Experiments on population growth of the predatory nematode *Prionchulus punctatus* in laboratory culture with observations on life history [1]

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Summary

A laboratory method was developed for culturing sufficient *Prionchulus punctatus* for use as a biological control agent in pot trials. Continued population growth required subculturing at approximately four week intervals and was greatest when old culture medium was transferred. Various additions to the agar media affected population development : soil or humus extracts increased predator population growth whereas aqueous extracts or dialysates of four week old stock culture agar reduced reproduction and survival.

Résumé

Croissance des populations du nématode prédateur Prionchulus punctatus *dans des élevages de laboratoire et observations sur son cycle vital*

Une méthode a été mise au point pour obtenir, en élevage au laboratoire, un nombre de *Prionchulus punctatus* suffisant pour être utilisé comme agent de traitement dans des essais en pots. La croissance continue de la population a nécessité des réinoculations sur milieu neuf (sous-élevages) toutes les quatre semaines et la croissance de la population a été maximum quand de petites quantités du vieux milieu de culture étaient incorporées aux sous-élevages. Huit milieux de culture gélosés ont été essayés. Les extraits de sol ou d'humus ont augmenté la croissance à la fois des populations élevées au laboratoire ou nouvellement extraites alors que les extraits aqueux ou les dialysats de gélose portant des élevages vieux de quatre semaines étaient défavorables aux deux catégories de populations. Les auteurs proposent une explication de ces faits reposant sur l'existence de facteurs agissant sur les stades les plus sensibles (particulièrement le 1er et le 4eme) et s'appuyant sur la biologie du prédateur.

Early attempts to culture mononchid nematodes, using simple media, were only partially successful (Steiner & Heinly, 1922 ; Cassidy, 1931 ; Esser, 1963). Nelmes (1974) studied feeding behaviour of *Prionchulus punctatus* (Cobb) cultured on a modification of a medium used by Springett (1964) for culture of enchytraeids ; Maertens (1975) used a similar medium to study reproduction and embryology. The medium is suitable for other predators e.g. *Mononchus aquaticus* Coetzee (Grootaert & Maertens, 1976 ; Small & Grootaert, 1977).

The method used by Nelmes and Maertens was adequate for the small numbers of adult predators their experiments required. To obtain sufficient numbers to conduct pot experiments (Small, 1979) we found it necessary to modify the method. This paper describes experiments

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to develop mass culture techniques for *P. punctatus* and discusses the effect of medium in terms of the predator's biology.

Materials and methods

Xenic stock cultures of *P. punctatus* were maintained on 1% soil-extract agar in 9 cm diameter Petri dishes at 25°. Soil extract was prepared by thoroughly mixing equal volumes of de-ionised water and soil (from Imperial College Silwood Park) and allowing the soil to settle over 24 h. The supernatant was filtered through asbestos wool covered by a filter aid (Celite Hyflo Super-cel). The resulting extract was free of soil particles and approx. pH 7 ; it was used to make 1% agar with «Davis» standard agar. Prey (*Panagrellus redivivus* (L.) or *Aphelenchus avenae* (Bastian)) were added to *P. punctatus* cultures at weekly intervals. Neither prey species reproduced or survived long in predator cultures even when added in excess, presumably because suitable food was lacking. Cultures were subcultured at four-week intervals by dividing the parent culture amongst three or four new plates. *P. redivivus* was cultured on a flour-paste medium (Ellenby & Smith, 1966) and *A. avenae* on an oat-grain and fungus culture medium (Evans, 1970).

The culture method for *P. punctatus* incorporated techniques suggested by experiments described below. Up to 4 000 *P. punctatus* (1 000 adults) per plate were regularly obtained in each four-week period. Experiments on the effect of temperature on *P. punctatus* populations generally confirmed Maertens' (1975) results : optimum temperatures were 22°-25° but our population declined at 10°. At 15°-25° our population always declined after 4 weeks, suggesting a need for reculturing.

