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Abstract

Male first instar larvae possess more germ cells in their gonads than female larvae of the same stage. To determine the earliest time point of sexual dimorphism in germ cell number, we have counted the germ cells of sexed embryos at different developmental stages. We found no difference in germ cell number of male and female embryos at the blastoderm and early gastrulation stage, or when germ cells are about to exit the midgut pocket. We find, however, that males have significantly more germ cells than females as soon as the germ cells are near the places where the gonads are formed and in all later stages. Our results show that germ cells are subject to a sex-specific control mechanism that regulates the number of germ cells already in embryos.

A sex-specific number of germ cells in embryonic gonads of *Drosophila*

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SUMMARY

Male first instar larvae possess more germ cells in their gonads than female larvae of the same stage. To determine the earliest time point of sexual dimorphism in germ cell number, we have counted the germ cells of sexed embryos at different developmental stages. We found no difference in germ cell number of male and female embryos at the blastoderm and early gastrulation stage, or when germ cells are about to exit the midgut pocket. We find, however, that males have significantly more germ cells than females

as soon as the germ cells are near the places where the gonads are formed and in all later stages. Our results show that germ cells are subject to a sex-specific control mechanism that regulates the number of germ cells already in embryos.

Key words: germ cells, germline, *Drosophila*, sex determination, sexual dimorphism

INTRODUCTION

In *Drosophila*, the genetic pathway that determines the sex of somatic cells is well understood (reviewed in: Baker, 1989; Steinmann-Zwicky et al., 1990; Belote, 1992; Burtis, 1993). In recent years, it was even possible to identify elements of the primary sex-determining signal, the X:A ratio, which had until then proved refractory to analysis (reviewed in: Cline, 1993).

The genes that determine the sex of somatic cells also participate in determining the sex of germ cells. Most of these genes, however, are not required within germ cells (Marsh and Wieschaus, 1978; Schüpbach, 1982). Their action is indirect: in somatic cells, they regulate the expression of inductive signals that direct XX germ cells into the male or the female pathway (Steinmann-Zwicky et al., 1989; Steinmann-Zwicky, 1994a).

Besides inductive signals, germ cells also require autonomous information to determine their sex (reviewed in: Steinmann-Zwicky, 1992a,b). XX germ cells become spermatogenic in a male host, but XY germ cells cannot become oogenic in a female host. Therefore, XY germ cells must possess an autonomous signal that makes them different from XX germ cells (Steinmann-Zwicky et al., 1989). The difference between XX and XY germ cells, however, is not provided by those genes that form the elements of the primary sex-determining signal acting in the soma, called the X:A ratio signal (Steinmann-Zwicky, 1993; Granadino et al., 1993). The gene *Sxl*, which in the soma is a target of the X:A ratio signal (Cline, 1993), is also required within XX germ cells for oogenesis (Schüpbach, 1985; Steinmann-Zwicky et al., 1989). It seems, however, that *Sxl* is not required for the early events of germ line sex determination (Steinmann-Zwicky, 1994b).

The question of when prospective germ cells first display

sexual dimorphism has occupied researchers long before anything was known about the genetic control of germ cell sex determination. Kerkis (1931) showed that the gonads of first instar larvae display a clear sexual dimorphism, male gonads being 3.5 times larger than female gonads in newly emerged larvae. This difference arises because male gonads contain more germ cells than female gonads, and not because of a sex-specific size difference in the somatic tissues of the gonads (Aboim, 1945). When males, however, first contain more germ cells than females, has not been determined. So far, the results obtained from the analysis of embryos are conflicting.

Pole cells, which are the progenitors of the germ cells, are formed at the blastoderm stage (3 hours AEL = after egg-laying), at the posterior pole of the embryo. At the beginning of gastrulation, they are moved dorsally and anteriorly. They are then internalized into the embryo and localised in a pocket formed by prospective gut cells. At the end of germ band elongation, the progenitors of the germ cells migrate through the walls of the posterior midgut primordium. During early germ band shortening (8 hours AEL), they become aligned in two lateral positions. By the end of germ band shortening (10 hours AEL), the germ cells have grouped at the positions where the somatic cells of the mesoderm will form the gonads, where they are closely associated with each other.

