Moulting of *Aphelenchoides hamatus*, with especial reference to formation of the stomatostyle

Kenneth A. WRIGHT* and Roland N. PERRY**

* *Department of Zoology, University of Toronto, Toronto, Ontario, Canada, M5S1A1, and*
** *Entomology and Nematology Department, AFRC Institute of Arable Crops Research, Rothamsted Experimental Station, Harpenden, Herts., AL5 2JQ, England.*

SUMMARY

Using differential interference contrast light microscopy, detailed observations were made on the behavioural and morphological changes during the moult from fourth stage juvenile (J4) to adult of *Aphelenchoides hamatus*. The process took 12-13 h to complete. Initial changes included the loss of knobs and shaft of the stylet as the head retracted away from the old conus. The new conus was completed rapidly compared to the longer period for formation of the new shaft. The nematode's volume and water content decreased, enabling it to retract within and away from the old cuticle. During the early period, the moulting worms were most quiescent. They then began to twitch, retract and bend their heads, and ultimately the entire body moved within the old cuticle. The oesophagus became active as the adult expanded rapidly to burst the old cuticle. Shrinkage and subsequent expansion of the worms that resulted in ecdysis is thought to be due to water loss controlled by the nematodes, followed by water re-entry. Worms that did not ecdyse immediately became trapped in the old cuticle as it softened and stretched. The first stage juvenile (J1) within the egg, did not develop a stylet, head skeleton, or oesophageal cuticle. Moulting in the egg to the J2 stage tooks less than half the time for the J4 to adult moult.

RÉSUMÉ

La mue chez Aphelenchoides hamatus, *avec référence particulière à la formation du stylet*

Le comportement et les changements morphologiques liés à la mue d'*Aphelenchoides hamatus* du quatrième stade juvénile au stade adulte ont été observés de façon détaillée en microscopie à contraste de phase interférentiel. Le processus est accompli en 12 à 13 h. Les premières modifications concernent la disparition des boutons basaux et de la hampe du stylet alors que la partie céphalique se détache de l'ancien cône du stylet. Le nouveau cône est formé rapidement en comparaison de la période plus longue de formation de la partie basale. Le volume et le contenu en eau du nématode diminuent, permettant ainsi au corps de se rétracter et de se détacher de l'ancienne cuticule. Durant la première période de la mue, le nématode demeure quiescent. Puis il commence à se contracter, se rétracter, à courber la tête et à bouger le corps à l'intérieur de l'ancienne cuticule. L'œsophage devient actif lorsque l'adulte commence à se gonfler rapidement pour faire éclater l'ancienne cuticule. Contractions et expansions du nématode durant la mue sont supposées résulter des sorties et des entrées d'eau, contrôlées par le nématode. Les nématodes qui ne muent pas immédiatement restent à l'intérieur de l'ancienne cuticule devenue molle et détendue. La cuticule de l'œsophage, le squelette céphalique et le stylet ne se développent pas pendant le premier stade juvénile, dans l'œuf. La mue conduisant de l'œuf au stade J2 dure moins de la moitié du temps de la mue du stade J4 à l'adulte.

Most observations of moulting in plant parasitic nematodes have been incidental to other studies, or were included with observations of the worms' developmental sequence. Anderson and Darling (1964) determined an approximate duration of 10-17 h for moulting in *Ditylenchus destructor*, while Roman and Hirschmann (1969) found moulting in *Pratylenchus* to last 3 days. Singh and Sulston (1978) studied the general moulting process in the free-living nematode *Caenorhabditis elegans*. Carter and Wright (1984) analysed the cytology of formation of the odontostyle of *Xiphinema americanum*. Endo (1985) has shown ultrastructural features of stylet formation during the moult of *Heterodera glycines*. The present study was carried out to determine the parameters of the moulting process from the J4 to adult in *Aphelenchoides hamatus* as background to an ultrastructural study of the formation of its stomatostyle and head skeleton.

Materials and methods

Aphelenchoides hamatus was originally collected from strawberries at Bristol, England, and maintained in the Entomology and Nematology Department, Rothamsted Experimental Station, on cultures of the imperfect fungus, *Botrytis* sp. growing on potato dextrose agar.

