egg masses (from *A. stolonifera* collected in September 2006) using evaporation dishes as collection trays (Southey, 1986) and were $\leqslant$1-day-old (*i.e.*, they had been in the collecting tray for less than a day). The motility of J2 was recorded at a range of temperatures (4-30°C) in wells (0.7 cm diam. $\times$ 1 cm deep) of a 1 $\times$ 12-well Microstrip (Flow Laboratories, Helsinki, Finland). Each well contained 50 μl of sterile tap water. The J2 were transferred from the evaporating dish to a well using a metal wire pick. Each well contained one J2, and only one J2 was transferred and observed at a time. The Microstrip was placed in a shallow water bath with a glass base to facilitate viewing. The temperature of the water bath was adjusted by circulating coolant through a loop of copper tubing from a recirculating water bath (Grant Instruments, Cambridge, UK). The temperature of the water in a well adjacent to the experimental well was monitored throughout the experiment. The J2 were observed using a dissecting microscope at $\times$40 magnification. The microscope was fitted with a cold (fibre optic) light source. The J2 were allowed 3 min to adapt before their movement was recorded. The rate of J2 movement was measured by assessing head movement *i.e.*, starting with the nematode's head in line with the rest of the body, a head movement occurred when the head moved $>$90° angle to the body in any direction. For example, from the centre to the left is one head movement, and left to centre is one head movement.

There were two experiments. In the first, J2 movement was recorded at 4, 6, 8, 10, 12, 15, 20, 23, 25 and 30°C. There were ten J2 per temperature treatment and each J2 was used once only. Each nematode was observed for 5 min. A second experiment was done to see if J2 that were inactive at lower temperatures were capable of activity when stimulated. Grass root extract, made from fresh creeping bentgrass roots, was used to stimulate activity. The roots (5 g) were ground in 2 ml tap water with a mortar and pestle. A pinch of sand was added during grinding to aid the breakdown of the cell walls. The sand and large debris particles were allowed to settle before the supernatant was used. Six temperatures (4, 6, 10, 12, 15, 20°C) were used in the experiment with 15 J2 per temperature. After a 3 min acclimation period each nematode's movement was recorded for 2.5 min at the target temperature. Grass root extract (10 μl) was slowly added to the well. The behaviour was recorded immediately for another 2.5 min. This was followed by an immediate third recording of 2.5 min.

Data analysis

Minitab 14.0 was used for all statistical analysis. Data were checked for normality using the Anderson Darling test. Treatments were compared using General Linear Model ANOVA. Two similar experiments were carried out for the effect of temperature on hatching. A General linear model ANOVA showed that temperature had a highly significant effect on the percentage hatch but experiment did not, and there was no interaction between the two factors. The same was found for the mean time to hatch. Therefore, the data for the two experiments were pooled for subsequent analysis. Where differences were detected, means were separated using Tukey's test at $\alpha = 0.05$.

Results

Hatching of J2 from egg masses collected at different times of the year

Eggs collected from the field at all times of the year hatched at 20°C. The number of eggs and percentage hatch varied between sampling dates (Table 1). The percentage hatch ranged from 47% (May 2006) to 88% (September 2005), with a significant difference between the highest and lowest values. In October 2003 and May 2006 the percentage hatch was less than 50% but at all other times it was 70% or higher.

The average number of eggs/egg mass varied from 22 to 139 (Table 1). The highest number, in September 2005, was significantly different from that of every other month except October 2003. The number of J2 that hatched at 20°C also varied between collection dates. There were significantly more J2 in September 2005 than in any other month (Table 1). In order to investigate seasonal trends, the data for total numbers of eggs and numbers of hatched J2 were plotted against month (assuming that seasonal factors determine reproductive output), irrespective of which year they were sampled (Fig. 1). This showed evidence of two peaks for total eggs, one in May and a larger one in September when the number of hatched J2 also peaked. Pearson Correlation failed to detect any significant relationship between percentage hatch and number of eggs/egg mass ($P = 0.649, R = 0.06$). While the viability of unhatched eggs was not rigorously

