Observations on Development of the Gonad and on Reproduction in Aphelenchus avenae[1]

J. M. FISHER and A. C. TRIANTAPHYLLOU[2]

Abstract: Nuclear changes occurring in male and female gonads of *Aphelenchus avenae* during postembryogenesis were studied in relation to time and feeding periods on *Rhizoctonia solani*. Development of the female gonad was similar to that in other nematode species, but development of the male gonad followed the growth pattern of female rather than male gonads. This deviation was explained by the assumption that males in amphimictic populations have appeared as the result of recent evolution of such populations to sexuality from originally parthenogenetic ancestors. A certain, period of feeding of larvae (16 h for L-2 & L-3, but 12 h for L-4) was required before molting. Cell divisions were confined to the periods of lethargus during the second and third molts, but started during the larval stage in fourth-stage larvae. Crosses in various combinations demonstrated that temperature-induced males do inseminate females of the amphimictic and some parthenogenetic populations, but their spermatozoa are nonfunctional. Similarly, males of the amphimictic population inseminated females of a parthenogenetic population, but the sperm did not penetrate the oocytes. *Key Words:* postembryogenesis, sexuality, hybridization.

Development of the female reproductive system in terms of nuclear changes during postembryogenesis is known for a number of plant-parasitic and mycophagous nematodes. In second-stage larvae, the gonad primordium contains three (two epithelial and one germinal) nuclei in *Ditylenchus triformis* Hirschmann and Sasser (5) or four (two epithelial and two germinal) nuclei in *Pratylenchus* spp. (7). Divisions of the epithelial nuclei in the developing reproductive systems may be limited to the periods of molting in *Helicotylenchus dihystera* (Cobb) Sher (6) or may occur also during the third or fourth larval stages in *Pratylenchus* (7). The germinal nuclei behave differently. No divisions take place till the fourth stage, and divisions may occur in the motile part of that stage, as in *Pratylenchus* spp. (7), or during the fourth molt, as in *D. triformis* (5) and *H. dihystera* (6).

Development of the male system shows some important differences. During the second molt, the anterior epithelial nucleus divides repeatedly to produce a number of nuclei anterior to the germinal nucleus, as in *D. triformis* (5), and the orientation of the system changes during the third molt when the primordium takes a turn of 180° and starts growing posteriad. Division of the germinal nucleus occurs during the third molt in *Pratylenchus* spp. (7) or early in the fourth stage in *D. triformis* (5).

Sex can be distinguished by the presence or absence of specialized ventral chord nuclei as early as second or third larval stages.

Extension of the developing reproductive system in *Aphelenchus avenae* Bastian was related to molting (2). Each stage was divided into three parts: prestimulus part of variable duration (depending on the availability of food), motile part of constant duration, and lethargus or molting, during which movement was arrested and the cuticle was shed. The determination of receipt of the stimulus was hypothetical and was based on the ability of larvae to molt without further ingestion of food. Extension of the gonad occurred shortly after the stimulus was received and the rate of extension was greater in the fourth than in the third stage. No information is available on nuclear changes in the developing reproductive system of *A. avenae*.

The fecundity of parthenogenetic females and their life span were greatly reduced when males of an amphimictic population were introduced into a culture (3), but there has been no examination of the factors involved. It is known that a thelytokous (giving female progeny only) population of *A. avenae* reproduces by meiotic parthenogenesis (8) and another population

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from California which normally has only females produces males under various conditions of stress (1, 4). It is not known whether males of the latter population are functional.

This paper reports the nuclear divisions in the developing reproductive system and the results of crosses between temperature-induced males or males of an amphimictic population and females of three parthenogenetic and one amphimictic population of *A. avenae.*

MATERIALS AND METHODS

Stock cultures of *A. avenae* were maintained on *Rhizoctonia solani* Kühn growing on one-fifth dilution of potato dextrose agar. When nematodes of a certain stage were required, a petri dish with a growing population was flooded with sterile water and, after the nematodes had emerged from the agar, those in the appropriate lethargus were hand-picked and placed in sterile tap water to complete the molt. The nematodes were then added to fresh cultures of the fungus. Timing for the study of their development was initiated from this transfer of the larvae to food. To study nuclear changes during development and detect insemination, nematodes were stained in 1.5% propionic orcein (5).