The nematodes were observed at room temperature (about 20 °C) by differential interference contrast microscopy in slide preparations as used by Sulston (1976) for *Caenorhabditis elegans* (i.e. worms were placed on a thin agar pad in M-9 buffer and the coverslip was sealed with microscope immersion oil). Twenty nematodes were followed to varying extents through the J4 to adult moult; most were females. They were examined under a × 100 oil immersion objective and photographed with an electronic flash at intervals of 1 to 1.5 h; observation periods were kept to 5-10 min. More active intermoult nematodes were first anaesthetized with 0.1 % sodium azide. The moulting process was also followed in several nematodes mounted in artificial tap water (ATW; Greenaway, 1970), to confirm that it was not influenced by salts in the buffer. Embryonation and subsequent development in the egg were examined in similar agar slab slide mounts. Freshly laid eggs were pipetted from condensation droplets on the Petri dish lid into which females had migrated from the fungus culture below.

Observations of the moulting sequence showed that a space developed between the worm and the old cuticle. This could occur by stretching of the old cuticle, or by shrinkage of the worm inside the cuticle. To examine this aspect, volume and water content changes during moulting were determined. For volume measurements, intermoult nematodes were classed, according to the sequence shown in Fig. 3, as being at the early (approximately 3-5 h), middle (7-9 h), or late (over 11 h) phase of the moult. Individual nematodes were transferred to ATW on a microscope slide and photographed at × 200 magnification. The length and maximum diameter of fifteen nematodes of each group were measured from photographic prints (enlarged × 5) and volumes were calculated using Andrássy's (1956) formula; a slide graticule was used for calibration. The measurements were made to the inside of the cuticle, and the tip of the retracted head in the moulting juveniles.

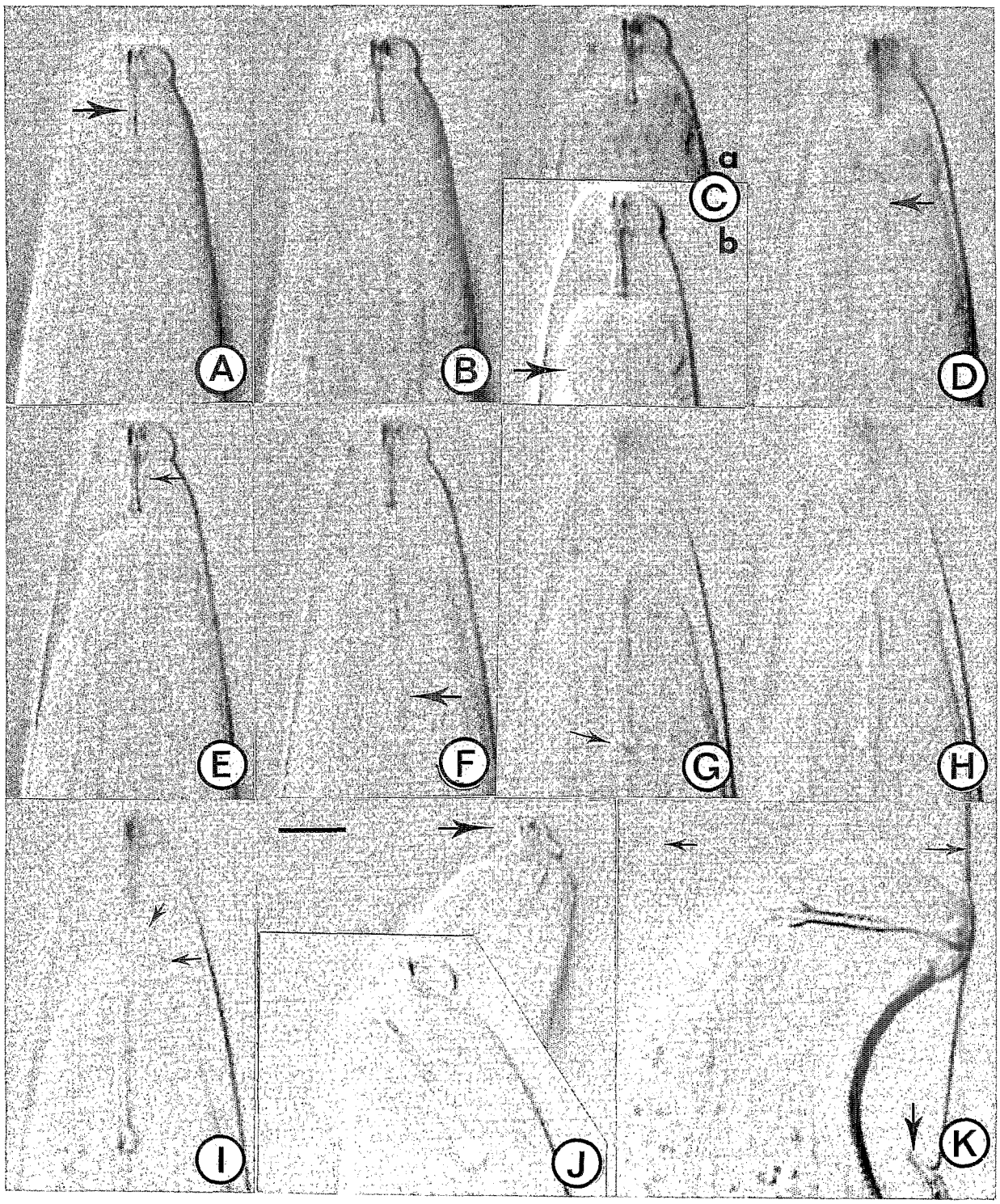
For experiments to examine changes in water content during moulting, individuals were selected from culture plates, transferred to ATW on a glass slide and classed as 3, 5, 6, 7, 9, and 12 or 13 h (see Fig. 3); their water content was then determined by quantitative interference microscopy techniques (Ellenby, 1968). The lengthy moulting period made it very difficult to follow the entire process continuously while measuring the water content at set time intervals. However, two individuals were observed over 7-8 h periods from the start of moulting and their water content was determined hourly; both individuals successfully completed the moult. The water content of individual juvenile and adult stages was also determined. Refractive index data, rather than water content values derived from them, were used for statistical analysis for reasons given by Ellenby and Perry (1976).