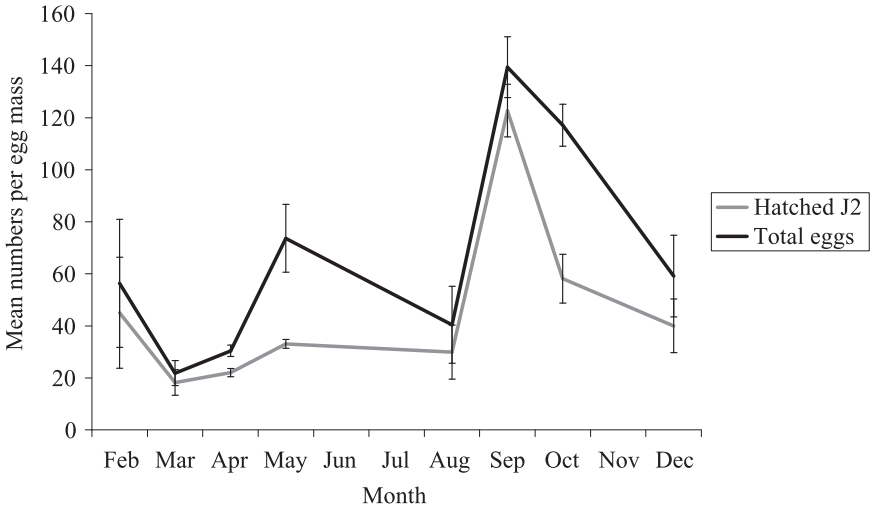

Fig. 1. *The total number (mean ± SE) of* Meloidogyne minor *eggs per egg mass and number (mean ± SE) of hatched second juveniles (J2) per egg mass (hatched at 20°C). Total eggs = number of J2 + eggs remaining once hatching ceased (empty eggshells were not counted). Months are displayed in order as in calendar year, not in order of collection.*

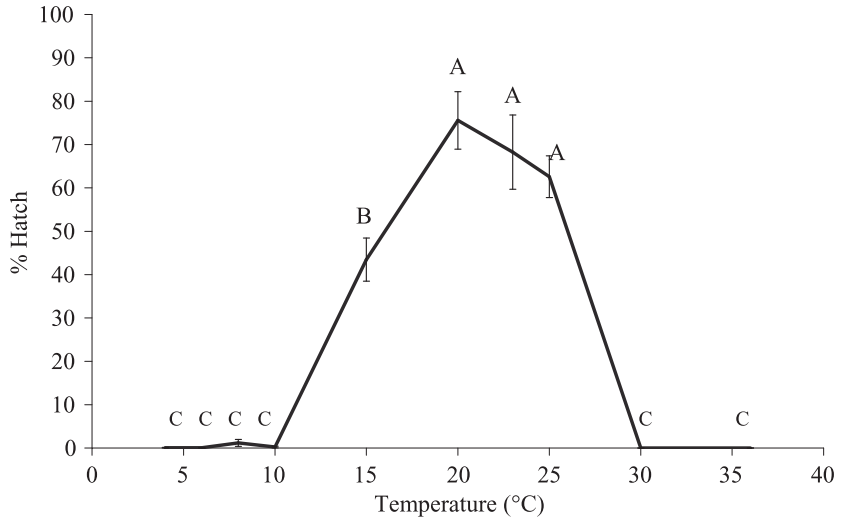

Fig. 2. *Percentage hatch (mean ± SE) of* Meloidogyne minor *after incubation for up to 63 days at various constant temperatures (N = 9). Points accompanied by the same letter are not significantly different* ($F_{9,71} = 54.37$, $P < 0.001$; *Tukey's test,* $\alpha = 0.05$).

assessed, most of them appeared to be degenerating when examined microscopically.