Subculturing (Experiments *i* and *ii*)

Plastic Petri dishes (3.5 cm diameter) containing approximately 4 ml soil extract agar were used. Unless stated otherwise, cultures were inoculated using agar discs cut from stock cultures with a 1.2 cm diameter cork borer : discs were placed in the centre of the dish when the agar was cool but still molten. Predators in each disc were counted. Cultures were supplied with abundant *P. redivivus* at the start of experiments and at weekly intervals thereafter so that availability of prey did not limit predator populations. Nematodes were extracted from cultures using a miniature tray technique (Whitehead & Hemming, 1965) and counted.

Experiment i) Effects of subculturing on population growth of P. punctatus : Thirty plates were each inoculated with 32 ± 1.8 ($\bar{x} \pm$ S.D) adult and 122 ± 8.3 juvenile *P. punctatus* ; fifteen replicates were maintained at both 15° and 25°. After four weeks nematodes from five replicates at each temperature were extracted and counted ; a further five replicates were subcultured by dividing the agar into three equal portions and transferring each portion to a new plate which was kept at the temperature of the parent cultures. A nematode trapping fungus (*Arthrobotrys* sp.) grew on some subcultured plates. Six weeks after initial inoculation the predators from the five remaining original cultures and four fungus-free replicates from each temperature were extracted and counted. Three uncontaminated subcultures at 25° were extracted and counted eight and ten weeks after initial inoculation.

Experiment ii) Effect of three methods of subculturing P. punctatus *cultures:* Twenty dishes were inoculated with agar discs from a ten day old stock culture. The number of *P. punctatus* in ten discs was adjusted by removal of excess nematodes to leave twenty adults and 68 juveniles (Treatment A). All *P. punctatus* were removed from remaining discs and rinsed in sterile, de-ionised water before twenty adults and 68 juveniles were replaced in each disc (Treatment B). Twenty adults and 68 juveniles from the stock culture were rinsed in sterile, de-ionised water and placed in drops of water on a further ten plates without agar from the stock culture (Treatment C). All cultures were incubated at 25°. The nematodes were counted, by direct observation of cultures, at weekly intervals for nine weeks and finally after extraction twelve weeks from inoculation. In a repeat experiment all experimental details were similar, except that the stock culture used for inoculation was four weeks old. The experiment was terminated after 6 weeks.

INFLUENCE OF MEDIUM ON POPULATION GROWTH OF P. punctatus (Experiments *iii* to *v*)

Small culture chambers (approx. 0.2 ml), made by heating square pieces of perspex (2.5 cm side × 1.5 cm thick) to 165° and pressing against a metal former in a vice to give four chambers per square, were used. With care distortion was avoided and visibility through the base was good. Five drops of agar were placed in each chamber. Cultures were incubated at 20° in a humid atmosphere which delayed dehydration of the agar for up to 3 weeks after which addition of fresh agar or reculturing of the nematodes was necessary. Cultures were fed only with *A. avenae* which disrupted the agar less than *P. redivivus*.

Individual gravid *P. punctatus* females were placed in drops of water on the agar surface of each culture. Approximately 150 washed and filtered *A. avenae* were added to each culture at the start of the experiment and at three to seven day intervals (depending on predator density) so that prey did not limit predator population growth. All life stages of *P. punctatus* were counted at intervals. First stage juveniles which had fed (indicated by pigmented intestinal walls) were grouped with second stage juveniles since they were not easily distinguishable and preliminary experiments had indicated that the majority of first stage juveniles moulted within 24 h of feeding. Nematodes were also periodically extracted by gently macerating the agar in 3 ml water until all the *P. punctatus* could be counted, active stages were replaced in fresh agar. These extraction-count data were analysed statistically ; full results are recorded in Small (1977).

The preparation of media described below ensured that their consistency differed little, except that untreated old S.E.A. (experiment *v*) was slightly softer than other media.

Experiment iii) Population development of P. punctatus *in four agar media.* Media were prepared as follows :

A. 10 ml of agar taken from a well established (i.e. > 3 500 *P. punctatus*), four week old stock culture was gently heated until molten and the debris allowed to settle. Excess water was removed and the clear agar decanted *(Old S.E.A.)*.

