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Ditylenchus dipsaci Infestation of *Trifolium repens*. I. Temperature Effects, Seedling Invasion, and a Field Survey[1]

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Abstract: Rates of development of stem nematode (*Ditylenchus dipsaci*) in white clover (*Trifolium repens*) seedlings were found to be linearly related to temperature. Basal developmental temperature (T_b) was 3 °C, and the thermal constant (S) for development of gravid adult females from freshly laid eggs was 270 accumulated day-degrees above the T_b. Only 12% at 20 °C and 4% at 4 °C of the gravid female nematodes inoculated into seedling axils successfully penetrated seedling epidermis. These nematodes slowly migrated within the seedling and after a lag of 5 days at 20 °C started to lay eggs. The maximal rate of egg production was temperature-dependent, being 0.8 and 3.1 eggs female^{-1} day^{-1} at 10 and 20 °C, respectively. Nematodes emigrated rapidly from infested stolons when they were immersed in water, with rates being highest at 25 °C and lowest at 4 °C. The sensitivity to temperature of many of the parameters that govern nematode population dynamics indicates that climatic changes will have a marked effect upon this host-parasite system. A study of infested stolons from the field indicated that nematode numbers increased up to 3,000 or more before tissue senesence, triggered by nematode damage, caused a mass emigration of nematodes from the stolon.

Key words: Ditylenchus dipsaci, development, life cycle, nematode, population dynamics, rates of development, temperature effects, thermal time, *Trifolium repens.*

In England and Wales approximately 40% of white clover (*Trifolium repens* L.)-perennial rye grass (*Lolium perenne* L.) swards are infested with stem nematode (*Ditylenchus dipsaci* (Kühn) Filipjev) (Cook et al., 1992b). The nematode is endoparasitic and feeds on the contents of host plant cells (Hooper, 1972). Its first-stage juveniles (J1) molt in the eggs and emerge as J2 (Griffith et al., 1996). Mating between males and females is essential for reproduction (Hooper, 1972; Yuksel, 1960). The pest can have a marked adverse effect on the establishment of white clover seedlings (Cook et al., 1992a; Williams and Barclay, 1972) and cause serious damage to established swards (Cook et al., 1992b). Symptoms of stem nematode attack include hypertrophy, stunting of infected leaves and petioles, and shortening of internodes (Cook and Yeates, 1993). Damage to infested plants is particularly severe in spring after mild winters. It has been hypothesized that this is a consequence of stem nematode activity continuing when white clover is inactive due to low temperatures and light intensity (Cook et al., 1992a; Sackville-Hamilton, 1990). Because of this, it is possible that predicted increases in mean annual temperature due to global warming may have a noticeable impact on the incidence and severity of this disease. The primary aim of this research was to elucidate and quantify the effects of temperature on population dynamics of *D. dipsaci* in white clover seedlings. This information was required for the construction of a predictive simulation model of the host-nematode system. In addition, an assessment was made of the structure of nematode populations in infested white clover stems (stolons) collected from the field. Under field conditions, stem nematodes (especially J4) are known to migrate out of infested stems during rainy periods (Wallace, 1962). This migratory behavior will affect the population structure, and hence dynamics, within infested tissues. We therefore also undertook experiments to quantify rates of migration of nematodes from infested stolons.

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Materials and Methods

Establishment of sterile white clover seedlings: White clover cv. S184 seeds were chemically

scarified and surface-sterilized by submersing them for 15 minutes in concentrated H_2SO_4 followed by rinsing in several changes of sterile distilled water. They were then immersed for 15 minutes in a solution of $HgCl_2$ (1,000 ppm in 30% ethanol), washed again in several changes of sterile distilled water, and left to soak in distilled water for 3 hours to remove leached tannins. The water was drained away, and seeds were transferred aseptically to the surface of B50 agar (3.875 g/liter Gamborg's B5 salts and organics, 20 g/liter sucrose, and 8 g/liter agar; pH 6) in 9-cm petri dishes and left for 4 days to germinate.