The effect of temperature on hatching

The highest percentage hatch (76%) was at 20°C, but this did not differ significantly from that at 23 or 25°C. At 15°C, fewer than 50% of J2 hatched from eggs (Fig. 2). Very few (<1%) J2 hatched ⩽10°C or ⩾30°C. Due to the low numbers it was not feasible to determine hatch time for these temperatures. The shortest mean time to hatch (approximately 7 days) was at 23°C. This was different from 20°C but not 25°C. Hatching took more than twice as long at 15°C as at 23°C (Fig. 3).

Effect of temperature and stimulation on J2 motility

J2 were active in water at all temperatures tested between 4 and 30°C (Fig. 4). Temperature affected motil-

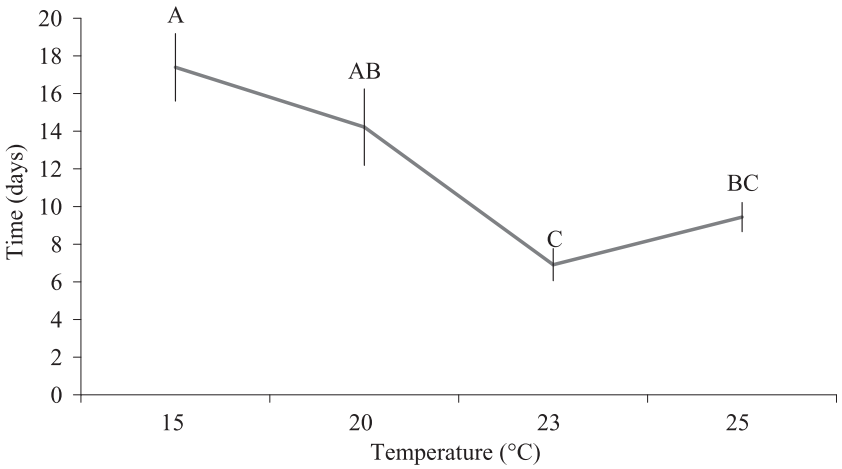

Fig. 3. *Time to hatch (mean ± SE) of* Meloidogyne minor *incubated at various constant temperatures (N = 9). Points accompanied by the same letter are not significantly different (*$F_{3,52} = 10.23$, $P < 0.001$; *Tukey's test,* $\alpha = 0.05$*).*

ity ($F_{9,90} = 34.16$, $P < 0.001$). All J2 were active at 10-30°C, but the highest rate of activity (head movements 5 min^{-1}) occurred between 15 and 25°C. Activity levels at these temperatures were nearly identical (approximately 57 head movements 5 min^{-1}) but decreased again at 30°C (Fig. 4). Below 10°C, not all J2 were active and their movement was slow. In a second experiment, grass root extract was used to stimulate J2 activity at various temperatures (4-20°C) with particular emphasis on the lower temperatures where J2 were relatively inactive in the first experiment. The J2 activity was recorded in three adjacent periods: immediately before and immediately after addition of the stimulus, and a third adjacent time slot immediately afterwards. A two-way ANOVA revealed that root extract ($F_{2,252} = 41.4$, $P < 0.001$), temperature ($F_{5,252} = 531.63$, $P < 0.001$) and their interaction ($F_{2,10} = 12.15$, $P < 0.001$), had a significant effect on J2 movement. As in the previous experiment, there was very little activity at 4, 6 and 8°C. At these temperatures the root extract did not have an effect on activity (Fig. 5). The addition of the grass root extract did have an effect on the J2 at the higher temperatures (10-20°C). There was an immediate increase in activity after the addition of the extract. This effect did not persist for the next 2.5-min observation period, except at 10°C.