Crossing tests involved one amphimictic population from Australia and three parthenogenetic populations from Australia, California, and North Carolina. To obtain virgin females of the amphimictic population, female larvae at the fourth lethargus were hand-picked and placed in sterile tap water to complete the molt. The young females were then transferred to new cultures of *R. solani* to which an equal number of temperature-induced males were added. Temperature-induced males were obtained from young cultures of the California population which was maintained for 4 days at 30 C and then transferred to 25 C for another 2-4 days during which period numerous males emerged. Young females of each of the parthenogenetic populations were hand-picked from young cultures and transferred to new cultures of *R. solani* to which an equal number of males of the amphimictic population or temperature-induced males were added for the appropriate crosses. In a cross between males of the amphimictic and females of the parthenogenetic Australia populations, the number of females which became inseminated and the number of those which had some glue-like material attached to the vulva were assessed 2 and 5 days after exposure to males.

RESULTS

DEVELOPMENT OF THE GONAD IN PARTHENOGENETIC POPULATIONS: *Second-stage larvae.*—The genital primordium contained three nuclei on hatching; one germinal and two epithelial. The posterior epithelial nucleus divided first, and this division occurred 28 h after the larvae were placed on food (Fig. 1). Nuclear divisions continued until about 40 h after the larvae were placed on food, but the germinal nucleus did not divide. Division of the nuclei in the hypodermal chords followed the same pattern.

At 4-h intervals, larvae were removed from food and placed in water to determine when they first had the ability to molt. After 16 h of feeding, all larvae in the sample had the ability to molt without further feeding. At 28 h, 2 of 11 larvae were in lethargus, and by 32 h, all were in lethargus. At 40 h, 1 out of 10 larvae had resumed motility after lethargus.

Four specialized ventral chord nuclei could be distinguished just before the onset of lethargus (24 h).

Third-stage larvae.—Immediately after the second lethargus, the primordium contained a single germinal nucleus, a cap cell nucleus, and about 10 other epithelial nuclei (Fig. 1). Four specialized ventral chord nuclei were seen. Nuclear divisions did not occur until the 28-h sample and continued until about 40 h. The germinal nucleus did not divide.

After 16 h, all larvae had the ability to molt without further feeding. At 28 h, lethargus was beginning, and after 40 h, motility was resumed.

Fourth-stage larvae.—Immediately after the third lethargus, the reproductive system contained a germinal nucleus, a cap cell nucleus, and about 56 other epithelial nuclei (Fig. 1). There were 12 specialized ventral chord nuclei arranged in 2 groups of 6 on opposite sides of the future site of the vagina.