Germinated seeds were aseptically transferred into 25-ml Sterilin tubes (4 seeds per tube) containing 10 ml of B50 agar. The seedlings were transferred to a growth room (day-night temperature cycle of 18 to 15 °C; photoperiod 12 hours) for 25 days until the first trifoliate lamina was present.

Preparation of inoculum for seedling experiments: White clover plants infested with stem nematode were collected from a white clover sward in Cae Glanrafon, IGER, Aberystwyth, U.K. (O.S. national grid reference SN 624838) and cut up finely. Nematodes were extracted from the tissue on sieves under automatic intermittent mist spray (Hooper, 1986). Accumulated adult and J4 *D. dipsaci* were washed in several changes of sterile tap water to remove traces of plant phenolics, and then resuspended in 10 ml of sterile tap water. Males and gravid females were transferred to 10 ml of aqueous (0.5% v/v) 20% hibitane acetate solution of chlorhexidine for a minimum of 3 hours, after which they were stored in the dark at 2 °C.

Seedling invasion: In the first experiment, 180 seedlings were inoculated with freshly prepared 7-µl droplets of sterilizing solution containing three gravid females and three males. These were aseptically placed at a petiole axil in the vicinity of each seedling's terminal meristem. Seedlings were then incubated at 16 °C and sampled at 8-hour intervals for up to 48 hours after inoculation, 12 seedlings per sample, to assess seedling invasion.

At each sampling, seedlings and nematodes were fixed by flooding the Sterilin tubes with 30% ethanol. Each seedling was removed from its tube by severing the stem at the stem root junction. It was then washed gently in distilled water and nematodes stained with acid fuchsin following clearing in NaOCl solution (17% chlorine) for 4 minutes following the technique of Byrd et al. (1983). Seedling washings were collected and free *D. dipsaci* counted. Stained seedlings were dissected to reveal the location of nematodes.

Temperature and population development: In a second experiment 600 seedlings were inoculated in the same way except that the inoculum droplet contained three gravid females and no males. Inoculated seedlings were then transferred to a range of constantly illuminated controlled-growth environments at 4, 8, 12, 16, and 20 °C. Temperature was monitored constantly throughout the experiment in which population development at the different temperatures was observed. Seedlings were sampled at various time intervals from each of the growth environments, eight per sample. Sampled seedlings were fixed, stained, and dissected to reveal the position and length of all nematodes. The body length, anterior to posterior tip of outer cuticle, of molting nematodes was measured. From these data the mean lengths of nematodes at molts was calculated and used to determine stage of development. The number of hours that elapsed before the first appearance of *D. dipsaci* eggs, eggs containing J2, J2, J3, J4, and males and females was determined in replicate seedlings.

Field survey: Eleven *T. repens* plants showing symptoms of *D. dipsaci* infestation were collected from the Cae Glanrafon site in June and July 1993. The form of each plant was recorded on diagrams and the stoloniferous stems cut into 5-mm-long pieces. Each piece was split longitudinally, immersed in 0.15 ml of tap water, and left in the dark at 4 °C for 17 hours to extract *D. dipsaci.* After extraction the number and, in some samples, the length and stage of development of *D. dipsaci* that had migrated from each of the stolon pieces was assessed. All

lengths were then extracted twice more in the same way.

Migration study: Infested white clover stolons (2 m cut from 25 plants) were collected from a sward at Trawscoed, IGER, Aberystwyth, U.K. (O.S. national grid reference SN 673736) in spring 1994. Stolons were washed in tap water and 150 randomly selected, 1-cm-long pieces were cut from them. Cut ends of each section were sealed with molten histological wax (56 °C), the ends being plunged into cold water immediately after sealing to minimize temperature damage. Sections were then equilibrated for 1 hour at one of five temperatures (4, 12, 18, 25, and 30 °C), 25 sections per temperature. After equilibration, a single longitudinal incision was made along the length of each section and the section was immersed in 1 ml of tap water (also pre-equilibrated at the appropriate temperature) for 45 minutes. After this period, nematodes that had migrated out of the section were counted. Nematodes remaining within sections were extracted on sieves in the mist chamber. Rates of migration (proportion of the original total population, migrated and extracted nematodes, per time interval) at the different temperatures were then calculated.