Discussion

Eggs were found in egg masses throughout the year, and a high percentage hatch was recorded when they were incubated at 20°C. This indicates that if there is diapause then it is not present in a high proportion of the population, unlike *M. naasi*. Ogunfowora and Evans (1977) incubated field-collected *M. naasi* eggs at 20°C and found that there was a higher percentage hatch from eggs collected in November than in August. This can be explained as the requirement of a large proportion of *M. naasi* eggs within a population for a period of chilling before hatch, a form of diapause (Evans & Perry, 2009). In our study, the low hatch (49%) in October might be interpreted as eggs being in diapause but not having experienced a long enough cold period. However, similar low hatch (47%) was also seen in May, so other factors must be involved, such as age of females, or condition of soil and/or plant (Huang & Pereira, 1994; Gaur *et al*., 2000; Wesemael *et al*., 2006). The *M. minor* eggs remaining once hatch had ceased appeared to be degenerating, but we cannot be certain that there were no viable eggs amongst them. It is possible that addition of root diffusate may have increased hatch rates; while most species of *Meloidogyne* hatch in water, hatch rate of some species may be enhanced by host root extract, especially at certain times of the year (Wesemael *et al*., 2006).

This study shows that the minimum temperature for *M. minor* hatch is between 10 and 15°C, and that hatch occurred from 15 to 25°C. Although suboptimal, 15°C was still quite suitable for hatching of *M. minor* J2: the percentage hatch was approximately 45% and the mean time to hatch did not differ from that at 20°C. The shape of the curve (Fig. 3) suggests that 20°C is the optimum temperature for percentage hatch, though

