Effects of killing, fixing and mounting methods on taxonomic characters of parthenogenetic adult female *Caenorhabditis elegans* (Nematoda : Rhabditidae)

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SUMMARY

A comparative study of several commonly-used killing, fixing and mounting techniques was made to identify the optimum procedure for preparation of parthenogenetic adult female *Caenorhabditis elegans* for taxonomic examination. Nematodes were either *i)* killed in hot water (95 °C) and fixed in cold fixative (22 °C) or *ii)* killed and fixed in hot fixative by Seinhorst's (1966) technique. Both the qualitative and quantitative effects of five fixatives (FA 4:1; FP 4:1; TAF; FG and FAA) on each of twenty body characteristics were studied in temporary mounts of specimens killed by each method. Although all the fixatives caused significant shrinkage and/or distortion, killing and fixing in hot TAF (95 °C) produced the least-affected specimens. Subsequent processing of hot TAF-killed and -fixed specimens to lactophenol or glycerol caused further significant shrinkage and/or distortion. Of the four techniques studied, processing of nematodes to glycerol by the slow method affected fewest parameters and produced the most acceptable specimens.

RÉSUMÉ

Effets sur les caractères taxonomiques de la femelle parthénogénétique adulte de Caenorhabditis elegans *(Nematoda : Rhabditidae) des méthodes utilisées pour tuer, fixer et monter les nématodes*

Il a été procédé à une étude comparative des méthodes courantes utilisées pour tuer, fixer et monter les nématodes, étude ayant pour but de déterminer la procédure optimale de préparation de femelles parthénogénétiques adultes de *Caenorhabditis elegans* en vue de recherches taxonomiques. Les nématodes ont été *i)* tués dans l'eau chaude (95 °C) et fixés à froid (22 °C) ou *ii)* tués et fixés dans du fixateur chaud suivant la technique de Seinhorst (1966). Les effets qualitatifs et quantitatifs de cinq fixateurs (FA 4:10; FP 4:1; TAF; FG et FAA) sur vingt caractères du corps ont été étudiés sur des nématodes tués par l'une et l'autre méthodes précitées, et montés temporairement. Bien que tous les fixateurs causent des affaissements et (ou) des distortions, les spécimens tués et fixés à l'aide du TAF chaud (95 °C) sont les moins affectés. Le transfert de ces derniers spécimens dans le lactophénol ou la glycérine provoque de nouveaux affaissements et (ou) distortions. Des quatres techniques étudiées, le transfert des nématodes dans la glycérine par la méthode dite lente est celle qui affecte le plus faible nombre de paramètres et produit les spécimens les plus satisfaisants.

Preparation of temporary or permanent microscope slides of nematodes for taxonomic studies may involve three procedures : killing, fixing and processing to mounting medium. Several methods are available for each (Hooper, 1986) but some are known to alter key taxonomic characters of particular groups of nematodes, especially tylenchids (Stone, 1971; Stynes & Bird, 1981; Olowe & Corbett, 1983), dorylaimids (Lamberti & Sher, 1969; Boag, 1982) and mermithids (Curran & Hominick, 1981). The comparative effects of different methods of processing rhabditid nematodes are little known. This paper describes a comparative study of several of the commonly-used methods of preparing slides of nematodes to identify the optimum procedure for parthenogenetic adult female *Caenorhabditis elegans*.

Materials and methods

Caenorhabditis elegans (Maupas) Dougherty was isolated from a sample of mushroom compost collected from Taunton, Somerset. The nematode was cultured on 3 % nutrient agar (Oxoid Ltd.) in Petri plates along with the associated bacterial floral at 22 °C. Only similar pre-egg-laying parthenogenetic adult females (with fully developed eggs in the uterus) from 6-day-old cultures were selected for this study. Males were extremely rare in this isolate.

KILLING AND FIXING

Nematodes were either killed in hot water and fixed in cold fixatives or killed and fixed in hot fixatives accord-

ing to Seinhorst (1966). Hot water-killed, unfixed nematodes served as controls. The following fixatives were used : (*a*) FA 4:1 (10 ml 40 % formaldehyde, 1 ml glacial acetic acid, 89 ml distilled water); (*b*) FP 4:1 (10 ml 40 % formaldehyde, 1 ml propionic acid, 89 ml distilled water); (*c*) TAF (7 ml 40 % formaldehyde, 2 ml triethanolamine, 91 ml distilled water); (*d*) FG (8 ml 40 % formaldehyde, 2 ml glycerol, 90 ml distilled water); (*e*) FAA (6 ml 40 % formaldehyde, 20 ml 95 % ethanol, 1 ml glacial acetic acid, 40 ml distilled water).

