

Reproductive Behaviour of the Free-Living Marine Nematode *Chromadorita tenuis*

P. Jensen

Marine Biological Laboratory, University of Copenhagen, Strandpromenaden, DK-3000 Helsingør, Denmark

ABSTRACT: Premating behaviour of the free-living marine nematode *Chromadorita tenuis* consists of movements of the male towards the adult virgin female stimulated by a pheromone released by the female. The vaginal gland cells are suggested as possible site of pheromone production. Sperm cells or an accompanying substance probably inhibit pheromone release after insemination. At close range, the attracted male shows pre-copulatory behaviour consisting of reverse locomotion which is not sex-specific since it also occurs between males. Mating itself consists of the male coiling the posterior part of the body around the female. The single ventral seta anterior to the male cloaca is suggested to act as receptor of mechanical stimuli (vibrations, touch) involved during, or preceding, mating. Insemination results in fertilization, egg shell formation, oviposition and loss of the capacity to attract males. Females mate only once during their life. Adult virgin females are continuously attractive to males; ovulation proceeds continuously; eggs are deposited in the proximal chambers of the uterus. There is no egg shell formation, nor are the unfertilized eggs deposited in the substrate; they are resorbed. At the age of 32 d, virgin individuals of both sexes have as high a fecundity/fertilization capacity as newly matured 16-d olds.

INTRODUCTION

While it is now well established that free-living nematodes are the most diverse, abundant and widespread marine metazoan taxon (Fenchel, 1978; Platt and Warwick, 1980), almost nothing is known about their reproductive mechanisms. For the first time we present here new pertinent information, based on results of laboratory experiments on *Chromadorita tenuis*, and attempt to relate structural properties with functional details during partnership events. Our results are not comparable to related studies on other marine species, but should be evaluated in the light of findings from parasitic and terrestrial nematodes.

The study is part of a project on the behaviour and ecology of Baltic Sea nematodes carried out at the Tvärminne Zoological Station, University of Helsinki, Finland.

MATERIAL AND METHODS

Chromadorita tenuis (G. Schneider, 1906) was collected from 11 different submerged macrophytes (Jensen, 1979) from brackish waters in the vicinity of Tvär-

minne Zoological Station, southern archipelago of Finland. Adult *C. tenuis* are about 1 mm in length; sexes are separate, reproduction continuous, and there is normally a sex ratio in the field of 1 male to 2 females. The nematode lives on submerged macrophytes, but in winter occurs sometimes in the detritus on the sea bottom (Jensen, 1981). Its food consists mainly of diatoms (Jensen, 1982). The species occurs in all European mesohaline waters.

At low magnification, the sexes can be clearly differentiated at the fourth juvenile stage on the basis of both primary and secondary sexual characteristics. Body length and organic carbon contents also allow separation at the third juvenile stage. Differentiation between an old juvenile and a young adult is primarily based on presence or absence of a replacement tooth in the buccal cavity and, more specifically whether or not the vulva of the female and the preanal supplements of the male are covered with cuticle. The reproductive systems are illustrated in Figs. 1 and 4.

The female gonad consists of 2 opposed and reflexed ovaries, oviduct and uterus; it opens through the ventral vagina and vulva. The uterus consists of a central muscular chamber with 2 pairs of spermathecae and 2 proximal thinwalled chambers. Vaginal gland cells are