B. The remaining 15 ml of agar from the stock culture was shaken with 60 ml sterile, deionised water and the suspension allowed to stand for 75 min at 27° before centrifugation. The 50 ml of supernatant recovered was mixed with 50 ml fresh, molten (but cool) 2% soil-extract agar *(S.E.A. + extract)*.

C. Fresh soil extract agar (1%) was prepared as described for *P. punctatus* stock cultures *(Fresh S.E.A.)*.

D. Water agar (1%) was prepared using distilled water *(Water agar)*.

P. punctatus females from a four week old stock culture were used to inoculate 24 replicates per treatment. Life stages of *P. punctatus* were counted at two to three day intervals. Every two weeks *P. punctatus* from six replicates of each treatment were extracted and counted. *P. punctatus* extracted at week 2 were not retained but thereafter predators were returned to the experiment. *P. punctatus* from all cultures remaining at sixteen weeks were extracted and counted.

Experiment iv) Population development of P. punctatus *extracted from the fermentation layer (F-layer) of a field of bracken* (Pteridium aquilinum *(L.) Kühn) and cultured in five agar media.*

This experiment investigated the possible adaptation of *P. punctatus* to stock culture conditions. The F-layer population was of interest because of its high density (approx. 250 *P. punctatus* per litre), the predominance of fourth stage juveniles (although all stages were present), and the presence of a male *P. punctatus* (Small, 1977). Nematodes were extracted from the F-layer material by the tray method (Whitehead & Hemming, 1965) over the 24 hr preceding inoculation.

The four media (A-D) of experiment *iii*) were used together with a further medium (E) prepared in the same way as soil-extract agar but using F-layer material, similar to that used to obtain the *P. punctatus*, in place of soil. The agar was pH 6.1 (F.L.E.A.). Each treatment had eleven replicates. *P. punctatus* from all replicates were extracted, counted and recultured on days 17, 35 and 70.

Experiment v) Population development of P. punctatus *in seven agar media:* The four

media (A-D) of experiment *iii*) were used, plus three others :

E. All *P. punctatus* were removed from a two week old stock culture ; some of the agar from this culture was used without further treatment *(Unlreated old S.E.A.)*.

F. A sealed dialysis membrane tube containing agar from the same stock culture was submerged in 15 ml sterile de-ionised water for 3 h. The dialysate was mixed with 15 ml cool, fresh 2% soil-extract agar *(S.E.A. + dialysate)*.

G. The dialysis membrane with the dialysed agar was suspended in a stream of tap water flowing over the entire surface for 22 h before placing it in three changes of 30 ml sterile de-ionised water for 1 h each. The 25 ml dialysed agar obtained was mixed with 25 ml of 2% soil-extract agar *(S.E.A. + dialysed agar)*.

P. punctatus females from a two week old stock culture were used to inoculate 50 replicates per treatment. Eggs and juvenile *P. punctatus* were extracted and counted after fourteen days. Some *P. punctatus* females in old S.E.A. and S.E.A. + dialysed agar treatments had migrated between cultures ; results presented are for unaffected cultures only.

Results

Experiment i): Predator populations reached means of 1 726 (15°) and 1 804 (25°) after four weeks. By week 6 non-subcultured populations had decreased to means of 1 454 (15°) and 1 467 (25°). At 15° subcultured populations increased from an estimated 575 predators (i.e. 1 726 divided amongst three subcultures) to 1 299 in weeks 4 to 6 ; similarly at 25° the subcultured population increased from an estimated 601 (1 804 : 3) to 774. Analysis of variance indicated no significant difference between subcultured and non subcultured *P. punctatus* populations at week 6 ($F : 0.41$; d.f. $= 4,18$) despite the reduction in subcultured populations caused by the division of the parent cultures amongst three new plates. Thus subculturing had a significant effect on predator population growth.

Experiment ii): Large differences in *P. punctatus* numbers between the three methods of subculturing were recorded. Populations in treatment C declined steadily (Fig. 1). Population changes in treatments A and B were broadly similar but a consistently smaller population occurred in treatment B for eight weeks. Populations in treatments A and B were significantly greater than in treatment C throughout the experiment ($p < 0.01$) ; the differences between treatments A and B were significant ($p < 0.05$) for weeks 1, 2 and 6.