Water and fixatives were contained in separate glass test tubes and were maintained at 95 °C in a water bath. Selected specimens, placed in a very small drop of saline (0.9 % w/v sodium chloride) in a glass cavity block, were flooded with 4 ml of hot water, or fixative, to ensure rapid death and fixation of nematodes. For cold fixation of nematodes, excess water from the cavity blocks containing hot water-killed nematodes was removed and the specimens were flooded with 4 ml of cold fixative (22 °C). Temporary mounts of nematodes (i.e. in water or fixative) were prepared using pieces of agar (2 g agar, Oxoid Ltd.; 50 mg $CuSO_4.7H_2O$; 100 ml distilled water) as cover-glass supports (Grewal, 1990) and were sealed with glyceel. Hot water-killed, unfixed nematodes were examined for taxonomic details and were measured immediately after killing whereas the fixed specimens were maintained at 22 °C for 10 days and then studied.

Processing to mounting medium

Nematodes were killed and fixed in hot TAF and, after 10 days in fixative at 22 °C were processed to lactophenol or glycerol using one of the following techniques : (*a*) Rapid lactophenol method (Franklin & Goodey, 1949); (*b*) Rapid method to glycerol (Baker, 1953); (*c*) Glycerol-ethanol method (Seinhorst, 1959); (*d*) Slow method to glycerol (Goodey, 1963). Permanent mounts were also prepared using agar pieces as cover-glass supports and were sealed with glyceel.

Data recording and analysis

A compound microscope was used for observations of both qualitative and quantitative effects. Drawings and measurements were made on twenty individual nematodes in each treatment using differential interference contrast optics. Observations on twenty different body parameters (Table 1), most of which are commonly used in rhabditid taxonomy (Andrássy, 1983) were made. Taxonomic ratios including, a, b, c, c', V, m (promesostom length/promesostom width) and t (distance from posterior flexure of gonad to tail end as a percentage of body length) were calculated and a two-way analysis of variance was made of the data recorded. Observations on qualitative features including the appearance of cuticle and hypodermis and the clarity of structures such as the nerve ring, glottoid apparatus, œsophageal collar and excretory duct were made using high magnification and oil immersion objectives.

Table 1

Effects of killing and fixing methods on morphometrics of adult female *C. elegans* : analysis of variance

Body parameters	*Significance*		
	HF	*CF*	*HF × CF*
A : LINEAR PARAMETERS			
1. Body length	**	***	*
2. Oesophagus length	**	***	***
3. Promesostom length	NS	***	***
4. Length from anterior end to œsophageal valve	*	***	***
5. Length from anterior end to vulva	**	***	***
6. Gonad length (anterior to posterior flexure)	NS	***	**
7. Length from posterior flexure of gonad to tail end	**	**	***
8. Tail length	NS	***	NS
B : WIDTH PARAMETERS			
1. At median bulb	**	**	*
2. At posterior bulb	***	***	***
3. At vulva	*	***	***
4. At anus	**	**	NS
5. Promesostom width	***	NS	*
C : TAXONOMIC RATIOS			
1. a (= body length/greatest body width)	NS	***	***
2. b (= body length/œsophagus length)	NS	***	***
3. c (= body length/tail length)	*	NS	***
4. c' (= tail length/body width at anus)	**	**	*
5. V (= length from anterior end to vulva as percentage of body length)	NS	**	***
6. m (= promesostom length/promesostom width)	***	***	*
7. t (= length from posterior flexure of gonad to tail end as percentage of body length)	NS	***	***

HF = killed and fixed in hot fixative (95 °C); CF = killed in hot water (95 °C) and fixed in cold fixative (22 °C); HF × CF = interaction between hot and cold fixatives; NS = not significant; * $p < 0.05$ ** $p < 0.01$ *** $p < 0.001$.

Results

KILLING AND FIXING

Quantitative effects

The effects of methods of killing and fixing on certain dimensions of adult female *C. elegans* are summarised in Figs 1, 2 and 3. Neither of the methods of preparation involving the use of fixatives produced nematodes that were similar to those killed in hot water and unfixed. All fixatives caused significant ($p < 0.05$) shrinkage in most characters measured and this was particularly marked with regard to : body length, length from anterior end to vulva (Fig. 1) and anal body width (Fig. 2). Most of the commonly-used taxonomic ratios that were considered were also significantly ($p < 0.05$) altered (Fig. 3).