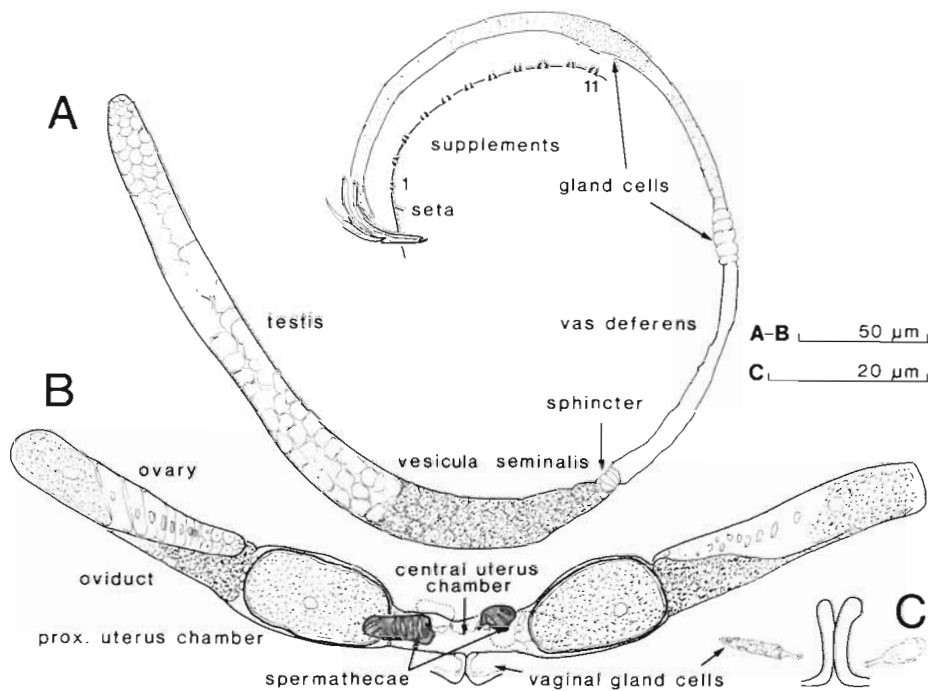


Fig. 1. *Chromadorita tenuis*. Right lateral views of gonads. A male; B fertilized female; C vaginal region of adult virgin female

present, very difficult to detect in fertilized animals but fairly evident in adult virgin females: 1 pair on each side of the vagina, each emptying through a fine duct and pore subventrally close to the vulva (Fig. 1 C).

The male gonad consists of a single outstretched testis, a coarsely granulated vesicula seminalis, a sphincter and a vas deferens differentiated into 3 zones with 2 different types of glandular epithelia. It opens, together with the intestine, into a cloaca at the ventral side. The copulatory apparatus consists of 2 curved spicules, each with a guiding piece (gubernaculum). In front of the cloaca, 11 ventral cup-shaped supplements are present, together with a single small stout seta just in front of the cloaca.

Nematodes were kept alive temporarily in small dishes with natural sea water and vegetation and long-term cultivated on a 0.8% Agar inoculated with the pennate diatom *Nitzschia* sp. (Jensen, 1982). By 1980/81, the information accumulated on the factors controlling the whole life span of *Chromadorita tenuis* permitted laboratory experiments to be carried out – using a large stock of eggs and individuals with known life history – independent of the season. Such stocks were used herein as well as original field material from and subsequent to 1978/79.

Sexual attraction experiments were conducted on sterile Agar plates 8.4 cm in diameter; 25 fertilized and 25 virgin adult females were incubated for 24 h in separate sterile Agar batches in order to produce "female agar". Knowing that previously identified pheromones are waterborne (Jones, 1967; Bone and Shorey, 1978) a small portion of this "fertilized female

agar" and "virgin female agar" was then used as attractants. A 24-h incubation is sufficient to produce a sexual response. Attraction of the male is elicited by the mature virgin female – not vice versa and not among specimens of the same sex. We used 25 mated males to test sex attractants released from the two types of "female agar". The males were placed together in the centre of the disc and allowed to migrate in all directions. The bottom of the disc was divided into 6 zones to the left and to the right of the inoculum. The "female agar" or the control was inoculated in Zone 4. The position of individual males was recorded 1, 2 and 3 h later. Each set of experiments was repeated 4 times with new individuals and dishes. The use of mature males, raised isolated from egg or first juvenile stage, often spoiled the experiments since many such individuals started pre-copulatory and mating behaviour with each other in the inoculum and did not migrate.