Results

Seedling invasion: The original inoculum droplet progressively decreased in size for up to 48 hours. By 16 hours a small proportion of the nematodes had penetrated the epidermis of seedlings (Table 1) and was embedded in tissues of the stipules, petioles, and lamina but not in stems. The proportion of seedlings with subepidermal infestations did not increase thereafter. Most nematodes failed to penetrate seedlings and were usually washed off the surface of the seedlings during preparations for staining. No eggs were recovered from seedling washes. However, a small proportion of the nematodes was sometimes retained, usually in sheltered locations such as axils surrounded by stipules where some had laid eggs. A maximum number of six eggs per subepidermally infected seedling was observed after 42 hours. Females that had penetrated the epidermis tended to lay eggs in clutches of 1 to 6 eggs before moving and laying other clutches. Sometimes eggs but not females were found within seedlings.

Effects of temperature on population development: The recorded temperatures in each of the incubation environments were: 3.9 ± 0.5, 8.7 ± 0.3, 11.0 ± 0., 16.8 ± 0.5, and 19.7 ± 0.5 °C. Free J2 molted to J3 after attaining a mean length of 436 ± 25 µm (95% confidence limits of mean, n = 25). J3 molted to J4 at a mean length of 648 ± 15 µm (n = 33) and J4 to adult at 927 ± 24 µm (n = 42). Nematodes failed to penetrate the seedling epidermis in more than 50% of the inoculated seedlings (Table 2). The mean proportion of seedlings from which nematodes and/or eggs were recovered from subepidermal positions differed significantly at different temperatures (ANOVA: $F = 3.0297$, df = 4, $P < 0.05$). Most of this effect was due to the mean proportion at 3.9 °C being signifi-

Table 1. Early stages of invasion of *Trifolium repens* seedlings by *Ditylenchus dipsaci* at 16.8 °C.

Time (hours)	Percentage of seedlings with subepidermal nematodes	N[a]	Percentages of original nematode inoculum recovered from various locations		
			Subepidermal	Axils	Seedling surface
0–8	0	36	2	0	53
9–16	27	48	7	7	51
17–24	37	48	6	10	52
25–32	17	12	14	4	42
33–40	33	12	4	7	67
41–48	29	24	6	10	45

[a] Number of seedlings sampled.

TABLE 2. Establishment of *Ditylenchus dipsaci* infestations in inoculated seedlings incubated at different temperatures.

Temperature °C	Mean proportion of seedlings with subepidermal infestations (±1 standard deviation)[a]
19.7	0.37 ± 0.11
16.8	0.39 ± 0.17
11.0	0.31 ± 0.11
8.7	0.43 ± 0.12
3.9	0.12 ± 0.10

[a] 160 seedlings sampled at each temperature.

cantly lower than at other temperatures (t = 2.5862, df = 1, $P < 0.05$; see Table 2). At all temperatures the mean number of seedlings with subepidermal infestations did not increase over time.

In later stages of invasion nematodes were often found in stem tissues but, as in earlier stages of invasion, eggs were laid in distinct clutches. Out of all the individual clutches of offspring identified in all seedlings at all times and temperatures, only 36% were spatially associated with female nematodes. The number of offspring in individual clutches ranged from 1 to 37. Larger clutches (>10 individuals) were found only in stem tissues. Occasionally, dead nematodes were recovered from seedlings at 3.9 °C. There was a delay between seedling inoculation and commencement of egg laying. This delay was most pronounced at low temperatures. The minimum observed delays at 3.9, 8.7, 11.0, 16.8, and 19.7 °C were 336, 384, 92, 70, and 51 hours, respectively.

For all stages the developmental rate (the reciprocal of the time taken from inoculation to the first observed occurrence of a stage in seedlings) was a linear function of temperature (Fig. 1, Table 3). Data from all samples at all temperatures in which mature first-generation adults were absent were analyzed to assess the range of rates of egg production. The observed range was greatest at higher temperatures, suggesting that rate of egg production was dependent on temperature (Fig. 2).