In the repeat experiment population changes were similar to those of the first trial but in treatments A and B maximum numbers were reached at week 4 (compared to week 6 in the first trial). Numbers in treatment C decreased rapidly to zero at week 5 ; from week 2 onwards there were significantly fewer predators than in other treatments ($p < 0.001$). Treatment B had significantly fewer predators than treatment A from week 2 onwards ($p < 0.001$). These results suggested that old culture medium affected predator population growth, and that the effect varied with the age of the medium.

Experiment iii): Culture medium had a significant influence on the populations obtained (Fig. 2 ; Table 1). Numbers of eggs and juveniles were generally fewest in old S.E.A. in which no nematodes survived longer than 65 days and none of the few fourth stage juveniles matured. Over the first week egg production in fresh S.E.A. and S.E.A. + extract was similar (approx. 2.8 eggs/♀/day) but fewer juveniles hatched and developed in S.E.A. + extract, and only four matured. Fewer eggs were deposited in water agar than in media containing fresh soil-extracts but survival was good and a total of 48 F_1 adults were recorded (day 86), compared to 46 in fresh S.E.A. (day 100). Treatments remaining at 84 days and 98 days were not significantly different but at 112 days significantly more unfed first stage juveniles occurred in fresh S.E.A. than in water agar ($p < 0.05$).

Rate of development was not greatly affected by medium. Eggs hatched seven days after deposition and first stage juveniles moulted two to five days later, depending on how quickly they found food. The second moult occurred approximately seventeen days after the first and the third after a further nine days. The