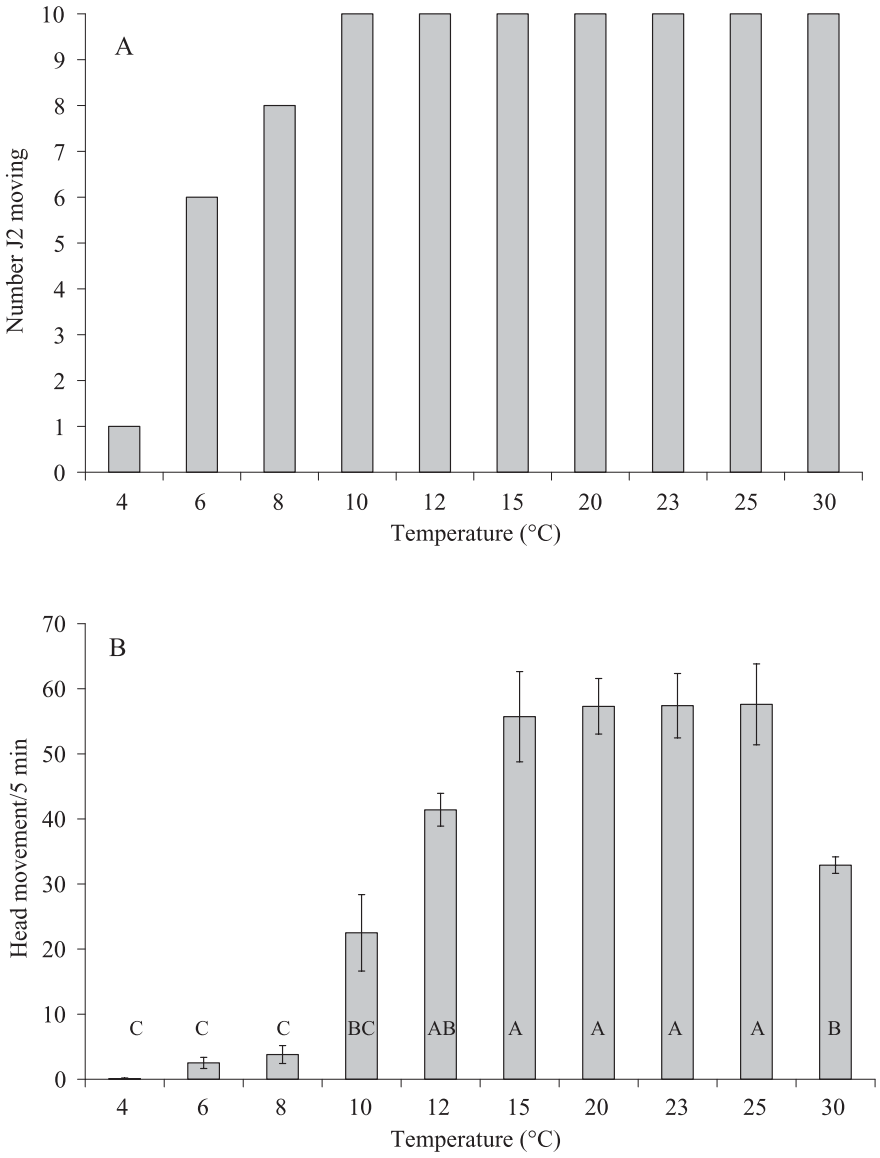

Fig. 4. *Movement of second-stage juveniles (J2) of* Meloidogyne minor *at various constant temperatures (N = 10). A: Number of J2 moving. B: Number (mean ± SE) of head movements 5 min*$^{-1}$*. Bars accompanied by the same letter are not significantly different (*$F_{9,90} = 34.16$*,* $P < 0.001$*; Tukey's test* $\alpha = 0.05$*).*

not significantly different from 23 and 25°C. The shortest mean time to hatch (*ca* 7 days) occurred at 23°C, which was significantly shorter than at 20°C. So, combining the percentage hatch and time to hatch, it would appear that the optimum temperature for hatch is close to 23°C, or more broadly between 20 and 25°C. This is typical of cold-adapted species (including *M. naasi* and *M. hapla*) where the optimum temperature is between 15 and 25°C, whereas 25-30°C appears optimal for warm climate species such as *M. javanica* (Bird & Wallace, 1965; Wallace, 1971; Bird, 1972; Ogunfowora & Evans, 1977). Nevertheless, the effective range for *M. minor* hatch in this study (15-25°C) appears skewed towards lower temperatures compared with a Welsh population of *M. naasi,* where there was negligible hatch at 15°C but up to 10% hatch at 30°C (Ogunfowora & Evans, 1977).

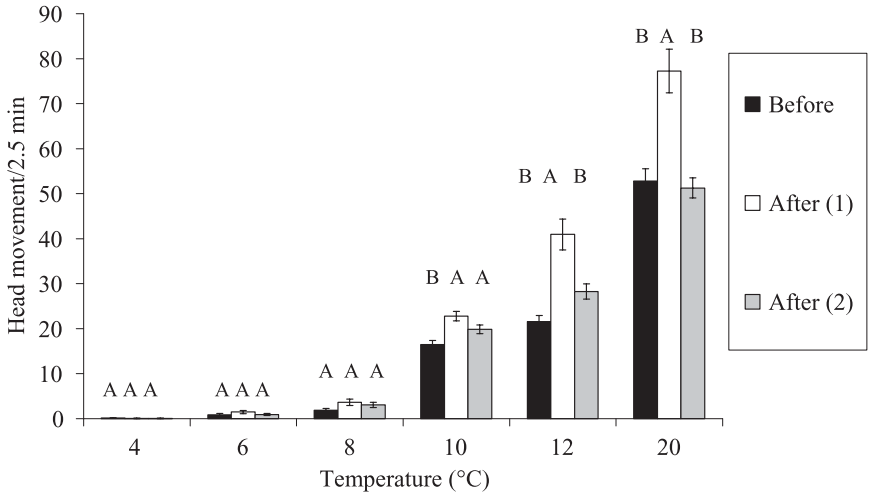

Fig. 5. *Movement of second-stage juveniles (J2) of* Meloidogyne minor *(mean ± SE head movements 2.5 min^{-1}) at various constant temperatures before and after application of grass root extract. After (1) = from time of application of extract; After (2) = from 2.5 min after application of extract (N = 15). Within a temperature, bars accompanied by the same letter are not significantly different (Tukey's test, α = 0.05).*

We used egg masses collected in February and March as it is from these that the first J2 of the season will emerge (Morris, 2008), and soil temperatures are most likely to be limiting. It is possible that egg masses produced at different times of the year might respond differently to temperature (*e.g.*, Ogunfowora & Evans, 1977) but this was not examined here.