RESULTS

Virginity

Males of *Chromadorita tenuis* are specifically attracted to neither "fertilized female agar" nor to the control (Fig. 2 A). The males did not move very much. Only a few individuals migrated far away from the centre, but never into a patch. "Virgin female agar" strongly attracted males, independent of a control or "fertilized female agar" (Figs. 2 B-C; 3 A). This

response was observable after 1 h. After 3 h, all males were found in the left part of the dish containing "virgin female agar", and more than 80 % of the males were found inside this inoculum. The males continuously moved about within this patch, but no pre-copulatory behaviour or mating behaviour were observed.

In 2 additional tests, 20 fertilized females plus 1 young mature, virgin female were kept together in a small dish with natural sea water and vegetation. A male was introduced. Apparently, it moved randomly not taking notice of the females. However, after about 1 h the male was so close to the virgin female that his

locomotion pattern drastically changed into pre-copulatory behaviour, followed by mating. The movement of the male towards the virgin female strongly indicates the release of a female-virgin substance and a perception of this substance of the male. Candidates for receptors are the amphids and setae or papillae of the cephalic sense organs.

A virgin female of *Chromadorita tenuis* is structurally characterized by distinct vaginal-gland cells accompanied with ducts emptying along subventral pores close to the vulva (Fig. 1 C). Spermathecae are indistinct. Sometimes many, irregularly-shaped eggs are present in the uterus (Fig. 4 C). They do not have a distinct shell, but the walls of the central uterus chamber are very distinct although the uterus volume is small (Fig. 4 C). When a virgin female is continuously kept isolated, the egg situated most distally in each proximal chamber, i. e. the first one released from the ovary, changes its colour from dark to pink. High magnification reveals that the contents of these eggs disappear (Fig. 4 C).

Pre-Copulatory Behaviour

The pre-copulatory behaviour is defined here as backwards movement of the male body and ventral curving of its tail (Fig. 3 B-C). No movements of the copulatory apparatus were observed. This type of pre-copulatory behaviour may be repeated several times. Movement of the female seems not to be influenced. The same behaviour is also observed when virgin males are brought together, suggesting that a sex-specific substance is not involved. Since the partners do not have physical contact with each other during this premating stage, tactile responses also seem to play no role. This leaves vibrations caused by the partner as the most plausible stimulus. Along the entire length of the body, 4 rows of somatic setae and pores are present, and ventrally in front of the cloaca 2 special types of sense organs: a single small stout seta and 11 cup-shaped supplements (Fig. 1 A). At present, it is not possible to decide whether these organs play a role as receivers of vibratory stimuli. However, it seems reasonable to search for such a site along the posterior body of the male, since the pre-copulatory behaviour is fully centred along this part of the body.

Mating Behaviour

Moving backwards, the male coils its tail around the posterior part of the female body (Fig. 3 D). In the following seconds, the loop consists of 2½-3 turns. It is