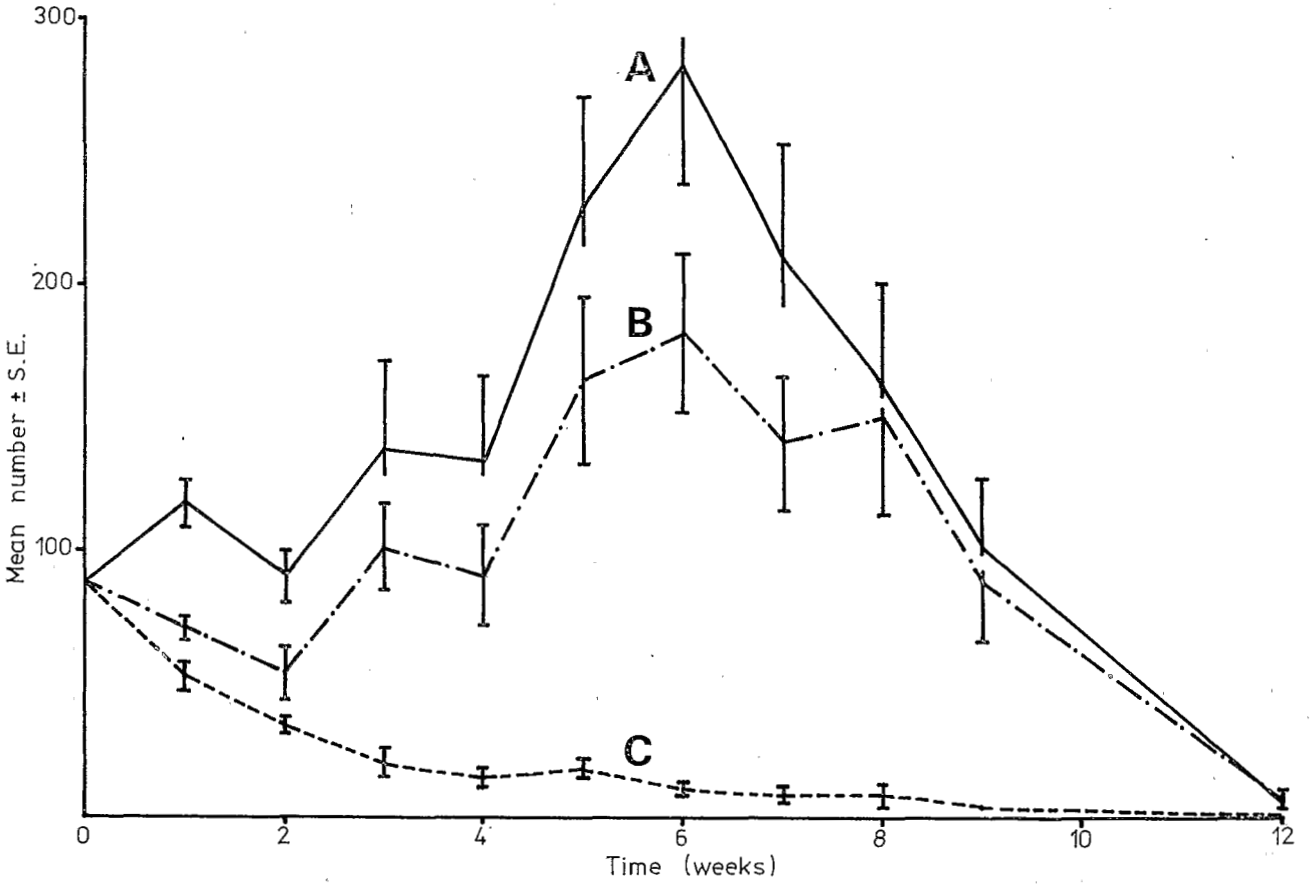

Fig. 1. Effect of method of subculturing on subsequent growth of *P. punctatus* populations maintained at 25°. A : predators transferred in agar ; B : predators washed and returned to agar ; C : predators washed, no agar transferred.

Table 1

Analyses of variance of numbers of life stages of *P. punctatus* recovered in experiment *iii*)

Week	*Eggs*	*Juvenile stages* *Unfed 1st*	*Fed 1st + 2nd*	*3rd*	*4th*	*Total offspring*	F_1 *Adults*
2	N.S.D.	A > B, D*	B > C*	—	—	N.S.D.	—
4	C > A, B*	C > D*	C > A** C > B, D*	N.S.D.	—	C > A, D** C > B*	—
6	—	—	N.S.D.	N.S.D.	N.S.D.	D > A**	—
8	—	—	—	C > A*	N.S.D.	C > A*	—
10	D > B, C**	—	—	C > B** C > D*	C > B**	C > B** D > B*	D > B*** D > C**

A : Old agar ; B : S.E.A. + extract ; C : Fresh S.E.A. ; D : Water agar.
* : p = 0.05-0.01 ; ** : p = 0.01-0.001 ; *** : p < 0.001 ; N.S.D. : No significant differences.