After sectioning embryos and analysing their gonads, Sonnenblick (1941) found smaller and larger gonads at 10 hours AEL. All germ cells seemed to be similar in size, but the number of germ cells within gonads varied. There appeared to be two groups of embryos: those with 5-7 germ cells in their gonads, and those with 9-13 germ cells. Thinking that the larger gonads were prospective testes and the smaller gonads prospective ovaries, Sonnenblick concluded that there is a sex-specific difference in gonad size already at 10 hours AEL and that this

difference results from a sex-specific number of germ cells. Sonnenblick was so convinced of this early sexual dimorphism that he wrote that this is “the earliest discernable feature of sex differentiation” in the whole animal. This, however, could not be tested, as no tools to sex embryos were available at that time.

When other authors later counted the number of germ cells contained within the gonads of embryos, they did not find two classes. Technau and Campos-Ortega (1986) reported that gonads have an average of 8-10 germ cells; Hay et al. (1988) found an average of 14 germ cells per gonad. In none of the experiments, however, was the sex of the embryos assessed to test whether males and females have a different number of germ cells.

We decided to reapproach this question, and to search for the earliest sexual dimorphism in germ cell number. Until 18 hours AEL when the cuticle is formed, germ cells of embryos can be specifically stained using an antibody recognizing the vasa protein (Lasko and Ashburner, 1990). Visualizing the germ cells with this antibody and sexing the embryos with sex-specific markers, we have counted the number of germ cells of male and female embryos at various embryonic stages. We found that males and females initially have the same number of germ cells, but that a sexual dimorphism in germ cell number appears as soon as the germ cells reach the places where the gonads are formed.

MATERIALS AND METHODS

To sex embryos at the blastoderm and early gastrulation stage and at the stage when germ cells are about to exit the midgut pocket, we used a female-specific β -galactosidase marker. We stained progeny from a stock carrying a *lacZ* gene driven by the early female-specific promoter of *Sxl* (Keyes et al., 1992). In this stock all XX embryos express β -galactosidase and become blue. To sex embryos at later stages, we crossed wild-type females (Oregon R) to males carrying on their X chromosome a *lacZ* gene driven by the *ftz* promoter (Hiromi et al., 1985). All XX progeny from such a cross show a blue *ftz* pattern after being stained with X-Gal. XY embryos remain white. In both types of experiments, embryos were stained with anti-vasa antibody to visualize the germ cells (Lasko and Ashburner, 1990).

Early stages of embryos were obtained by collecting eggs for 6 hours. After staining, we selected the embryos that were in the blastoderm stage (stage 5 of Campos-Ortega and Hartenstein, 1985), the early gastrulation stage (stage 6) and the stage at which germ cells are about to exit the midgut pocket (stage 10). The germ cells of early gastrulation embryos have started to migrate dorsally and are therefore not located at the very posterior pole, as they are at the blastoderm stage. This stage was chosen for two reasons. (1) After germ cells are formed, they undergo a few mitotic divisions before the blastoderm stage. It is therefore possible that the full complement of germ cells is not yet present in some early blastoderm stage embryos. (2) The sex-specific marker *Sxl-lacZ* is first expressed in blastoderm-stage embryos. Some early blastoderm embryos classified as XY animals could therefore be XX embryos that did not yet express the marker. At early gastrulation, this can be excluded.

Later stages of embryos were obtained by collecting eggs for 2 hours. Embryos were stained after 8 ± 1 , 10 ± 1 , 12 ± 1 , 14 ± 1 and 16 ± 1 hours. The stage-specific pattern of expression of the *ftz-lacZ* marker was helpful when embryos of the same stage were chosen (Hiromi et al., 1985). The age of our embryos at a specific stage does not exactly correspond to the age of embryos of the same stages described by Campos-Ortega and Hartenstein (1985). An 8 hours old embryo, however, corresponds to stage 11 (5:20-7:20 hours old according to

these authors) and a 10 hours old embryo corresponds to stage 12 (7:20-9:20 hours old). All experiments were performed at 25°C.