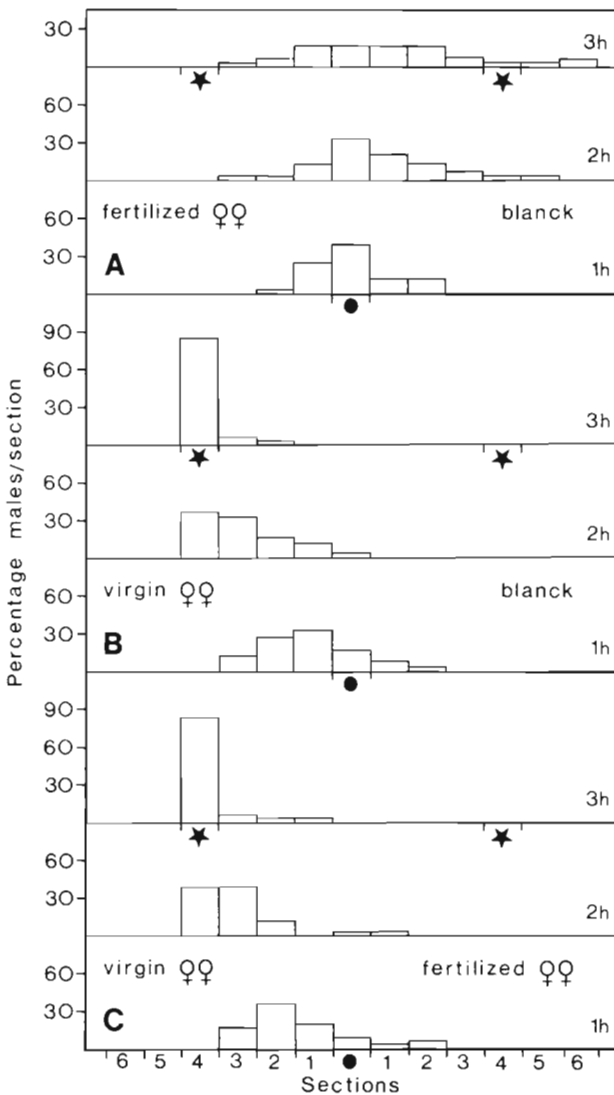


Fig. 2. *Chromadorita tenuis*. Distribution of male specimens in sectors of an Agar dish after 1, 2 and 3 h of testing attractive stimuli from fertilized females (A), adult virgin females (B), both (C). Section 0: inoculation zone of males; Section 4: inoculation zone of female material. Each histogram is the mean of 4 replicates

accomplished by continued backward moving of the male body. Apparently in order to locate the vulva, the male continues to spiral around the female body, although the number of turns remains constant. This spiralling is often interrupted while the female may continue to feed. The male seems to be fully occupied with mating activities. Unfortunately, during spiralling it was not possible to observe any possible actions of supplements, single seta or copulatory apparatus.

Spiralling and female gliding within the coil is stopped when the male inseminates (Fig. 3 E). Both partners hardly move at all during insemination, apart from a few head movements. Insemination lasts 3 to 5 min; it is terminated by the female initiating wriggling and twisting. While the male does not uncoil, the female glides forward so that the male must release any contact of preanal supplements.

The female is inseminated only once in her life; this results in 1 brood. We have tested this several times, using different male densities around a female which had mated before, and different temperatures and time schedules of illumination simulating different seasons.

Isolated males were sometimes observed coiling their tail around their own oesophageal region, with the body moving therein for a few seconds. When previously isolated males were brought together, their initial pre-copulatory behaviour also developed into mating behaviour with coiling. However, in most cases the advancing partner was rejected very soon.

The 11 cup-shaped supplement of the male most likely facilitate close contact with the female during

search for her vulva and during mating. Since the ventral seta of the male must necessarily touch the female during coiling, it is possible that it acts as tactile receptor for the vulva, and thus evokes spicule movements when the vulva is located.

On attaining maturity no differences were observed in the male's reproductive system independent of its life history.

A newly inseminated female has all 4 spermathecae filled (Figs. 1 B; 3 F; 4 A); also its central uterus chamber is expanded and totally filled with very finely granulated sperm cells and possibly an additional substance. No sperm cells or other materials were observed in the proximal uterus chambers. Sometimes sperm cells were left on the vulva in the form of a mucous mass, but more often this substance was released by the female after she had emptied the sperm mass from the central uterus chamber in the substrate.

Fertilized eggs are oval and have an ornamentated shell (Figs. 1 B; 4 B). They were never observed in the oviducts. Eggs are stored in the proximal uterus chambers. The eggs become strongly deformed when they pass through the closing cells between proximal chamber and central chamber and through the vagina. This process is completed within seconds. Eggs were never observed in the central uterus chamber of fixed females. Mostly, only 1 fertilized egg is present in 1 or in each of the 2 proximal chambers. On 2 occasions a fertilized egg was observed deposited in the substrate, where it became more spherical.