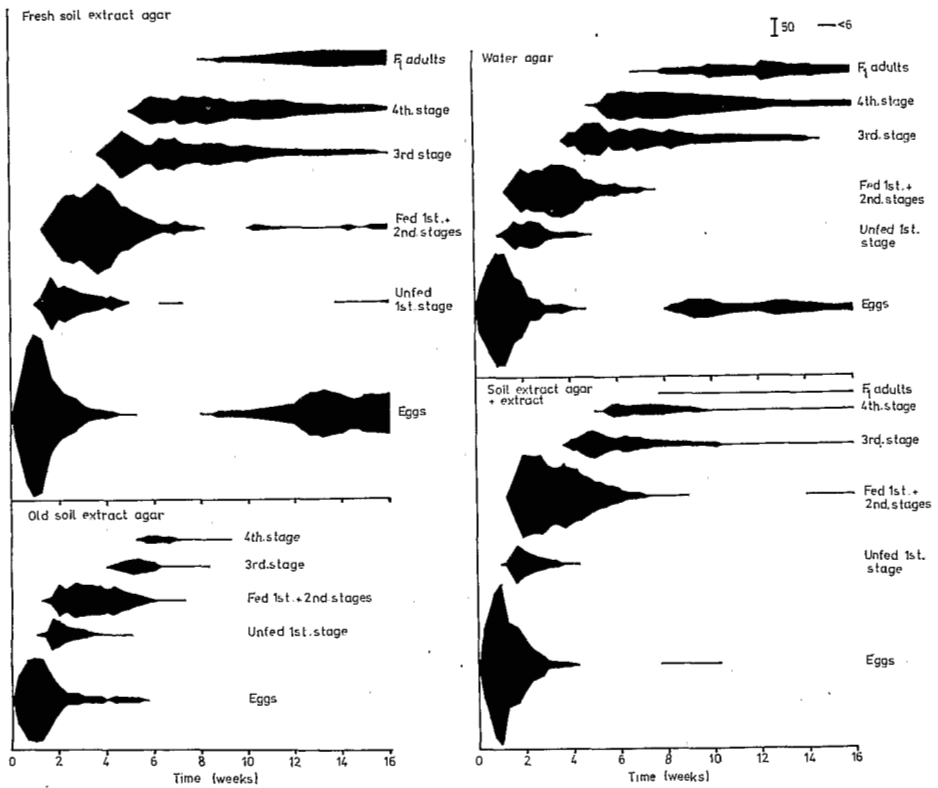

Fig. 2. Population development from *P. punctatus* females cultured individually at 20° (experiment *iii*).

Table 2

Analyses of variance of numbers of life stages of *P. punctatus* recovered in experiment *iv*)

Day	*Eggs*	*Unfed 1st*	*Juvenile stages* *Fed 1st + 2nd*	*3rd*	*4th*	*Total offspring*	*F_1 Adults*
17	D > B** D > C, E*	A > D, E*** A > B, C*	N.S.D.	—	—	N.S.D.	—
35	—	C > A, B*	E > D** C > D* E > A*	A > D**	—	C > D** E > D* A > D*	—
70	C > A, E*** B > A***	B > E*** B > C*	N.S.D.	C > A, D*	C > A, B, D** E > A, B, D*	C > A, D, E*** B > A, D*** E > A, D* B > E*	C > A, D*** B > A, D*** E > D** E > A*

Significance levels and treatments A-D as for Table 1 ; E : F.L.E.A.