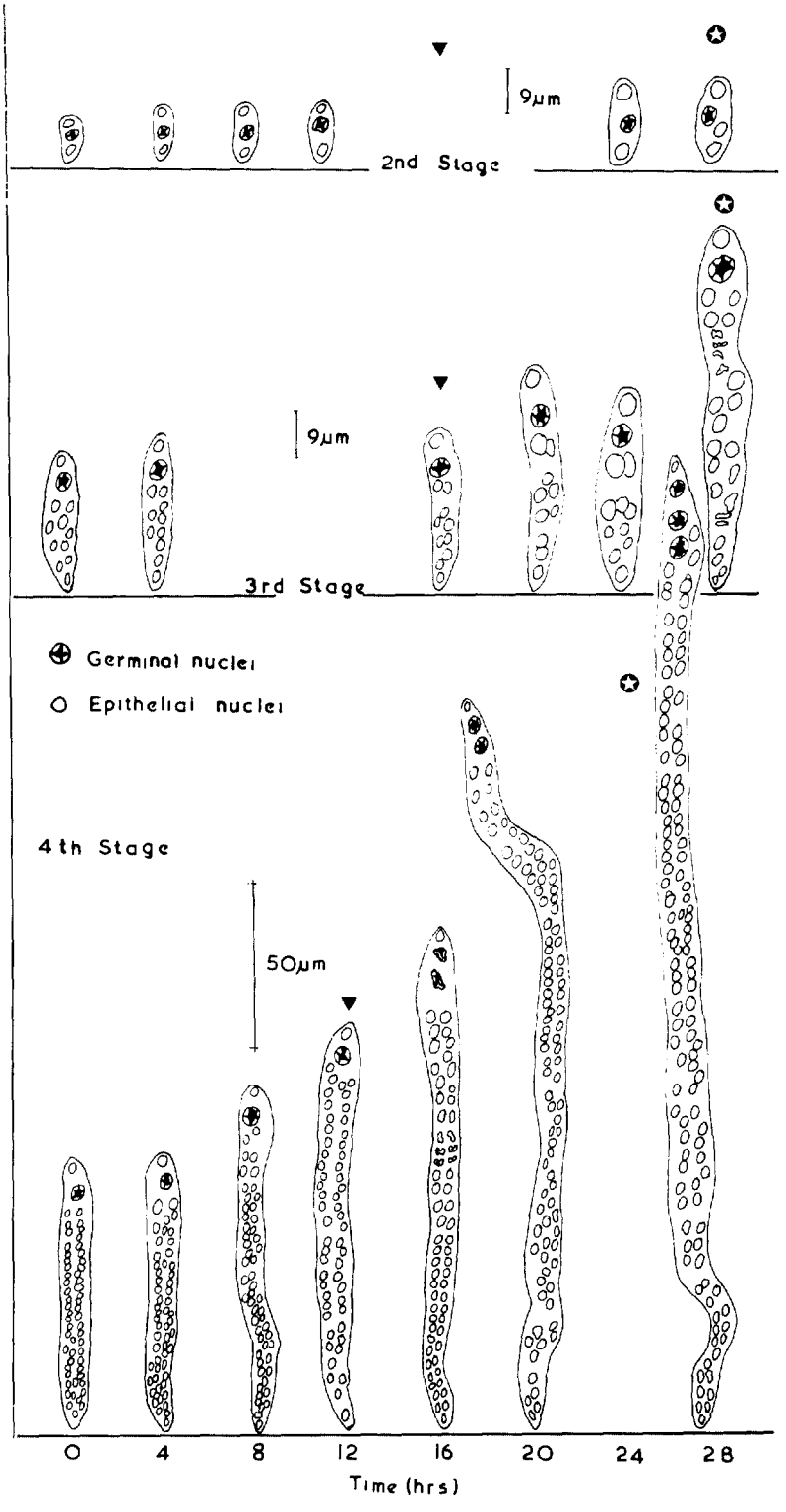

FIG. 1. Nuclear changes in the developing reproductive system of parthenogenetic females of *Aphelenchus avenae* in relation to molting. Arrows indicate the receipt of the molting stimulus; stars indicate the onset of lethargus.

No division of nuclei occurred until 16 h after the larvae were placed on food. At this time, the germinal nucleus and the epithelial nuclei were dividing, and both types continued to divide until about 36 h after the larvae were placed on food. About 10 to 15 epithelial nuclei of the anterior part of the ovary started to increase in size late in this stage (Fig. 3). They eventually became the "giant epithelial nuclei" of the growth zone of the ovary of adults (9).

Most larvae acquired the capacity to molt after 12 h of feeding; lethargus began shortly after 24 h; and motility was resumed shortly after 36 h.

DEVELOPMENT OF THE GONAD IN THE AMPHIMICTIC POPULATION: FEMALE.—Development of the reproductive system of the amphimictic female was similar to that of the parthenogenetic female.

MALE: *Second-stage larvae.*—Sex of larvae could not be distinguished until late in the second stage (during lethargus) when specialized ventral chord nuclei could be seen for the first time in female, but not in male, larvae. All primordia (male and female) had one germinal nucleus and two epithelial nuclei (Fig. 2). The posterior epithelial nucleus of male larvae began to divide during lethargus.

Third-stage larvae.—Immediately after the second lethargus, there were about 12 nuclei in the primordium—11 epithelial nuclei and one germinal nucleus—arranged as in female larvae, with the germinal nucleus at the anterior end. No change in number of nuclei occurred until the onset of the third lethargus when both epithelial and germinal nuclei divided (Fig. 2).