Results

Generally, moulting nematodes were first recognized by their reduced mobility and smoothly arched body form (not sigmoid). However, when prodded during pickup, the worms always showed some activity, though not as an effective sinuous locomotory response, but rather as a dorso-ventral thrashing movement. More coordinated movement was associated with either very early or late stages in moulting.

Since the loss and replacement of cuticle is clearly a continuous process, there are few discrete stages that can easily be used to begin timing of events. The earliest such " stage " was found to be when head tissues had separated from the old cuticle and retracted from the old head skeleton (Fig. 1 C). At this time, the shaft and knobs of the stylet had already disappeared and no cuticle was evident in the anterior oesophagus. The conus of the old stylet, apparently still attached to the old cuticle, was pulled forward in the " mouth " and was surrounded by a wider space than normal. The lip region was clearly demarcated by a pronounced constriction. This " head retraction stage " was used to time both earlier and later events. For example, at least one hour earlier (Fig. 1 B), tissue had also separated from head

Fig. 1. Moulting in the same female specimen (except Cb) of *Aphelenchoides hamatus* photographed at hourly intervals. — A : The earliest recognizable stage of moulting. The conus of the stylet (arrow) remains in the mouth, but the shaft has disappeared; B : After 1 h, little change is detectable by light microscopy; Ca : After 2 h, head tissues retract from the head cuticle, pulling the conus out of the mouth; a neck constriction (arrow) is characteristic; Cb : Another worm, about 30 minutes later in the moult; D : After 4 h, new conus (arrow) is detectable in the head; the old cuticle shows an impression of its annulation; E : By 5h, there is little evidence of shaft, but the new conus is better formed; an impression of cuticular strands (arrow) extend down from the old head skeleton around the old conus; F : By 6 h, new shaft material (arrow) is evident, but knobs are not seen; G : By 7 h, small knobs (arrow) can be seen on the new shaft; H : By 8 h, the stylet is well formed, but seems " soft "; I : About 11 h, jerking movements of the head inside the old cuticle pull the cuticular strands out of the amphids (arrows; note the enlarged ends of the strands; see also Fig. 2 B); the stylet is more completely formed; J : About 12-13 h, the adult's body fills the old cuticle, and as the worm moves its head, the collapsed old head skeleton moves to either side (pictures taken only seconds apart); K : By 14 h, the old cuticle (small arrows) stretches and thins (no annulations evident), and the adult moves within it; the stylet remains stationary, and the worm did not ecdyse; the cuticle of the excretory pore is noted (large arrow). *(Bar equivalent, A-K = 5 µm.)*