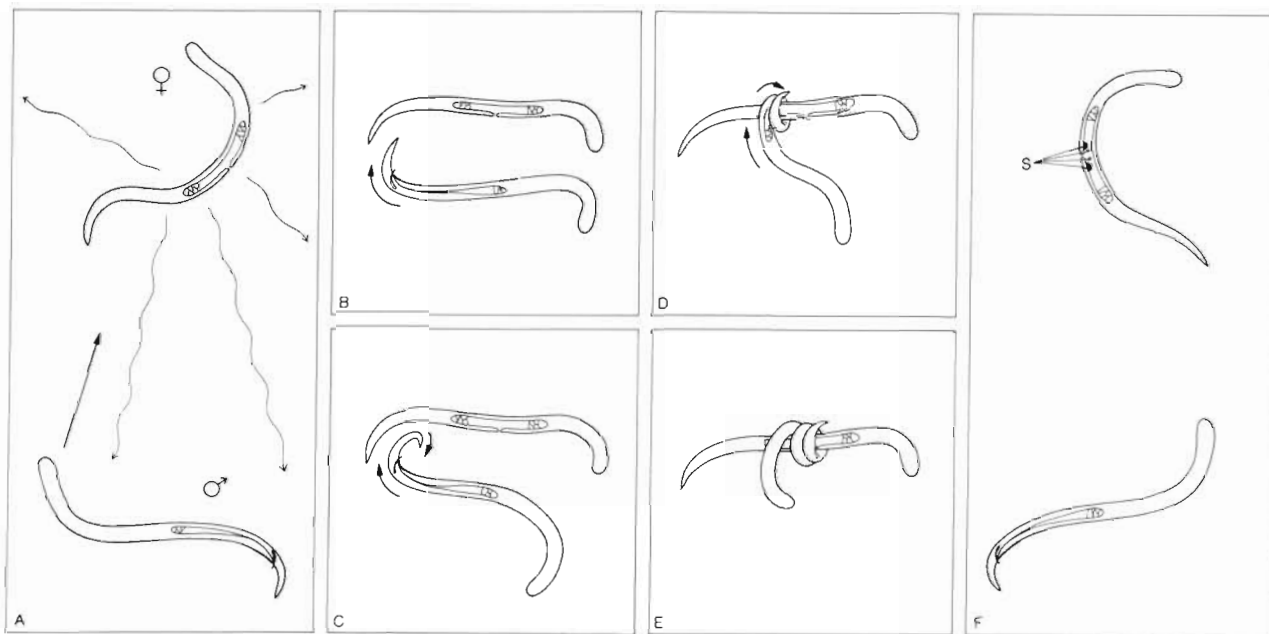


Fig. 3. *Chromadorita tenuis*. Sequence of events in sexual behaviour. A sexual attraction at long distance; B-C pre-copulatory behaviour; D-E mating behaviour; F disintegration (S: spermathecae). Not to scale

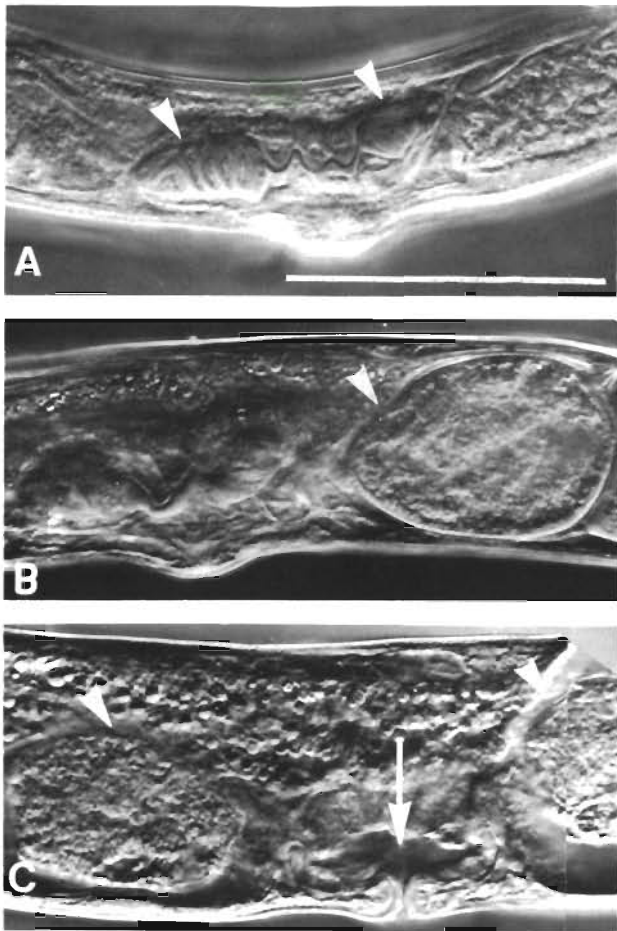


Fig. 4. *Chromadorita tenuis*. Right lateral views of the female reproductive system. A filled spermathecae; B fertilized egg in the proximal uterus chamber; C central uterus chamber and 1 egg in each proximal uterus chamber in an adult virgin. Scale = 50 μ m