Fourth-stage larvae.—Immediately after the third lethargus, there were four germinal and about 56 epithelial nuclei present. No further divisions of nuclei occurred until after 13 h of feeding when both germinal and epithelial nuclei began to divide and continued dividing throughout the remainder of the stage. The fact that the two most anterior germinal nuclei stained differently from the remainder of the germinal nuclei (Fig. 1, 4) was an indication that differentiation had taken place between spermatogonia and spermatocytes.

CROSSING TEMPERATURE-INDUCED MALES WITH FEMALES OF THREE PARTHENOGENETIC AND ONE AMPHIMICTIC POPULATION: Temperature-induced males of the parthenogenetic California population freely inseminated females of their own population. Numerous spermatozoa were observed in the spermatotheca and along the gonoducts of inseminated females, but of 150 oocytes examined, only 5 contained a sperm nucleus. Actual fertilization, i.e. fusion of sperm and egg pronuclei, was not observed in any case and probably did not occur.

Forty of the 60 females of the parthenogenetic Australia population and none of 50 females of the North Carolina population exposed to temperature-induced males became inseminated. Sperm was not observed inside any oocyte of the Australia population.

Of the 30 virgin females of the amphimictic Australia population, 20 became inseminated by temperature-induced males. Three oocytes of three different females contained one sperm nucleus each, and one oocyte in a fourth female contained two sperm nuclei. The fact that none of another 30 females exposed to temperature-induced males gave any progeny indicated that actual fertilization did not occur.

CROSSING MALES OF THE AMPHIMICTIC POPULATION WITH FEMALES OF THE AUSTRALIAN PARTHENOGENETIC POPULATION: Eight of 15 parthenogenetic females examined 2 days after exposure to males were inseminated, and 25 of 27 females were inseminated 5 days after exposure to males. Two females in the first observation and 5 in the second had some adhesive material on the vulva; thus the absence of a copulation plug is not proof that copulation has not taken place. Because no sperm was seen inside any oocyte of inseminated females, actual fertilization probably did not occur. Some of the parthenogenetic females were dead by the fifth day of exposure to males and others were dying. In the latter, sperm had migrated into the posterior part of the ovary; such a condition has not been seen in amphimictic females, except in very old cultures.