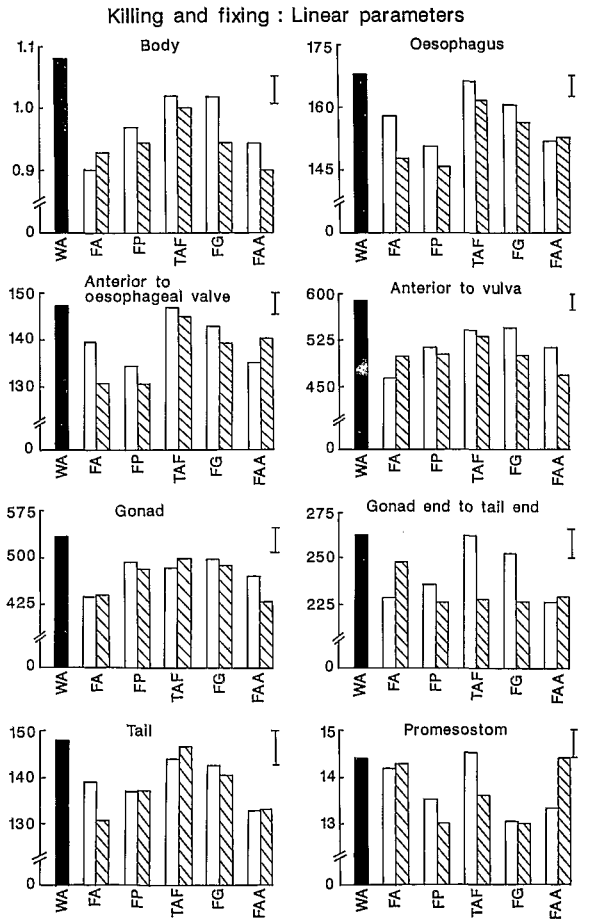

Fig. 1. Effects of killing and fixing methods on linear dimensions of parthenogenetic adult female *C. elegans*. For a key to methods refer to Fig. 2 and for the fixatives see " Materials and methods ". Bars represent L.S.D. ($p < 0.05$). All body measurements are in µm except body length (mm).

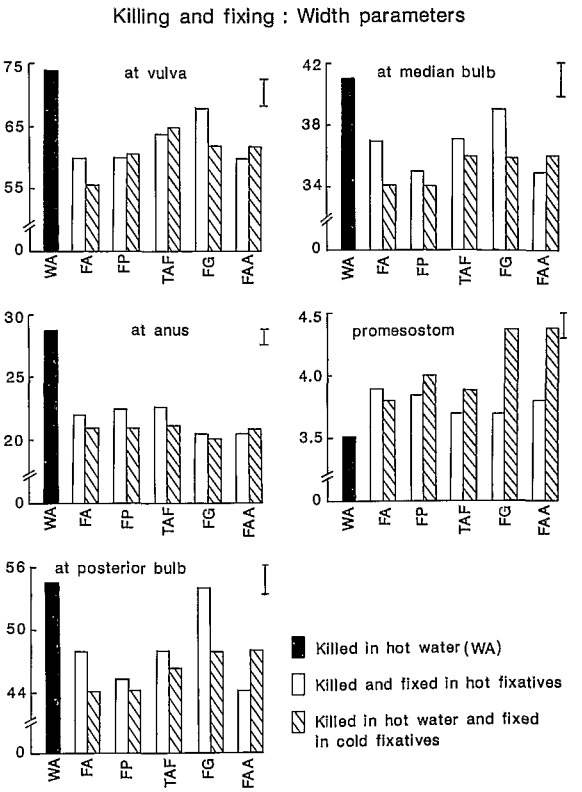

Fig. 2. Effects of killing and fixing methods on width parameters of parthenogenetic adult female *C. elegans*. For key to the fixatives see " Materials and methods ". Bars represent L.S.D. ($p < 0.05$). All body measurements are in µm.

Killing and fixing in hot fixative gave better results overall than killing with hot water and fixing in cold fixative (Table 1). Out of the 20 parameters evaluated, the former process had significant ($p < 0.05$) adverse effects on thirteen of them whereas the latter process significantly ($p < 0.05$) affected eighteen characters. Interactions between the two methods were significant for most characters indicating that a particular fixative had different effects when used hot or cold.