Fecundity and Fertilization Capacity

In order to test the fecundity of isolated 32-d old (from time of deposition) virgin females and the fertilization capacity of 32-d old virgin males they were mated as well as with newly matured 16-d old males and females. The 32-d old virgin individuals were not significantly different in their capacity to fertilize and produce offspring. The duration from insemination to the first egg laid was slightly higher in old females compared to newly matured females, 2.9-d versus 2.0 d at 18 °C.

DISCUSSION

The results presented strongly support the hypothesis of Greet (1964) that sexual attraction and copulation are independent events requiring separate

stimuli. The attracting substance, a pheromone (Bone and Shorey, 1978), released by the adult virgin female of *Chromadorita tenuis* is sex-specific and serves only to bring the males close to her, after which other stimuli take over. *C. tenuis* has a distinct pre-copulatory behaviour: initiating mating the male moves backwards ("reversing" sensu Croll, 1971) coiling its tail and posterior body, without physical female contact. The stimulus evoking this behaviour is not sex-specific (some males also elicit this behaviour). Presumably, vibratory stimulation is involved. The stimulus evoking the mating behaviour is clearly tactile: physical contact immediately triggers the backwards coiling of the male tail around the partner. Since in this phase the male does not make any distinction between the sex of the partner, it is likely that no chemical compounds are involved.

Marchant (1970), Cheng and Samoiloff (1972) and Bone et al. (1978) provided evidence that the female reproductive system is the site of pheromone production. However, Croll and Wright (1976) failed to confirm Marchant's results. The findings reported here also support that the female reproductive system is the site of pheromone production, probably the vaginal gland cells of adult virgins (Fig. 1 C, Table 1). While this conclusion is based on microscopical observations and on the fact that only virgin females of *Chromadorita tenuis* attract males, sperm cells and additional substances released by males inhibit further pheromone production after copulation, either because the substances enter the pores of the vaginal gland cells or simply by blocking the pores. The female receive far more sperm cells during insemination than can be stored in her spermathecae; also her central uterus chamber is filled, but soon afterwards this sperm mass is rejected through the vulva and may, in this way, come in contact with the pores of the vaginal gland cells.

Following copulation in the plant-parasitic nematode *Scutellonema cavenessi* Sher, 1964, Demeure et al. (1980) observed a mucus-like plug on the vulva. Unfortunately, it is not clear whether this was due to male activities after copulation or to the sperm mass released from the female as in *Chromadorita tenuis*.

Green and Greet (1972) suggested the hypodermal gland cells as site for pheromone production; especially the body surface of the postvulvar region was shown to release pheromones. *Chromadorita tenuis* also has hypodermal gland cells associated with cuticular pores along the body, although their presence in both sexes does not support sex-specific characteristics. However, it could be that the 2 pairs of vaginal gland cells in *C. tenuis* are just modified hypodermal gland cells.