X-Gal and antibody double staining

Embryos of all stages except blastoderm and early gastrulation stages were stained as follows. Embryos were dechorionated with sodium hypochlorite, washed with water containing 0.1% Triton and fixed in 12.5% formaldehyde (in 100 mM sodium phosphate buffer pH 7.0 with 1 mM $MgCl_2$) in the presence of heptane (shake for 20 minutes). Embryos were collected on a filter, washed with 0.1% Triton and brushed into Eppendorf tubes containing 1 ml of X-Gal solution (10 mM sodium phosphate buffer pH 7.0 with 0.08% X-Gal, 0.1 % Triton, 5 mM each of $K_3[Fe(CN)_6]$ and $K_4Fe(CN)_6 \cdot 3H_2O$, 0.015 mM $MgCl_2$, and 2.2 mM NaCl) preincubated at 37°C. The tubes were rotated for 6-7 hours at room temperature in the dark. Embryos were collected on a filter, washed with 0.1% Triton and refixed in 12.5% formaldehyde in the presence of heptane (shake for 20 minutes). To devitellinize embryos, fixative was removed and cold methanol was added (shake for 1-2 minutes). Embryos were transferred into Eppendorf-tubes, washed with cold methanol, washed 3 times with PBS (130 mM NaCl, 7 mM Na_2HPO_4 , 3 mM NaH_2PO_4), 3 times with PT (0.3% Triton X-100 in PBS) and incubated for 2 hours in 10% goat serum in PT. The goat serum was changed once and antibody was added (leave overnight at 4°C). Embryos were washed 3 times with PT, incubated for 30 minutes in 10% goat serum in PT, which was changed once. The secondary antibody (peroxidase-conjugated Affini Pure Goat Anti-Rabbit, Jackson Immuno Research Laboratories) was added (leave for 3 hours at room temperature). The embryos were washed 3 times with PT, 3 times with PBS and stained with DAB (2 mg/ml diaminobenzidine). The staining reaction was stopped by adding PBS. Embryos were progressively dehydrated with ethanol, treated with methyl salicylate, transferred to a slide and covered with Canada balsam.

Largely the same staining protocol was used for blastoderm and early gastrulation embryos, except that the vitellin membranes were

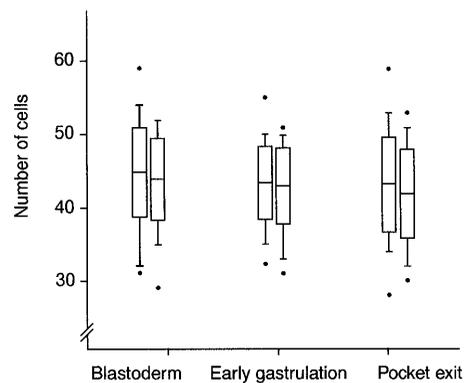


Fig. 1. Number of germ cells of sexed embryos at the blastoderm and early gastrulation stage, and when germ cells are about to exit the midgut pocket. At each stage, females are represented on the left side and males on the right side. The difference between all stages and between the sexes are small and not significant (Wilcoxon two sample rank test, $Z < 0.64$, $P > 0.52$). Boxes indicate the mean value with the standard deviation, bars show the intervals that contain 90% of the values, dots show values that are outside of this interval. 26 females and 26 males of the blastoderm stage were tested, and 30 embryos of each sex were tested in the case of the other two stages. The exact values (mean and standard deviation) are: blastoderm females 44.8 (±6.1), blastoderm males 44.0 (±5.6), early gastrulation females 43.4 (±5.0), early gastrulation males 43.0 (±5.2), pocket exit females 43.2 (±6.5), pocket exit males 41.9 (±6.1).

DISCUSSION

Early sex-specific loss of germ cells?

The number of pole cells we counted at the blastoderm stage is comparable to the numbers reported by others. Sonnenblick

(1941) found an average of 40 pole cells, Hay et al. (1988) counted 41 at the beginning of gastrulation. For the first time, we now report the number of pole cells of sexed embryos. We find no sex-specific difference in pole cell number at the blastoderm and at the early gastrulation stage. At these stages,