Meloidogyne J2 are sensitive to temperature as indicated through monitoring of J2 mobility (Robinson, 1994). This can also influence infectivity (Bergerson, 1959; Van Gundy *et al.*, 1967; Roberts *et al.*, 1981). Both nematode movement through soil and penetration into the plant require that the environmental temperature be above a certain threshold, otherwise nematode muscular activity is greatly reduced (Prot & Van Gundy, 1981; Roberts *et al.*, 1981). The present study indicates that *M. minor* J2 are capable of movement at all temperatures from 4 to 30°C, but at varying rates. From 10 to 30°C all J2 moved (100%) but they were most active between 15 and 25°C. The temperature at which movement is inhibited varies among species and between strains from different geographical regions; for example, *M. incognita* movement was inhibited at 18°C (Prot & Van Gundy, 1981), whilst *M. hapla* can penetrate alfalfa roots at 10°C (Griffin, 1969). In our study, all *M. minor* J2 were active at 10°C, but moved at only half the rate seen in the optimal temperature range. Below 10°C, the rate of activity was very low. This could be either because the J2 were unable to move (*e.g.*, the temperature was too low for neural or muscular activity) or because they responded to low temperature as a cue to become inactive in the absence of other stimuli for activity. If they were capable of activity, then we suggest that they should be activated by cues associated with their host plant (Perry, 1997). At 8°C and below, the addition of grass root extract had no significant effect on J2 activity confirming that the ability to move rather than the 'motivation' to move is severely reduced at these temperatures. At 10, 12 and 20°C there was an increase in activity after the addition of the grass root extract. However, the effect of the stimulus was short lived. A rapid return to baseline activity following a period of increased activity provoked by stimulation is typical of the infective juveniles of parasitic nematodes (Croll, 1972) and presumably conserves energy.

In general, the optimum temperature for a root-knot nematode corresponds to that of the host plant (Luc *et al.*, 2005). *Agrostis stolonifera* is a cool season turfgrass species that grows well in cool humid regions (Warnke, 2003) including Britain and Ireland (Sell & Murrell, 1996) and is one of *M. minor*'s natural hosts (Lammers *et al.*, 2006). Most cool-season turfgrasses have optimal root growth at soil temperatures between 10 and 18°C (Landschoot, 2007). Pote *et al.* (2006) found root zone temperatures of 23°C or above were detrimental to root activities of creeping bentgrass. In the present study, the optimum temperature for *M. minor* hatch was 20-25°C,

and for J2 activity was 15-25°C, somewhat higher than the preferred temperature for *A. stolonifera* root growth.

We show here that the minimum temperature for hatch of *M. minor* is between 10 and 15°C, and this accords with field collections from turfgrass where J2 were absent in winter, and first recovered once soil temperature exceeded 10°C in March (Morris, 2008). As temperatures in the current geographic range of *M. minor* are expected to increase by 1.4-1.8°C by the 2050s (Sweeney *et al*., 2008), we may expect an extended season for hatch of the species. It is unclear to what extent different *Meloidogyne* activities such as hatching, invasion, growth and embryogenesis have different thermal requirements. Bird and Wallace (1965) found that the optimal temperatures for growth and development of *Meloidogyne* spp., both in the egg and in the plant, were higher than the thermal optima for stages in the soil (*i.e*., J2 motility), but similar to each other, while Trudgill (1995) showed that embryogenesis and the whole life cycle of *M. javanica* had similar base temperatures. Assuming similar thermal responses of all life cycle activities, soil temperatures up to at least 25°C are likely to favour the species and exacerbate its pest status.

Acknowledgements

The work was funded by a Teagasc Walsh Fellowship. The authors thank Dr G. Karssen (Plant Protection Service, Wageningen, The Netherlands) for confirming the identification of *M. minor*.

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