A
B
a
C
b
D
E
F
G
H
I
J
K

cuticle but this was discerned only by subsequent electron microscopy (Wright *et al.*, unpubl.). As much as 2 h earlier (Fig. 1 A), much of the shaft and all of the knobs had already disappeared. It was not possible to identify any earlier moulting stage; this is taken as the first hour into moulting. Apparently loss of knobs from the stylet is rapid. By hour four, the head had retracted further from the old cuticle so that the old conus was entirely withdrawn from the mouth. At this time (Fig. 1 D), a new conus (about 3-4 µm long), although not as refractive as the fully formed conus (6 µm long), was detectable in the head. Conus material was formed prior to this, but was detected only by electron microscopy. The full length of the conus was present by about hour 4.5 (Fig. 1 E). Shaft material was detectable from 4 h (Fig. 1 F); the more anterior shaft appeared rigid as the worm moved its head. The full length of the shaft was present by about hour 6 and small knobs were then present (Fig. 1 G). The shaft continued to thicken and knobs enlarged until about 8 h (Fig. 1 H). After this time, knobs became more refractive (Fig. 2 A). After hour 4, the space between the head tip and the old head skeleton cuticle remained at about 8-10 µm. Fine strands, apparently the cuticle lining of the amphidial canals, extended from the amphids to the head skeleton (Fig. 2 B, C). The ends of these strands, inserted in the amphid, were expanded and slightly refractive (Figs 1 I, 2 B). As the worm's activity increased about hour 11, these strands were pulled out of the amphids (Fig. 2 D). In some specimens, it seemed that a fine strand of cuticle also connected the old head skeleton to the mouth opening.

Undisturbed worms were least active from hour 3 to 4. From hour 4, the head showed increasing twitching movements inside the old cuticle. With increasing coordination of these movements the entire head, including the old cuticle, began to move back and forth laterally, then the whole head would be pulled backward and relax forward again. The space between the head and the tip of the old cuticle gradually increased. By hours 11 or 12, the body began to move backward and forward in the old cuticle. These strenuous backward jerks " unplugged " the cuticular strands from the amphids. Later still, body movements gave a writhing action and, finally, coordinated body waves resulted in forward or backward locomotion.

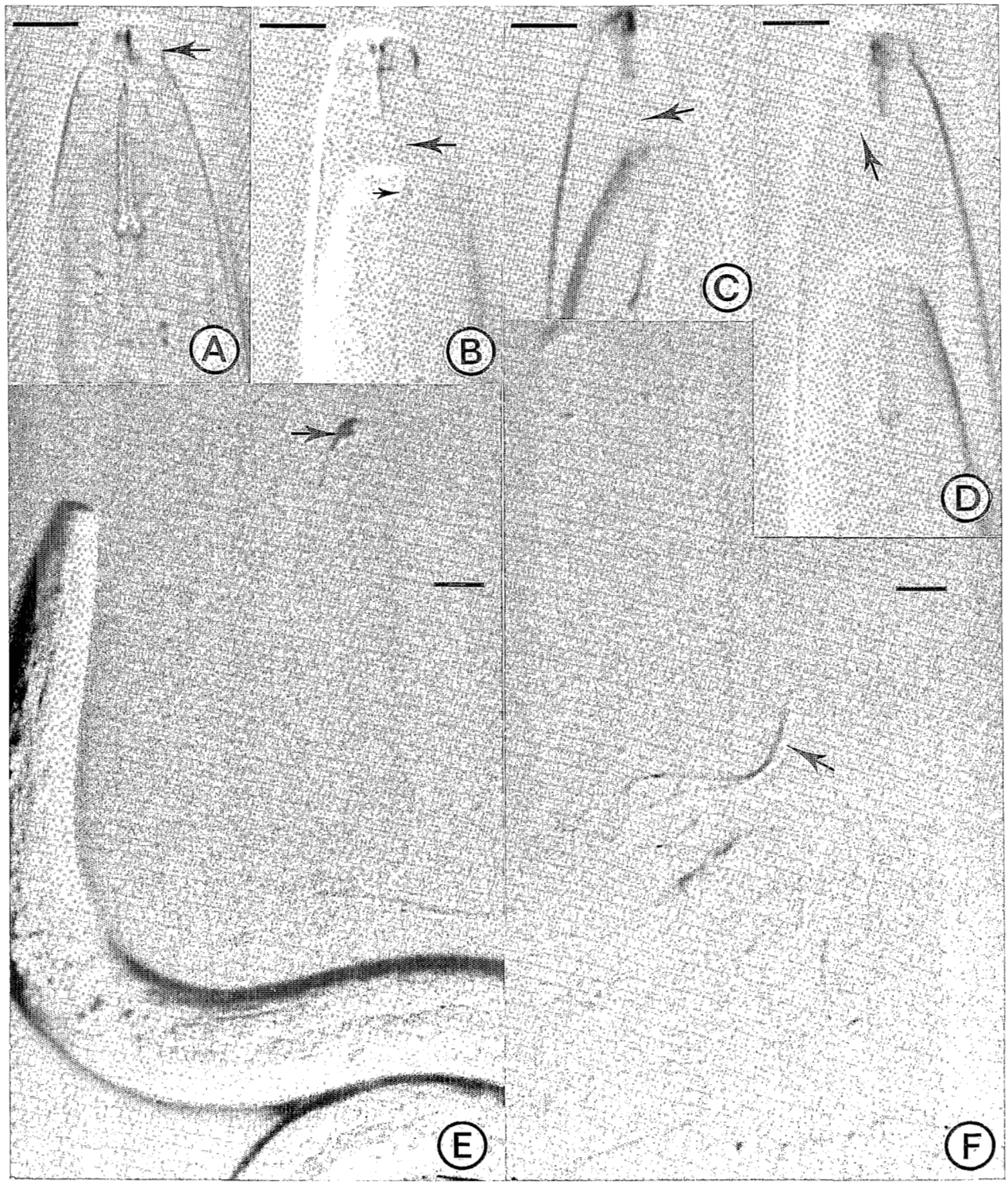
About hours 11-12, the metacorpus became active with long bursts of rapid " pumping " action (opening and closing of the pump chamber). Later on, bursts of pumping activity were shorter with initially rapid action becoming markedly slowed. The anterior oesophagus (procorpus) seemed to lengthen, and its lumen became straightened just before pumping, then with the last few slower pump actions, the oesophagus lumen undertook a few whip-like waves that travelled from the back to the front. This movement slightly joggled the stylet but did not result in the stylet tip being projected through the mouth. The metacorpus showed this activity over a period of about one hour. Initially the intestinal lumen was tightly closed, but after hour 11, the posterior intestinal lumen was expanded. About this time, or just after activity of the metacorpus began, the old cuticle thinned and lost its annulations. At hours 12 to 13, the worm's body expanded to fill completely the old cuticle. It was then evident that the old cuticle was more pliable and the old head skeleton was softened and collapsible. The worms began a " writhing " kind of activity and a few ecdysed from the old cuticle at this time. The old cuticle of those that did not ecdyse stretched, leaving a large space around the worm in which the worm was free to move. In this old cuticle, the linings of the excretory pore and the cloaca could be seen easily. At no time was the stylet seen to be thrust through the mouth, although the head often abutted the cuticle at right angles (Fig. 1 K). Ecdydis was seen only twice. Once the cuticle broke into two pieces explosively just behind the excretory pore (Fig. 2 E). The worm slid quickly backwards out of the front part of the cuticle, then moved forward out of the back part (Fig. 2 F). In the second ecdysis observed, it was not apparent where the cuticle burst. The moulting sequence and activities are summarized in Figure 3.