Field survey: Most plants had several *D. dipsaci*-infected regions separated from each other by pieces of uninfested stolon more than 5 mm long. Infested regions were typically composed of the proximal ends of several branches and their originator axes. The observed range of lengths of infested regions was from 1 to 68 mm (median value 9, n = 42). The population density of nematodes in infested regions ranged from 0.2 to 40.8 nematodes/mm length of stolon. Tissue senescence was often observed in infested regions with higher nematode densities. Nine of the eleven regions with nematode densities exceeding 7/mm length had senescing regions of stolon, whereas such senescence was observed in only 3 of the 31 infested regions with lower population densities. In one of the latter, tissue senescence was advanced and the stolon epidermis was split longitudinally in several places. This piece also contained a large number of bacteria-feeding nematodes and dead *D. dipsaci*.

The nematode population structure was assessed in 10 of the infested regions. The body-length distributions of juveniles and adults are shown in Fig. 3. The ratio of adult females to adult males ranged from 0.3 to 4 (mean 1.8, σ = 1.0898, n = 10). The proportion of adults to juveniles ranged from 0.04 to 0.80 (mean 0.30, σ = 0.2471, n = 10).

Total nematode populations in infested regions were assessed from three successive extractions. Few nematodes were recorded in the last extraction. The rates of nematode migration out of split stolon lengths in the first extraction were calculated and plotted against the total nematode population of each length. A regression analysis indicated that the number of nematodes migrating per hour was a linear function of the total population ($y = 0.0465x - 0.1172$, $R^2 = 0.9972$, n = 36). Thus, over the population density range of 0.2 to 40.8 nematodes/mm, proportionally more nematodes migrated as initial population increased.

Migration study: Sections that had a total nematode population of less than six were omitted from the data analysis. An ANOVA analysis of arcsine-transformed data indi-