TAF significantly ($p < 0.05$) affected the fewest parameters. Eleven parameters were affected when nematodes were killed and fixed in hot TAF and fourteen were altered when the specimens were killed in hot water and fixed in cold TAF (Figs 1, 2, 3). The greatest changes were found with FP 4:1 and FAA : each fixative significantly ($p < 0.05$) affected eighteen parameters when specimens were killed and fixed in hot fixative and nineteen parameters when hot water-killed nematodes were fixed in cold fixative.

Qualitative effects

Cold and hot fixation in FA 4:1, FP 4:1, FG or FAA caused considerable swelling of the cuticle, and in

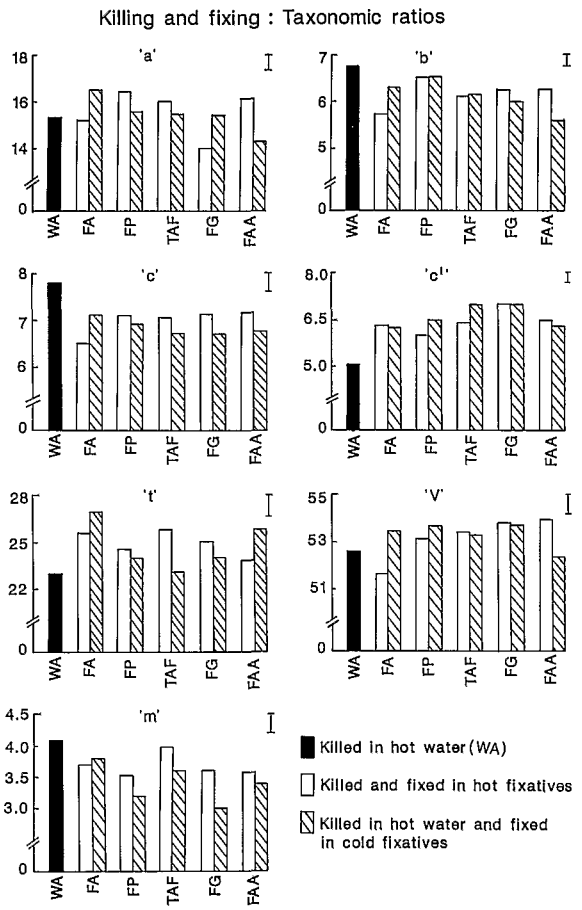

Fig. 3. Effects of killing and mounting methods on taxonomic ratios (see Table 1 for details) of parthenogenetic adult female *C. elegans*. For key to the fixatives see " Materials and methods ". Bars represent L.S.D. ($p < 0.05$).

extreme cases resulted in the cuticle tearing away from the hypodermis, distortion of the hypodermis and darkening (browning) of the specimens. However, TAF caused clearing of the specimens and improved the appearance of most features. Formalin-glycerol fixative (FG) had the most pronounced effects on cuticle thickness and this was reflected in mean width of the specimens (Fig. 2). TAF caused no such qualitative distortion and produced the most acceptable specimens. Structures including the nerve ring, promesostom, glottoid apparatus (metastom), denticles and tri-radiate œsophageal valve were most distinct in TAF-fixed specimens (observed after 10 days in fixative) but the excretory duct and œsophageal collar were more distinct in all other fixatives.

Processing to mounting media

Quantitative effects

When compared with nematodes killed and fixed with the optimal method (i.e. in hot TAF) all subsequent processing methods caused significant shrinkage ($p < 0.05$) in most characters measured (Figs 4, 5, 6). Characters including the lengths of promesostom, œsophagus, and tail which were not significantly ($p < 0.05$) affected by the killing and fixing in hot TAF were affected adversely by all the mounting methods. Processing of nematodes to glycerol by the slow method had significant ($p < 0.05$) adverse effects on the fewest parameters — 13 out of 20 studied. The rapid lactophenol method significantly ($p < 0.05$) affected the most parameters — 17 out of 20 evaluated.

Table 2 lists the dimensions (including mean ± standard error and range) of adult female *C. elegans* when hot TAF-killed and -fixed specimens were processed to mounting medium (lactophenol or glycerol) by four different techniques.