Volume determinations showed that there is no difference in volumes of J4 intermoult nematodes, or J4 nematodes at the early or middle phases of moulting (475×10^{-7} mm^3 $\pm$ 139×10^{-8} and 476×10^{-7} mm^3 $\pm$ 142×10^{-8}, respectively) but there is a significant decrease ($P < 0.05$) in the volume of nematodes at the late phase (425×10^{-7} mm^3 $\pm$ 204×10^{-8}). This is a decrease of 10.7 %.

The mean water content (%) of juvenile and adult stages is as follows :

J2 : 74.5 $\pm$ 0.7 (n = 45)
J3 : 74.7 $\pm$ 0.7 (n = 33)

Fig. 2. A : Same female as in Fig. 1; by 12 h, the stylet shaft and knobs are refractive; note that the worm entirely fills the old cuticle; the old head skeleton (arrow) is not yet collapsed; B, C, D : Moulting worms showing the streched cuticle of the amphidial canals; B : At 5 h, a fine strand (large arrow) extends from the old head skeleton to an enlargement in the amphid (small arrow); C : At 8 h, the amphidial strand remains connected, although the worm's head is beginning to move; D : At 11 h, strenuous movement of the worm, inside the cuticle, pull out the amphidial strands (arrow) that dangle from the old head skeleton (see also Fig. 1 I); E, F : A successful ecdysis; the old cuticle ruptured just below the excretory pore, the worm backed out of the anterior cuticle, and then moved forward out of the posterior cuticle; arrows note the old head skeleton and cuticle of the excretory pore. *(Bar equivalent = 5 µm.)*