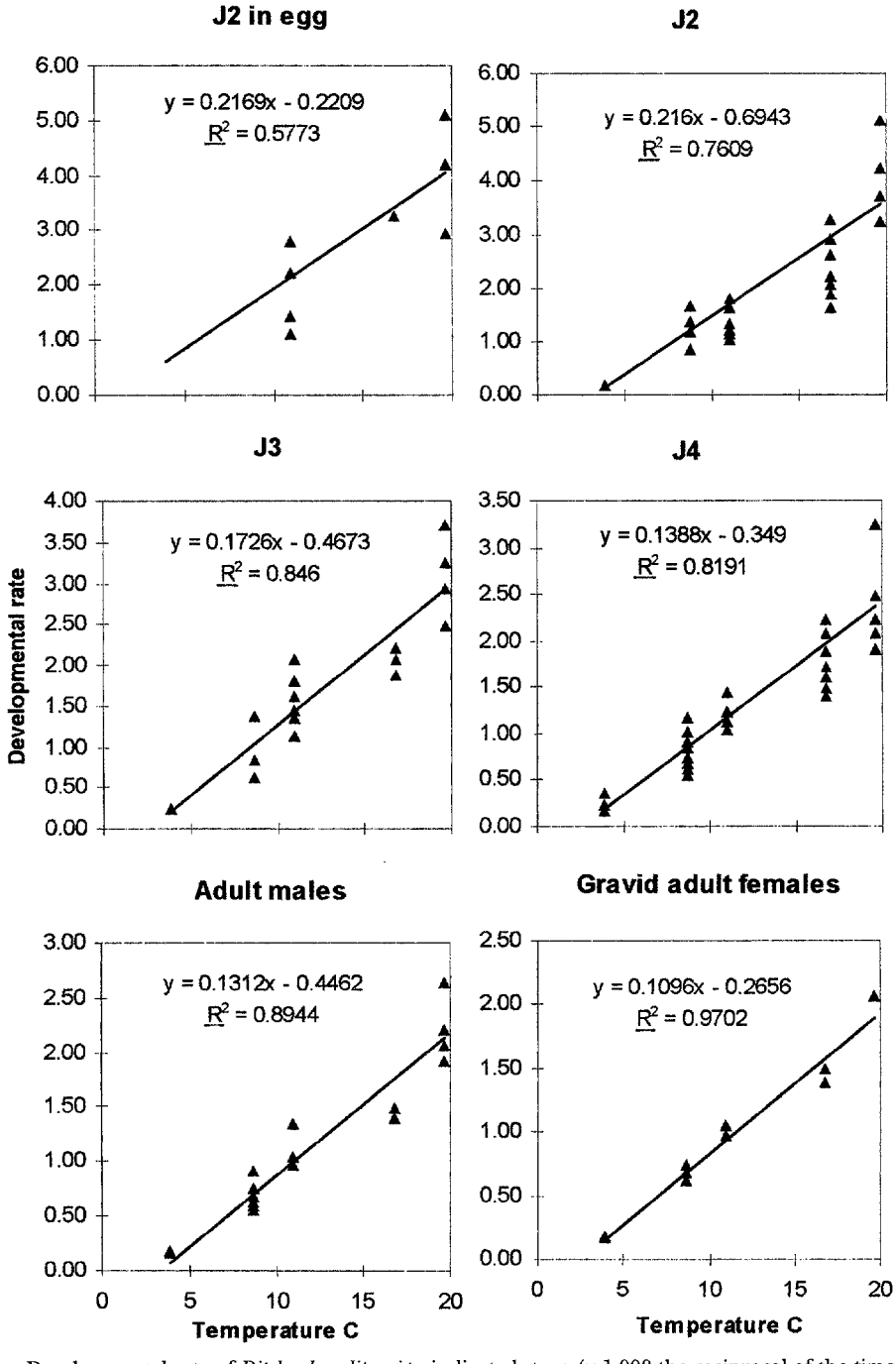

FIG. 1. Developmental rate of *Ditylenchus dipsaci* to indicated stage (× 1,000 the reciprocal of the time taken to the first appearance of the stage in *Trifolium repens* seedlings) plotted against temperature. Lines fitted by linear regression (GLM; see Table 3). For egg with J2 to gravid adult, N = 11, 42, 20, 46, 28, and 10.

cated significant differences among migration rates out of infested tissues at different temperatures. The rate of migration increased to a maximum at 25 °C and declined at higher temperatures (Table 4). There were no correlations between initial population density within sections and rates of migration.

TABLE 3. Linear regression analyses of developmental rates at different temperatures for different developmental stages of *Ditylenchus dipsaci*.

Stage	Regression equation	ANOVA			95% confidence intervals	
		F value	d	*P*	Gradient	Constant
J2 in egg	y = 0.2169x – 0.2209	13.6551	1	<0.01	0.0861 to 0.3478	–2.0764 to 1.6346
J2	y = 0.2160x – 0.6943	130.4823	1	<0.001	0.1778 to 0.2542	–1.2382 to –0.1503
J3	y = 0.1726x – 0.4673	104.3503	1	<0.001	0.1372 to 0.2080	–0.9969 to 0.0623
J4	y = 0.1388x – 0.3490	203.4994	1	<0.001	0.1192 to 0.1584	–0.6045 to –0.0898
Male	y = 0.1312x – 0.4462	228.5635	1	<0.001	0.1134 to 0.1490	–0.7045 to –0.1880
Gravid female	y = 0.1096x – 0.2656	292.6351	1	<0.001	0.0951 to 0.1241	–0.4364 to –0.0948

DISCUSSION

Seedling invasion: Most nematodes that penetrated seedling epidermis did so within 16 hours of inoculation. If we assume females inoculated onto the same seedling acted independently, then the probability of a seedling being invaded is the product of the number of inoculated females and the probability of an individual female initiating an infection. We can therefore calculate the latter probability from the data provided in Table 2. The probability of a female initiating an infection was small and temperature dependent, being 0.12 at 8 °C to 20 °C but only 0.04 at 3.9 °C. Similarly low-penetration probabilities have been observed for seedlings of susceptible red clover (*T. pratense* L.) inoculated with *D. dipsaci* (Dijkstra, 1957). Our observations suggest that seedling epidermis can be penetrated only at certain specific sites, primarily at axils. The method of inoculation presumably resulted in only a small proportion of nematodes being located at such sites, and these nematodes effected a rapid penetration. Nematodes not originally at such sites could not migrate over the external surfaces of the plant to axils thereafter; hence, the