Qualitative effects

Both the rapid lactophenol method and the rapid method to glycerol caused the greatest distortion of specimens and the loosening of cuticle away from the hypodermis. Although maximum distortion occurred in the region of the œsophagus the effects extended to the

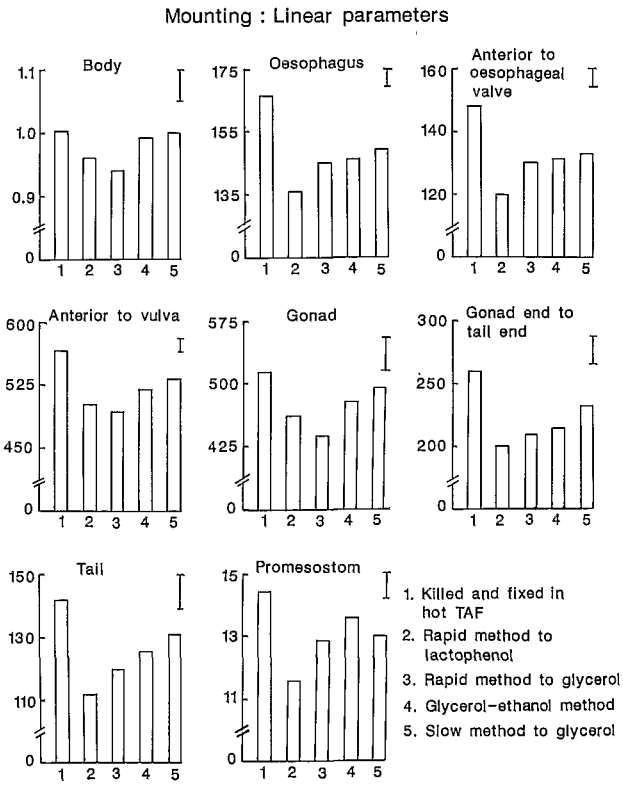

Fig. 4. Effects of mounting methods on linear dimensions of parthenogenetic adult female *C. elegans*. Bars represent L.S.D. ($p < 0.05$). All body measurements are in µm except body length (mm).

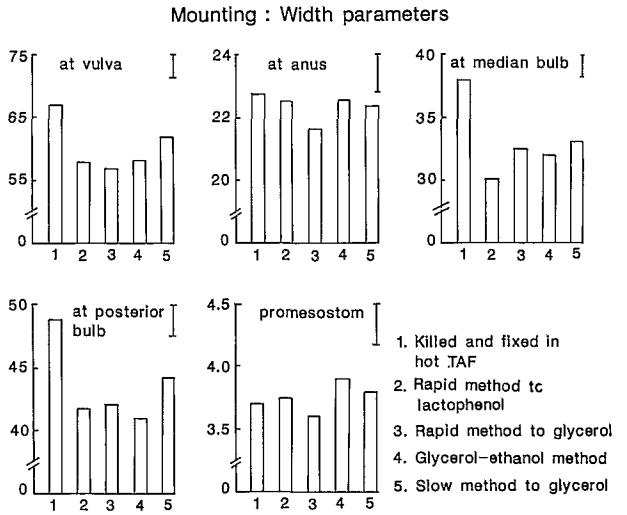

Fig. 5. Effects of mounting methods on width parameters of parthenogenetic adult female *C. elegans*. Bars represent L.S.D. ($p < 0.05$). All body measurements are in µm.