A
B
C
D
E
F

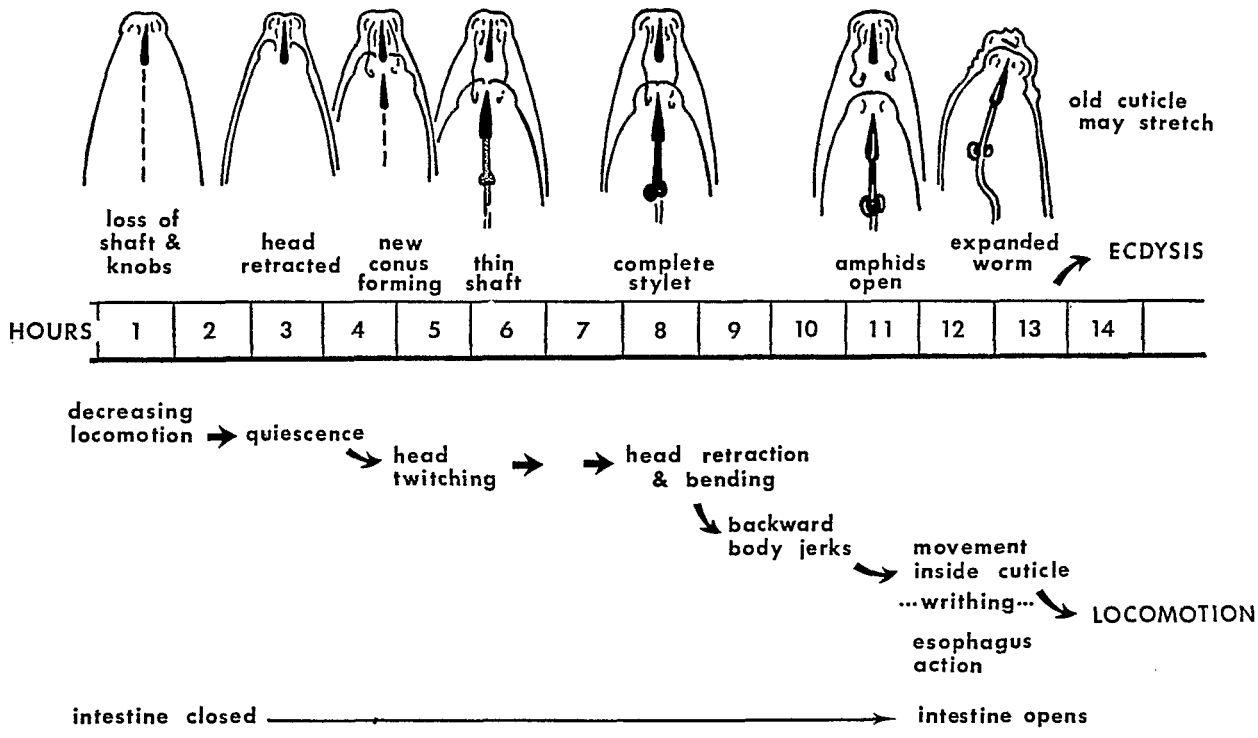


Fig. 3. A schematic representation of events in the moult sequence of *Aphelenchoides hamatus.*

J4 : 76.1 ± 0.7 (n = 29)
Male : 75.5 ± 0.7 (n = 24)
Female : 75.7 ± 0.5 (n = 26)
Adults (♂ + ♀) : 75.6 ± 0.5 (n = 50).

There was no significant difference in the water contents of any intermoult stages, or between males and females ($P > 0.05$). By contrast, analysis of variance shows a significant decrease ($P < 0.01$) in the water content of individuals later in the moult (Fig. 4 A). Between 3 and 9 h periods, the water content remained stable ($P > 0.05$), but there was a significant reduction ($P < 0.01$) in water content from 76.2 ± 0.6 % at 9 h to 73.4 ± 0.6 % and 71.9 ± 1.0 % at 11 and 13 h respectively. The mean water content for adults was 75.6 ± 0.5 %. The water content of the two individuals followed continuously during the moult showed a similar pattern (Fig. 4 B) with a marked reduction in water content after 9 h. The water content of one of them (solid circles and star) immediately before the old cuticle burst, was 77.9 %, while it had been 73.1 % at 12 h, and mean adult values were 75.6 %.

The first stage juvenile developed in eggs within about 48 h. Although the cuticle lining of the rectum was detectable, no stylet or oesophagus cuticle was seen. The head lacked a " neck " shape. A moult to the second stage juvenile occured about 55-60 h from the beginning of embryonation, and a stylet, oesophagus cuticle and bulb were then visible. The moult process may take as little as 4 h. In the ecdysed cuticle within the egg, no clear head skeleton was visible, though cuticle that would have extended into the mouth, and traces of amphidial canal cuticle could be seen. At hatching, the egg shell became thinner and more pliable, and the juvenile used strong stylet thrusts to puncture holes in it.