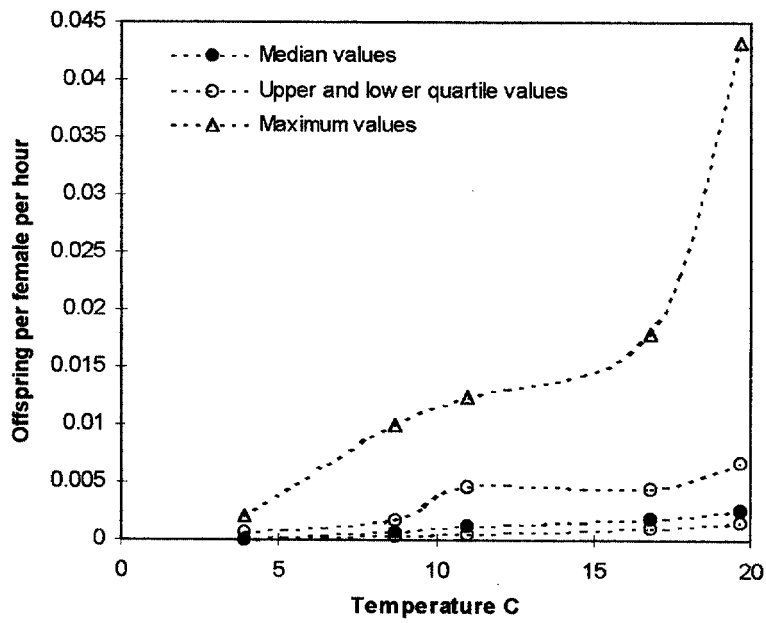

FIG. 2. Range of observed rates of egg production by *Ditylenchus dipsaci* in *Trifolium repens* seedlings at different temperatures. Data used were for all sampled infested seedlings in which mature first-generation adults were absent. For 3.9, 8.7, 11.0, 16.8, and 19.7 °C, N = 15, 48, 43, 42, and 48, respectively. Minimum values were zero in all cases.