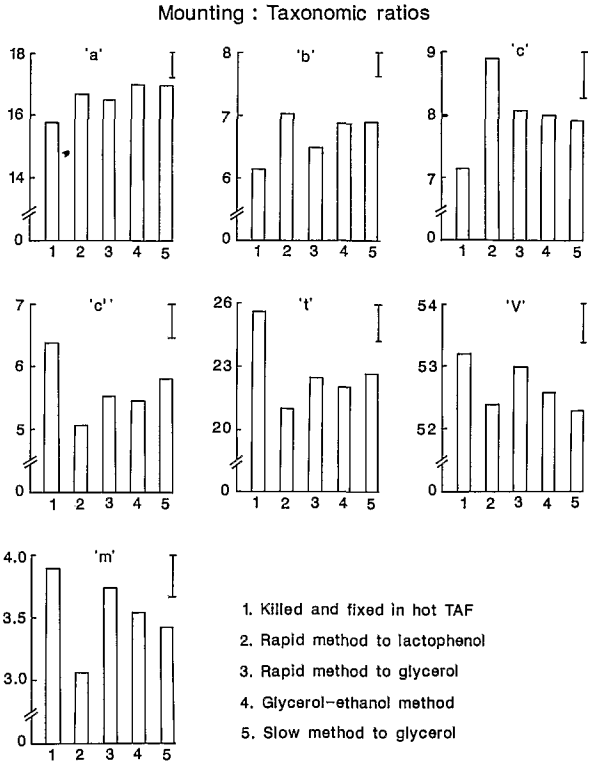

Fig. 6. Effects of mounting methods on taxonomic ratios (see Tab. 1 for details) of parthenogenetic adult female *C. elegans*. Bars represent L.S.D. ($p < 0.05$).

vulval region. The slow method to glycerol did not cause any such cuticular distortions. The glycerol-ethanol method (Seinhorst, 1959) also produced acceptable specimens.

Discussion

During this study our aim has been to compare different methods of killing, fixing and mounting to identify the optimum procedure for the preparation of permanent slides of *C. elegans*. Because all the methods studied in this investigation involve dead nematodes we used hot-water killed specimens for all comparisons, not anaesthetised or narcotised nematodes.

As expected there is no single combination of methods ranging from killing to mounting without any adverse effects on the specimens. Every stage in the process results in some degree of shrinkage and/or distortion of the specimens. Killing and fixing with hot TAF by Seinhorst's method and mounting in glycerol by the slow method produced the best specimens with measurements closest to those that were killed in hot water and not fixed. Lamberti and Sher (1969) recommended the same method to process *Longidorus africanus* for taxonomic study, but they used FAA for killing and fixing the specimens.

Curran and Hominick (1981) obtained the most life-like specimens of *Romanomermis culicivorax* and *Gastromermis* sp. by killing in water at 65 °C for three seconds, fixing in TAF for one week and processing to glycerol by Seinhorst's technique. However, the present study revealed that killing and fixing (in one process) with hot TAF produced significantly ($p < 0.05$) better specimens when compared with those killed in hot water (by Seinhort's method) and fixed in cold TAF. This may have been due to two factors : *i*) killing with hot water distorted some specimens (as the genital tracts came out through the vulva) probably due to osmotic differences, and *ii*) fixing the water-killed specimens in cold TAF caused shrinkage of specimens (due to the action of formaldehyde; Fagerholm, 1979).

Body measurements expressed as ratios are used in nematode taxonomy as they are presumed to reduce biological variability and hence are considered useful for discrimination between taxa (Cobb, 1913). However, we have observed that body measurements, even when considered as ratios, show significant differences ($p < 0.05$) when specimens of the same species are processed by different mounting techniques. Differential shrinkage of body tissues during the various processing methods may be the cause of changing ratios. For instance, killing and fixing with hot TAF (our best method) resulted in specimens with a mean c ratio (mean ± S.E.) of 7.08 ± 0.11 (for water-killed specimens it was 7.77 ± 0.26), while further processing by the rapid lactophenol method produced specimens with a mean c ratio of 8.77 ± 0.37. Furthermore, differential

Table 2

Dimensions of parthenogenetic adult female *C. elegans* killed and fixed in hot TAF (95 °C) and processed to mounting medium by four different techniques

Parameters	*Mounting methods*			
	Rapid method to lactophenol	*Rapid method to glycerol*	*Glycerol-ethanol method*	*Slow method to glycerol*
Body length (µm)	957 ± 22* 728 — 1 070**	937.2 ± 18 734 — 1 015	984.3 ± 8.7 900 — 1 037	1 016 ± 12 878 — 1 097
Ratio a	16.7 ± 0.39 13.4 — 19	16.5 ± 0.26 13.8 — 17.9	17.0 ± 0.19 15.6 — 18.5	17.0 ± 0.29 14.9 — 19.6
Ratio b	7.0 ± 0.14 5.7 — 7.8	6.5 ± 0.12 5.6 — 7.8	6.7 ± 0.06 6.2 — 7.3	6.8 ± 0.11 6.1 — 7.8
Ratio c	8.8 ± 0.37 6 — 12.5	8.1 ± 0.32 7 — 12.5	8.0 ± 0.14 7 — 8.9	7.9 ± 0.13 7 — 9.2
Ratio c'	5.7 ± 0.22 3 — 6.6	5.6 ± 0.23 3 — 7.2	5.5 ± 1.0 4.4 — 6.2	5.8 ± 0.11 5.0 — 6.8
Ratio V	52.4 ± 0.44 49.5 — 57.5	53 ± 0.74 44.6 — 62.8	52.6 ± 0.36 47.3 — 54.9	52.3 ± 0.39 49.5 — 54.6

* Mean ± standard error
** Range

shrinkage sometimes produces ratios that have no comparative value. For example, the ratio between mean body length and mean spicule length for *Hydromermis conopophaga* has been reported to be 44 (Poinar, 1968); 35 (Mulvey & Nickle, 1978) and 64 (Hominick & Welch, 1971).