Discussion

Under the conditions used here, moulting in *Aphelenchoides hamatus* took about 12 to 13 hours to complete. Initial separation of the cuticle from the epidermis (apolysis) could not be recognized by light microscopy. However, the subsequent separation of head tissues from the old cuticle became obvious about 2 h after the shaft of the old stylet began to disappear (such separation did not occur at the tail of the worm). Formation of the stylet and head skeleton progressed while the worm was thus retracted. It seems likely that this separation of the head of the worm from its original cuticle would be due to shrinkage of the worm tissues due to water loss. However, such small reductions in volume and water content would not be measurable until their magnitude increased later in the moulting period. It is also possible that, since both water content and volume decrease together, there is no net reduction of water content until

late in the moult when tissues may not be further compressed. Significant reductions in volume and water content were measured after 9 hours into the moult. The nematodes must actively reduce their water content during this time. At completion of the stylet and head skeleton, the worm again expanded to fill the cuticle and seemingly to burst it. This was likely accomplished by a rapid return of the nematode's water content to that of the intermoult/adult level or higher (see Fig. 4 B). The increase in water content might have been effected by the bursts of muscular activity seen in the metacorpus. *Aphelenchoides* does not use the stylet to punch holes in the old cuticle at ecdysis as it does in the egg shell at hatching (Doncaster & Seymour, 1973 and confirmed here). Probably in nature the worm's writhing activity promotes mechanical interaction with the environment that aids in rupturing the old cuticle. In microscope slide preparations, many worms became trapped in their old cuticles which had thinned and stretched around them. The thinning of the old cuticle occurred just after the pumping activity of the metacorpus. This suggests that esophageal secretions may have aided in the thinning process, though no movement of gland granules was noted. Perhaps secretions were released during the rapid pumping action, while the slower action followed by the whip-like anteriad motion of the procorpus lumen might have produced suction that could be important in swallowing the remains of the old procorpus lumen cuticle.

Although the excretory system is frequently implicated as the source of moulting fluid for ecdysis (Sommerville, 1982), there were no changes or activities seen in this system during the moulting of *A. hamatus*. Instead, just prior to ecdysis, the metacorpus of the esophagus was very active. In *Caenorhabditis elegans*, the dorsal esophageal gland was implicated in ecdysis as granules flow anteriad in its duct to its opening into the procorpus lumen just below the buccal capsule, only near the end of moulting (Singh & Sulston, 1978). Perhaps in aphelenchids, where the dorsal esophageal gland opens into the anterior metacorpus, secretions are released from the mouth during the pumping action of the metacorpus. As noted above, they might serve to thin or soften the old cuticle, though successful ecdysis seems to follow quite quickly and perhaps relies on internal pressure rather than enzymatic degradation.

Breakdown of the old stomatostyle shaft progressed from posterior to anterior. Formation of the shaft, however, progressed from the anterior to posterior. Conus was formed first, and apparently also from anterior to posterior. It may have been formed at a rate of about 3-4 µm per hour, while the shaft may be formed at about 1 µm per hour. The overall rate of formation of the stylet could be 1-2 µm per hour, similar to the rate of formation of the odontostyle in first stage juveniles of *Xiphinema diversicaudatum* (Flegg, 1968).

The amphids of *Aphelenchoides* remain connected to the old cuticle until just prior to ecdysis. It is nevertheless unlikely that they are functional, as electron microscopy shows that the cuticular strands (cuticle lining of the amphidial canals) are collapsed with almost no patent lumen. Such reduced chemosensitivity may be related to the characteristic lack of mobility of moulting worms. It was noted that mechanical probing led to thrashing activity, but not locomotion. Moulting nematodes may display uncoupling of their sensory and locomotory control mechanisms.