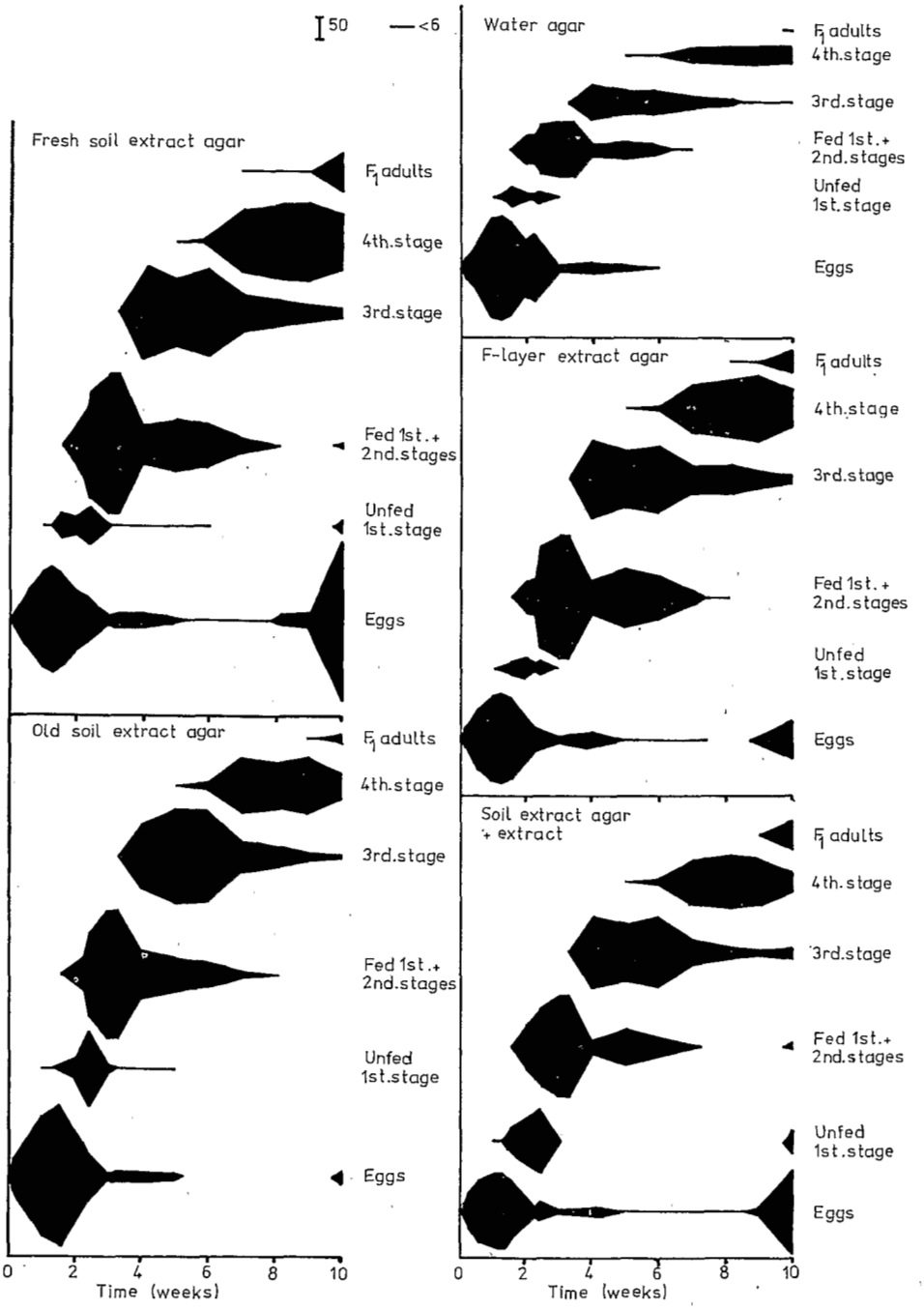

Fig. 3. Population development from *P. punctatus* females extracted from the bracken F-layer and cultured individually at 20° (experiment *iv*).

Table 3
Mean numbers of the life-stages of *P. punctatus* recovered in experiment *v)*

Treatment	*Number of replicates*	*Eggs*	*Unfed 1st stage juveniles*	*Fed 1st and 2nd stage juveniles*	*Total offspring*
A. Old S.E.A.	50	16	15	32	64
B. S.E.A. + extract	50	12	14	22	47
C. Fresh S.E.A.	50	14	13	29	56
D. Water agar	50	12	7	21	41
E. Untreated old S.E.A.	15	36	9	14	59
F. S.E.A. + dialysate	50	16	12	9	37
G. S.E.A. + dialysed agar	20	29	12	16	53
L.S.D.s ($p < 0.05$)					
Any pair of A, B, C, D, F		5.7	3.3	4.9	10.2
*Ev*G		9.7	5.6	8.3	17.4
Ev remaining treatments		8.4	4.9	7.2	15.0
Gv remaining treatments		7.6	4.4	6.5	13.5

final moult occurred nearly seven weeks after egg-deposition and F_2 eggs were deposited within two days, a generation time of approximately seven weeks at 20°.

Experiment iv): Culture medium again had a significant effect on population development (Fig. 3 ; Table 2). Egg production rates over the first week varied from approx. two eggs/♀/day in S.E.A. + extract to three eggs/♀/day in old S.E.A. but were similar in the remaining treatments (2.6-2.7 eggs/♀/day). Early juvenile stage durations did not vary between treatments and were similar to those of experiment *iii)* but significantly fewer survived to the second and subsequent stages in water agar than in other treatments. F_1 adults occurred in all treatments but numbers varied from three (water agar) to 86 (fresh S.E.A.). Variation in fourth juvenile stage duration was marked : thirteen days in S.E.A. + extract, fourteen days in fresh S.E.A., eighteen days in old S.E.A., 22 days in F.L.E.A. and 28 days in water agar. The life cycle and number of offspring was very similar in F.L.E.A. and fresh S.E.A.

Experiment v): Addition of an extract, and particularly a dialysate, of agar from an old culture appeared to depress reproduction (Table 3). Population structure differed between heated and untreated old S.E.A.