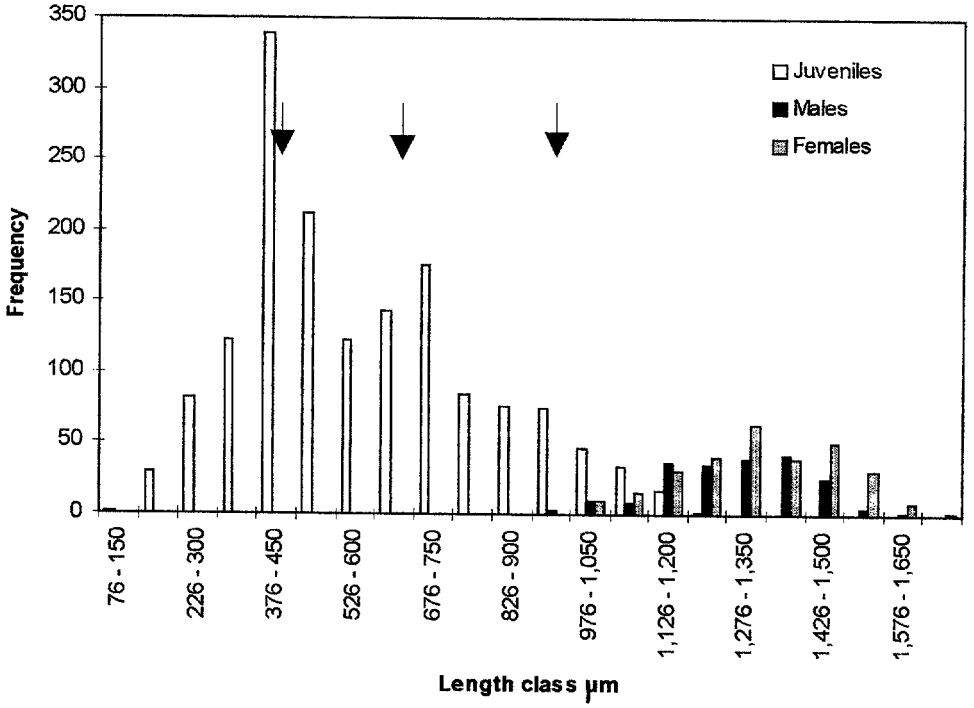

FIG. 3. Length distributions of *Ditylenchus dipsaci* juveniles, adult females, and adult males extracted from infested regions of *Trifolium repens* stolons collected from the field. Arrows indicate molt lengths.

proportion of infested seedlings did not increase 16 hours after inoculation.

Egg laying: Once nematodes penetrated the seedling epidermis, they started to migrate slowly into and through subepidermal tissues and, after a short delay probably to restore energy expended on penetration and migration, started to lay eggs. Eggs typically were laid in clutches. Clutch size was presumably dependent on the suitability of the tissues in which the females were located. The largest clutches were laid in parenchymous stem tissues, which are known to be the preferred food for stem nematode (Hooper and Southey, 1978). Sometimes eggs but not females were found within seedlings, indicating females sometimes egressed through the seedling epidermis after having laid eggs. Some of these females emerged in axils and could therefore migrate back again into subepidermal regions. The fact that eggs were never found in seedling washes indicated females located at non-axillary locations on the external surface of seedlings did not lay eggs. This was presumably because they could not penetrate the epidermis and feed. Yuksel (1960) showed that stem nematodes lay eggs only when feeding on host tissue.

Egg-laying rates estimated from the number of observed offspring produced over time indicated a broad range of egg-laying at each temperature, with the rates usually be-

TABLE 4. Rates of migration of *Ditylenchus dipsaci* out of stolons at different temperatures.

Temperature (°C)	Number of stolon pieces[a]	Mean proportion of total nematode populations migrating out of stolons in 45 minutes
4	8	0.097b
12	4	0.405
18	11	0.350c
25	14	0.578bcd
30	8	0.293d

One-way ANOVA of arcsine-transformed data indicated significant differences between means ($F = 3.49$, $P < 0.0154$). Overall standard error of the means from ANOVA was 0.186.

[a] Only stolons with nematode populations of greater than five individuals included. Means labelled with a common letter differed significantly from one another (*t*-tests, $P < 0.05$).

ing low (Fig. 2). The actual number of females that laid eggs in any seedling was uncertain because some females egressed out of seedlings and were not recovered. The rates recorded in Fig. 2 assume all the observed offspring in each infested seedling derived from all three inoculated females. This is unlikely, given the small probability of an individual female successfully penetrating the epidermis. Actual egg-laying rates per female are therefore likely to be up to three times the estimated values. Maximum rates of egg-laying would have occurred in seedlings where females had successfully invaded suitable regions of subepidermal tissues, especially the stem parenchyma, and started laying eggs after a minimum delay. Thus, under otherwise optimal conditions, no eggs are laid at temperatures less than approximately 3.5 °C. At 10 °C and 20 °C the respective egg-laying rates are probably of the order of 0.89 and 3.12 eggs/female/day. These estimates agree well with estimates of rates of egg production by the onion race of *D. dipsaci* provided by Yuksel (1960).