DISCUSSION

The pattern of nuclear changes in the female gonad of the parthenogenetic and

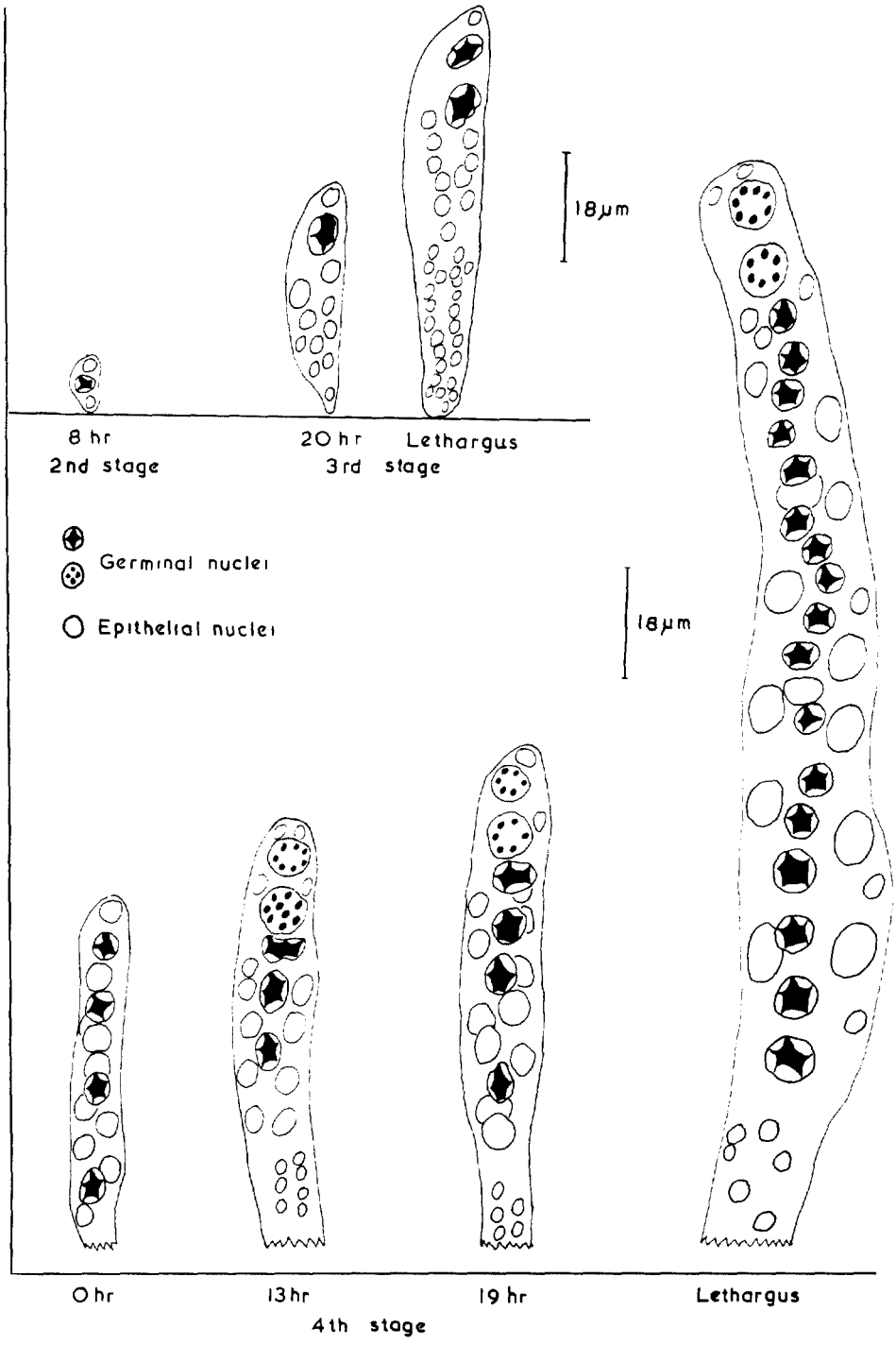

FIG. 2. Nuclear changes in the developing reproductive system of males from an amphimictic population of *Aphelenchus avenae* from Australia.