Discussion

Experiments *i)* and *ii)* indicated that subculturing was important in maintaining the growth of *P. punctatus* populations ; if not subcultured numbers usually declined from week 4. Onset of this decline appeared independent of inoculum, subsequent population growth and number of prey added. Only when the stock culture used for inoculation was two weeks old was the period extended. It seems possible that high concentrations of toxic metabolites may cause the decline but experiment *ii)* indicated that low concentrations in subcultures receiving some old agar increased population growth. Subculturing every four weeks by transfer of old agar containing predators allowed stocks to increase over three years, and provide sufficient predators for pot experiments.

Addition of a soil-extract to the culture medium also benefits *P. punctatus* population growth (Maertens, 1975 ; Small, 1977) and earlier attempts to culture mononchids without soil-extract were unsuccessful. This beneficial effect may result from a direct action on *P. punctatus* or via other organisms in the cultures. Steiner and Heinly (1922) considered that a component of soil might be necessary to mononchid development as first stage *Clarkus papillatus* (Bastian) juveniles apparently ingested soil particles.

These observations and the results of experiments *i)* and *ii)* led to experiments *iii)* and *iv)* in which the importance of culture medium was confirmed. Experiment *iii)* suggested either that particular factors in old agar were water insoluble or that dilution with soil-extract agar allowed population growth. Experiment *iv)* indicated differences between stock and newly-extracted populations ; possibly the F-layer nematodes were tolerant of biologically active molecules, and long term culture had reduced this tolerance. The properties of these factors were further investigated in experiment *v)* in which heating old S.E.A. may have expelled detrimental volatile compounds which could also be removed by dialysis.

Good reproduction in two week old but not in four week old agar suggested that the concentration of water soluble, dialysable molecules increased with time until a toxic level was reached at approximately four weeks. Ammonia, urea and carbon dioxide are excreted by nematodes (Wright & Newall, 1976) ; these compounds are consistent with the physical characteristics of the proposed population-influencing factors, and are known to affect nematode behaviour (Green, 1971 ; Shepherd & Clarke, 1971 ; Ward, 1973). However McNamara (1980) suggested that micro-organisms may extend survival of *Xiphinema diversicaudatum* (Micol.) in plant-free soil. It is difficult to directly relate McNamara's results with our own because of the different food of the two nematode species, and the different approaches adopted ; thus increased survival (e.g. by reduced movement and hence metabolism — McNamara's mode of action No. 3) would result in decreased feeding and hence reproduction in *P. punctatus*. Whilst our results neither confirm nor contradict the specific results for *X. diversicaudatum*, we agree with McNamara's general conclusion that results of experiments conducted in heated culture media should not be extrapolated to the field without caution.

Nonetheless differential reproduction in various agar media may elucidate some aspects of the biology of *P. punctatus*. Reduced egg production in poor media probably prevents wasted egg production in environments where juvenile mortality would be great. First stage juveniles were particularly sensitive to environmental conditions and experienced the greatest mortality. Second stage juveniles were comparatively long-lived ; the growth and accumulation of food reserves may allow rapid development through to the fourth stage in good conditions, or represent a survival strategy for adverse conditions. Similar, but more obvious strategies are found in other nematodes e.g. dauer larvae of some rhabditids or the infective stage of some plant parasites (Evans & Perry, 1976). Second stage juvenile nematodes have exhibited a particular plasticity in the course of evolution to become a critical stage in the life cycle of many species including perhaps, in a relatively unspecialised way, *P. punctatus*.

The final moult was delayed in poor media, perhaps paralleling the survival strategies of fourth stage juveniles of other nematodes (Evans & Perry, 1976). This population-limiting effect might be expected for a predator and may account for the predominance of fourth stage juveniles in the dense *P. punctatus* population of the bracken F-layer, and the generally small proportion (< 5%) of mononchids in many soils (Thorne, 1927 ; Overgaard-Nielsen, 1949 ; Banage, 1963 ; Nelmes & McCulloch, 1975). Maintenance of a constant population, slow development, delayed reproduction, large body size and long life of *P. punctatus* are features characteristic of K-selected animals (Dobzhansky, 1950 ; Pianka, 1970) which are rarely effective biological control agents (Force, 1974). A single addition of large numbers of *P. punctatus* to soil is therefore unlikely to effect a lasting control, but the culture method described may be applicable to other predatory nematodes which are more promising biological control agents.

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