Length and width of stoma are important characters in rhabditid taxonomy (Andrássy, 1983). Our observations revealed, however, that processing methods are liable to alter these parameters. For instance, the mean length and breadth of the promesostom ranged from 11.5-14.5 µm and 3.5-4.4 µm respectively in specimens of *C. elegans* processed through different mounting techniques. Significant differences ($p < 0.05$) were observed even when the ratio between length and breadth of promesostom was used as a parameter for comparing various processing techniques.

The validity of the use of ratios in nematode taxonomy has been discussed extensively (Roggen & Asselberg, 1971; Fortuner, 1984; Roggen, Revets & Van den Berghe, 1986). According to Fortuner (1984) a ratio is considered as taxonomically valid when the characters which constitute it are biologically related. This relationship must be verified by the study of the significance of the correlation between the two characters. In addition to its validity, a ratio is considered useful when its variability in a sample is lower than the variability of its constituent characters. It is evident from the present study that the processing of nematodes through various mounting techniques differentially alters the constituent characters of a ratio and thereby affects the ratios significantly.

Mounting techniques may alter the specimens to the extent that processing artifacts may sometimes be interpreted as taxonomic characters. For example, *R. culicivorax* is morphologically similar to *R. iyengari* and has been distinguished primarily by the more acute angle of the papillary tract to the œsophagus and a tendency for a thinner cuticle and egg-shell (Ross & Smith, 1976). However, Curran and Hominick (1981) have concluded that the above characteristics are processing artifacts rather than real differences and considered *R. culicivorax* as a *species inquirenda*. In the present study, such effects of processing were also evident. Hot formalin-glycerol fixative (FG), for example, increased the cuticle thickness considerably and thus resulted in an apparent increase in mean width at the vulva (Fig. 2).

Geographically isolated populations of the same species show considerable morphometric variations. For instance, the present population of *C. elegans* (processed by the optimal method) was much smaller (L = 878-1 097 µm; a = 14.9-19.6; b = 6.1-7.8; c = 7-9.2) when compared with the originally described (Maupas, 1900) Algerian population (L = 943-1 700 µm; a = 20-22; b = 5-8.5; c = 7-10). While van den Berg (1988) observed great variation in body measurements of various populations of two rhabditid nematodes, *Elaphonema messinae* and *E. mirabile* collected from different regions in South Africa. Body measurements of *Helicotylenchus dihystera* are also known to

vary with the host (Fortuner & Quénéhervé, 1980) and those of *Aphelenchoides composticola* with the amount of nutrition and population density (Franklin, 1957). Stephenson (1942) found that the measurements of *Rhabditis terrestris* varied so greatly because of culture conditions that individuals from opposite ends of a specific range could be mistaken for different species. These population variations coupled with the differential effects of mounting techniques further complicate the situation and may lead to false conclusions.

Fortuner and Wong (1984) developed a computer programme (NEMAID) for the identification of species of *Helicotylenchus* in which intraspecific variability of measurements were taken into account. The present study tends to widen the definition of intraspecific variability to include not only naturally-occurring differences caused by culture methods, host type or geographic locality, but also induced variability (i.e. the effects of handling and mounting techniques). We suggest therefore that measurements, if used for diagnostic purposes, indicate specific differences only if those differences exceed natural and induced (including fixation artifacts) intraspecific variability.

The effects of methods of killing, fixing and mounting on particular groups of nematodes vary greatly. Effects of processing techniques also depend on the species involved, the combination of methods used, the concentration of the preservatives and the time of preservation (Lamberti & Sher, 1969; Stone, 1971; Boag, 1982; Olowe & Corbett, 1983). The results of this work further support the need for concise and detailed accounts of the methods used by taxonomists when describing new species or real differences between nematode populations.

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