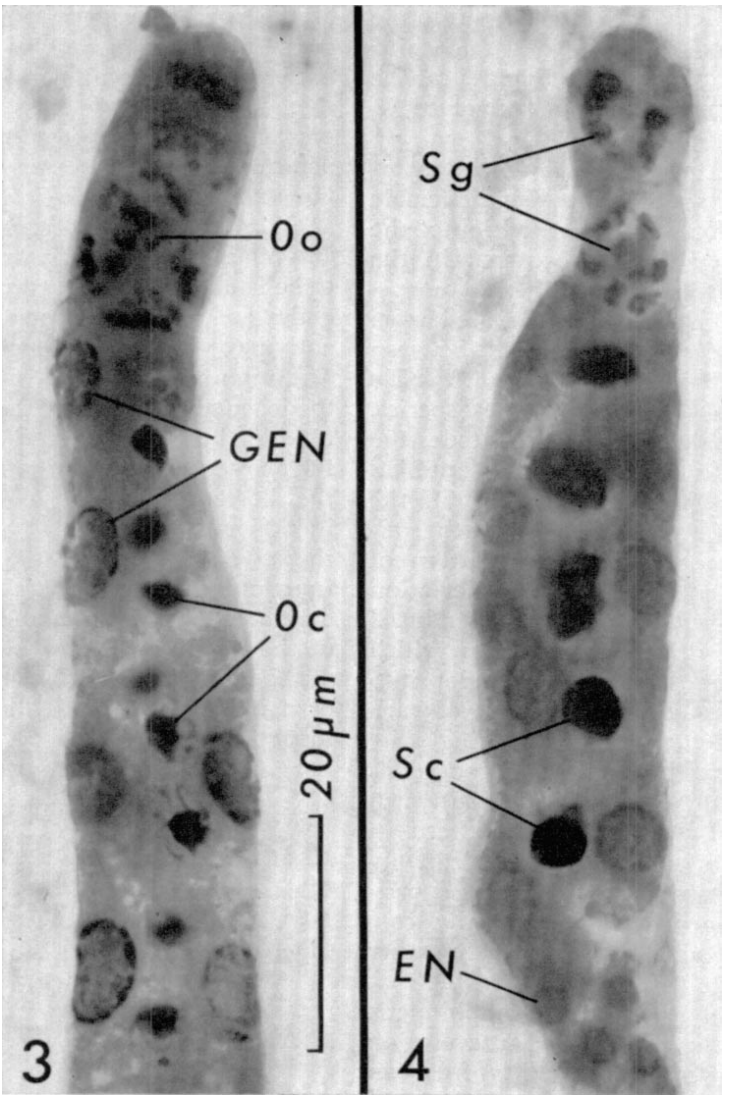

Fig. 3-4. Photomicrographs of anterior portion of gonad of advanced 4th-stage female and 4th-stage male larvae, respectively, of *Aphelenchus avenae*. EN, epithelial nucleus (normal size); GEN, giant epithelial nucleus; Oc, oocyte nucleus; Oo, oogonial nucleus (dividing); Sc, spermatocyte nucleus; Sg, spermatogonial nucleus (dividing). Scale for Fig. 4 as in Fig. 3.

amphimictic populations of *A. avenae* was similar to that of *Ditylenchus triformis* (5) in the early larval stages. All nuclear divisions were confined to the periods of lethargus of the second and third molts, and only the epithelial nuclei divided at these times. In the fourth stage, divisions of both germinal and epithelial nuclei began shortly after the larvae had acquired the ability to molt without further feeding. Such divisions continued throughout the remainder of the motile part of the stage and through lethargus. This developmental pattern coincided with the changing rates of extension of the gonad already reported (2). The increased rate of extension in the fourth stage occurred when the nuclei were dividing.

The nuclear changes in the fourth stage relate the onset of the developmental changes to the stimulus for molting and indicate that the underlying mechanism for control is changed in the fourth stage from the previous two stages. The period from receipt of the stimulus for molting in the

fourth stage to lethargus is analogous to the lethargus period in the second and third stages. Presumably, nuclear changes can be related to hormonal changes involved in molting.

The overall pattern of development of male larvae was similar to that of female larvae except for the earlier division of the germinal nucleus, which occurred in the third lethargus, and the absence of specialized ventral chord nuclei in male larvae. A striking difference was detected between development of male gonad in *A. avenae* and that of other nematode species. Whereas, in males of other nematodes the gonad primordium starts growing anteriad and turns posteriad later (5, 6, 7), the primordium of *A. avenae* males starts growing posteriad, i.e. follows the pattern of growth of female gonads. This deviation can be explained in temperature-induced males of the California population, which genotypically are females and develop into males only through the influence of various environmental factors during late embryogenesis and early postembryogenesis (4). It is difficult, however, to explain such a deviation of developmental pattern in the normally bisexual, amphimictic population from Australia, in which sex expression is presumed to be genetically controlled, and should follow the male rather than the female pattern. It is possible that the presence of males in the amphimictic population is the result of recent evolution of this population to sexuality from an originally parthenogenetic population, the type that is most common around the world. This then is the first case of suspected reverse evolution from parthenogenesis to amphimixis in nematodes (10).

Temperature-induced males of the California population freely inseminated females of their own population and a high percentage of females of the parthenogenetic and amphimictic Australia populations. Although sperm occasionally entered oocytes of inseminated females, no fusion of sperm and egg pronuclei was observed, and it is suspected that it did not occur. The failure of females of the amphimictic population to give progeny after they were inseminated by temperature-induced males suggests that actual fertilization did not occur. All progeny of inseminated females of the parthenogenetic populations developed into females. This developmental pattern is consistent with the assumption that inseminated females reproduced by parthenogenesis and that the sperm of temperature-induced males were nonfunctional. However, this response is not a proof that amphimixis did not occur. Even following amphimixis, the progeny would be expected to be females since the temperature-induced males are sex-reversed females and apparently have the female genotype.

Amphimictic males inseminated parthenogenetic females of the Australia population, but the sperm did not penetrate the oocytes. The reduction in fecundity and the shortened life span of females reported in previous tests (3) may be attributed to the adverse influence of the foreign, nonfunctional sperm inside the female gonoduct on gonad function and the normal life of the inseminated females. The influence may be mechanical, chemical, or behavioral, or it may be a combination of various factors.

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