The precise location of pheromone receptors in the male remains unknown. Two possible sites have been proposed: the tips of the spicules (Clark et al., 1973; Samoiloff et al., 1973) and the cephalic nerve fibres (Ward, 1973; Croll, 1977). However, as pointed out by Bone and Shorey (1978), it is likely that anterior chemoreceptors control orientation toward a pheromone source from a distance, and that posteriorly located receptors control short-range copulatory reactions. The results reported here strongly support the above hypothesis. At some distance from the female, the behaviour of *Chromadorita tenuis* male is very different from that at close range. It is doubtful whether the tips of the spicules act as receptors for the pre-copulatory stimulus in *C. tenuis* since they were never observed protruding or moving at all during this event. Tips of the spicules are known from other studies to play a role while searching for the vulva (Chitwood and Chitwood, 1950; Greet, 1964; Chin and Taylor, 1964; Lee, 1973; Clark et al., 1973), but neither von Thun (1968) nor we were able to observe the spicules during coiling around the female body. SEM photographs of *C. tenuis* may provide new information about specific pores on the spicules or on the ventral border of the tail not observable by light microscopy, as shown by Clark et al. (1973) and Clark and Shepherd (1977). The ventral seta located just in front of the cloaca in *C. tenuis* (Fig. 1 A) is a possible receptor site for vibratory and tactile stimuli during the pre-copulatory behaviour and/or during mating. This seta, found only in males, is shorter and stouter than other somatic setae on the body and its position is unique, indicating a specific function.

Insemination of sperm cells and possibly of additional substances from the vas deferens (Fig. 1 A) causes not only fertilization of the eggs and stops the female's attractiveness to males, but triggers other mechanisms in the reproductive system of *Chromadorita tenuis*, compared to a virgin female (Table 1). Both types of females produce eggs from the

time of maturation. Since eggs were never observed in the oviducts, the period from ovulation to egg deposition in the proximal uterus chambers is very short, therefore fertilization presumably takes place in the proximal chambers in addition to the formation of a definite ornamentated egg shell. However, the problem then arises as to how sperm cells from the presumed spermathecae in the central uterus chamber enter the proximal chambers to fertilize the eggs. Indeed, one would expect the spermathecae opening at the junction between the oviduct and the proximal chamber, fertilizing the eggs when passing from the ovary. Structurally, this might be deduced to be the case in the majority of Chromadoridae observed, but these species do not have the 4 distinct pouches in the central uterus chamber as found in *C. tenuis* and also in *C. guidoschneideri* (Filipjev, 1929) (unpubl. obs.). In *C. tenuis* there are some (6?) irregularly shaped and grouped cells at the junction between the oviduct and the proximal uterus chamber and the central chamber; these cells form without any doubt the closing mechanism and are not a store for sperm cells (Fig. 1 B).

Virgin females do not form a shell around the non-fertilized eggs in the proximal uterus chambers. They do not release the energy stored in the eggs by depositing them in the substrate, although ovulation continues and food conditions are favourable. The contents of the eggs are resorbed in the proximal chambers. This can be observed as a colour change of the eggs and a slow disappearance of the contents (Fig. 4 C), rather similar to the observations in *Dipetalonema viteae* (Chabeaud, 1952) Johnson et al. (1974) and *Panagrellus redivivus* Goodey by Duggal (1978). Such a mechanism can be of great energetic advantage.

Jennings and Deutsch (1975) thought to have found in matured female specimens of a closely related species (both regarding taxonomy and trophic type), *Chromadorina germanica* (Bütschli, 1874), a high arylamidase activity in a spermatheca, which they interpreted to indicate absorption of sperm cells left over

Table 1 *Chromadorita tenuis*. Supposed trigger mechanisms involved in female reproductive system upon insemination or virginity

Location	Ovary	Oviduct	Uterus				Central chamber	Vaginal gland cells
			Proximal chambers	Central chamber	Central chamber	Central chamber		
Event	Ovulation	Fertilization	Fertilization	Egg shell formation	Egg presence	Egg reabsorption	Oviposition	Sex pheromone production
♀ (inseminated)	+	?	+	+	+	-	+	-
♀ (virgin)	+			-	+	+	-	+

from an earlier copulation. However, it seems strange that only one spermatheca should have been observed, because spermathecae normally are paired in Chromadoridae. Could it be that Jennings and Deutsch in reality stained an unfertilized egg? Then the speculation of Warwick (1981) has no basis, that energy from surplus sperm helps the female to produce eggs. This would imply repeated copulations, which have never been observed in this material of *Chromadorita tenuis* nor by Tietjen and Lee (1973) in the related species *Chromadora macrolaimoides* Steiner, 1915.

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