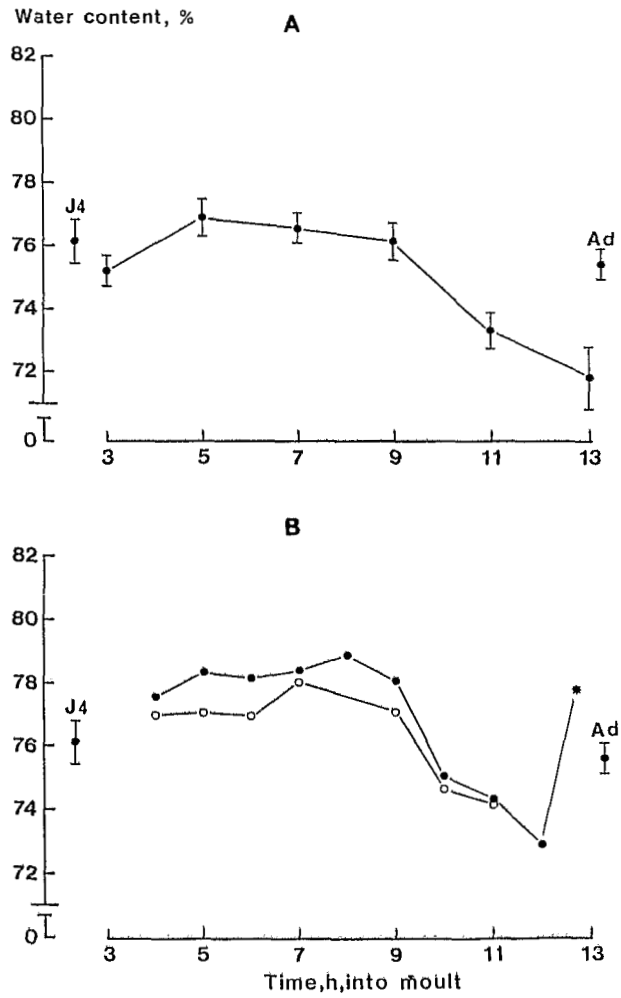


Fig. 4. A : The water content of *Aphelenchoides hamatus* at various time during the moult from fourth stage juvenile to adult (vertical lines represent the standard error of the mean; n = 20); B : Water content changes of two *Aphelenchoides hamatus* followed continuously through the moult from fourth stage juvenile to adult; the star notes the unusually high water value of one individual just before ecdysis; values for mean water content of fourth stage juveniles and adults (Fig. 4 A) are included for comparison.

It is interesting that the first stage juvenile within the egg does not form a well differentiated esophagus, buccal capsule (stylet), or head skeleton. While the complete moulting cycle of nematodes is conserved, differentiation of the first stage juvenile seems to be abbreviated.

References

ANDERSON, R. V. & DARLING, H. M. (1964). Spear development in *Ditylenchus destructor.* Thorne. *Nematologica,* 10 : 131-135.

ANDRÁSSY, I. (1956). Die Rauminhalts- und Gewichtsbestimmung der Fadenwürmer (Nematoden). *Acta zoologica Academiae Scientiarum hungariensis,* 2 : 1-15.

CARTER, R. F. & WRIGHT, K. A. (1984). Formation of the odontostyle during moulting of the nematode *Xiphinema americanum* (Nematoda : Dorylaimoidea). *Journal of Ultrastrure Research,* 87 : 221-241.

DONCASTER, C. C. & SEYMOUR, M. K. (1973). Egg transport in the genital tract and egg-laying by *Aphelenchoides blastophthorus. Nematologica,* 19 : 379-388.

ELLENBY, C. (1968). Determination of the water content of nematode worms by interference microscopy. *Experientia,* 24 : 84-85.

ELLENBY, C. & PERRY, R. N. (1976). The influence of the hatching factor on the water uptake of the second stage larva of the potatoe cyst nematode *Heterodera rostochiensis. Journal of experimental Biology,* 64 : 141-147.

ENDO, B. Y. (1985). Ultrastructure of the head region of moulting second stage juveniles of *Heterodera glycines* with emphasis on stylet formation. *Journal of Nematology,* 17 : 112-123.

FLEGG, J. J. M. (1968). Embryogenic studies of some *Xiphinema* and *Longidorus* species. *Nematologica,* 14 : 137-145.

GREENAWAY, P. (1970). Sodium regulation in the freshwater mollusc *Limnaea stagnalis* (L.) (Gastropoda : Pulmonata). *Journal of experimental Biology,* 53 : 147-153.

ROMAN, J. & HIRSCHMANN, H. (1969). Embryogenesis and postembryogenesis in species of *Pratylenchus* (Nematoda : Tylenchida). *Proceedings of the helminthological Society of Washington,* 36 : 164-174.

SINGH, R. N. & SULSTON, J. E. (1978). Some observations on moulting in *Caenorhabditis elegans. Nematologica,* 24 : 63-71.

SOMMERVILLE, R. I. (1982). The mechanics of moulting in nematodes. In : Meerovitch, E. (Ed.). *Aspects of Parasitology.* Montreal, Institute of Parasitology, McGill University : 407-433.

SULSTON, J. E. (1976). Postembryonic development in the ventral cord of *Caenorhabditis elegans. Philosophical Transactions of the Royal Society, B,* 275 : 287-298.

Accepté pour publication le 7 août 1990.