Developmental rates and temperature: For all developmental stages of *D. dipsaci* the rate of development (the reciprocal of the time in days to reach a well-defined developmental point) was directly proportional to temperature (Fig. 1). Given these linear relationships, it is possible to apply the concepts of thermal time to *D. dipsaci* development (Trudgill, 1995; Trudgill and Perry, 1994). It follows that there is a minimum temperature (T_b) below which no progress will be made along a developmental path. Further, it is possible to derive a temperature-independent developmental constant (S), which is the number of accumulated day-degrees above the basal temperature (T_b) required to complete a developmental pathway. The derived S and T_b for the various developmental stages of *D. dipsaci* are provided in Table 5 along with data derived from Yuksel (1960) for the onion race of *D. dipsaci*. The low T_b and high S values are appropriate for nematode populations from temperate climates (Trudgill and Perry, 1994). To determine the S values for individual stages, it was necessary to estimate the mean delay (α) between seedling inoculation and commencement of egg-laying at each temperature. These delays were not directly measured since it was not possible to tell by direct observation when eggs were actually laid in seedlings. However, it can be reasonably assumed that at each temperature the variation in time taken to the first appearance of a particular stage in seedlings (β, which was symmetrical about the mean value) was a direct result of the original variation in α. Thus, at each temperature mean α could be estimated by adding half the maximum observed range of β to the observed minimum α. In this way, means α at 19.7, 16.8, 11.0, 8.7, and 3.9 °C were estimated as 158, 229, 357, 864, and 1,968 hours, respectively. A regression analysis of these data (taking reciprocals of α) yielded S and T_b for commencement of egg-laying by inoculated nematodes of 113.3 days and 3.8 °C. The estimated S value for production of one gen-

TABLE 5. Accumulated effective day-degrees required for completion of different stages of *Ditylenchus dipsaci* development.

Stage of development	White clover race		Onion race[a]	
	Basal temperature T_b °C	Thermal constant S	Basal temperature T_b °C	Thermal constant S
Egg to hatched juvenile stage 2	3.2	80.0	3.0	132
Hatched juvenile stage 2 to juvenile stage 3	2.7	48.2	3.0	27
Juvenile stage 3 to Juvenile stage 4	2.5	58.5	3.0	39
Juvenile stage 4 to adult	3.4	17.4	3.0	54
Egg to adult	3.4	204.3	3.0	252
First generation adult to second-generation egg	2.4	64.0	3.0	60

[a] Estimated from Yuksel's data (1960) for *D. dipsaci* onion race at 15 °C, assuming a T_b of 3 °C.

eration used below does not include this delay.

These results enable an estimate of the likely effect of global warming on nematode population development to be made. At present in Wales, bearing in mind that white clover is a perennial plant, the nematode can produce nine generations in 1 year (mean annual daily temperature of 10 °C). An increase in mean annual daily temperature of +1.8 °C by the years 2060–2070 (Murphy and Mitchell, 1995) would allow development of three more generations in 1 year. Further, during winter and spring, host growth is limited by low light intensity rather than temperature (Sackville-Hamilton, 1990); therefore, in warmer winters and springs host growth will not accelerate to compensate for more rapid turnover of the nematode population.

If the rate of egg production is also directly proportional to temperature and the apparent deviation from linearity evident in Fig. 2 is a product of sampling error, then the relationship of maximum observed egg-laying rate to temperature would be: $y = 0.0022x - 0.0097$, $R^2 = 0.8042$. From this equation it is possible to derive the rate of egg production in terms of eggs per day-degree. Thus, the maximum rate of egg production per female per day-degree under optimal conditions would be 0.158, the basal temperature for egg production being 4.41 °C (an overestimate since we know eggs were laid at 3.9 °C).

Field survey: Populations of up to 3,000 *D. dipsaci* per infestation locus developed under field conditions. As population density increased within stolons, longitudinal splits in the stolon epidermis occurred that exposed underlying infested tissues. It is highly probable that water would trigger a mass emigration of nematodes from such stolons, leaving few *D. dipsaci.* Interestingly, a particular piece of stolon that we observed also contained a large number of dead *D. dipsaci* and living bacteria-feeding nematodes. Other researchers have shown that *D. dipsaci* migrate rapidly out of dying plant tissues, presumably because of the presence of toxic, water-soluble metabolites (Robertson, 1928). Observations of nematode migration out of artificially split stolons indicated that at 4 °C the probability of a nematode migrating out of infested tissue during a 1-hour time span was approximately 0.05.

This study has shown that temperature has a marked effect on several important parameters, including rates of development and egg production, that govern the population dynamics of stem nematode in white clover. It also has shown that the concepts of thermal time are applicable to stem nematode population development. Information derived from this study is being combined with information from other studies of this host-nematode system (Griffith et al., 1996, 1997) to construct a simulation model of nematode population development in infested host stems.

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