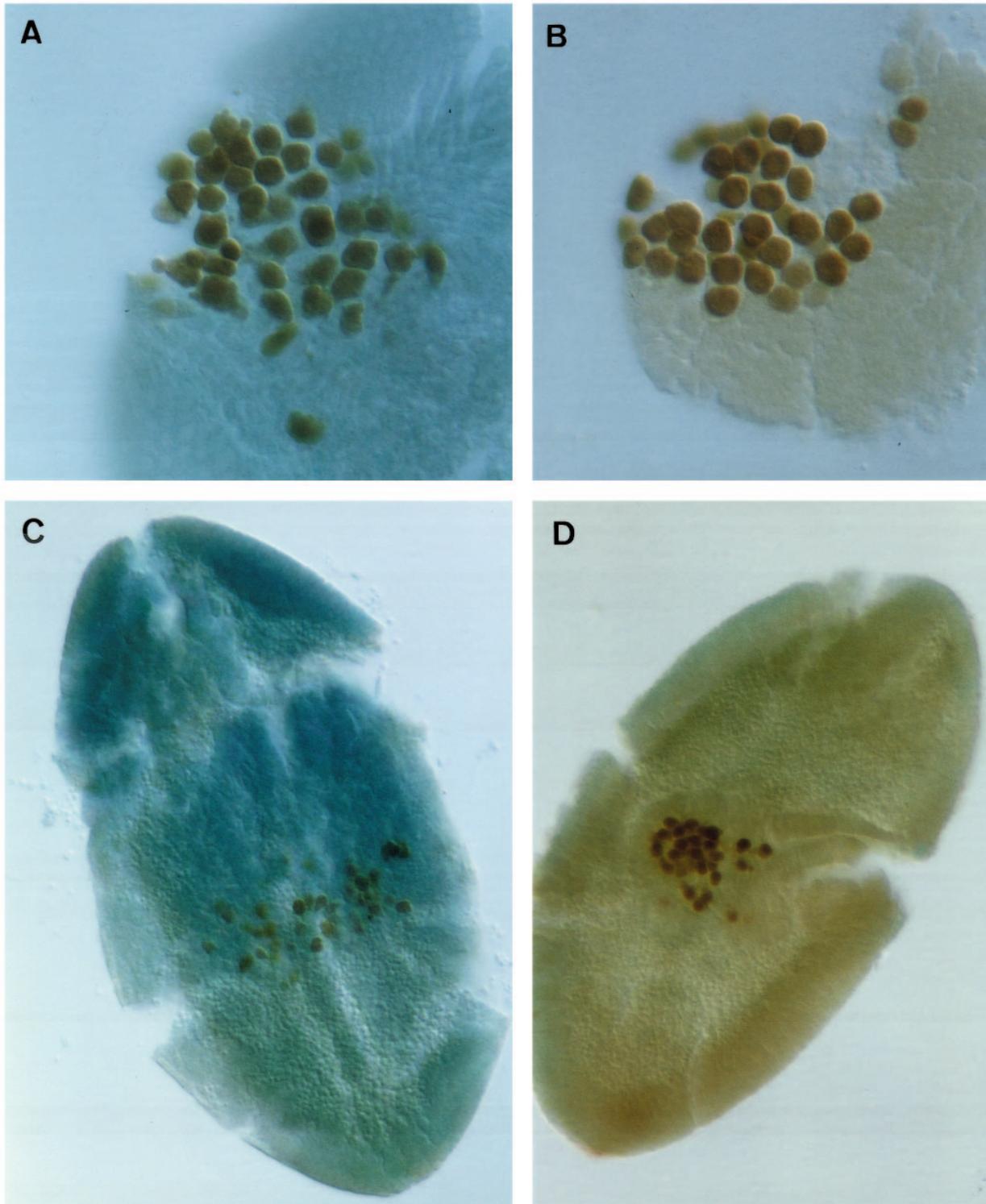


Fig. 2. Germ cells of sexed embryos in early development. (A) Female blastoderm stage; (B) male blastoderm stage; (C) female pocket exit stage (slightly squashed); (D) male pocket exit stage. Note that the somatic cells of females are blue.

females and males had an average of 44.8 and 44.0 pole cells and 43.4 and 43.0 pole cells, respectively, per embryo. These numbers are statistically not different.

Germ cells are internalized into the forming midgut and they remain in the midgut pocket for some time without diminishing in number. At the end of germband elongation, Hay et al. (1988) counted 43 germ cells that were about to pass through the walls of the posterior midgut primordium. In our sexed embryos, we counted 43.2 germ cells in females and 41.9 germ cells in males. These numbers are statistically the same.

After the germ cells have migrated through the midgut towards the sites where the gonads are being formed, we find a highly significant sex-specific difference in germ cell number. We do not observe what Sonnenblick (1941) had postulated i.e. that males have an average of 9-13 germ cells per gonad and females 5-7 germ cells per gonad. Rather, our results show that males and females largely overlap with respect to their germ cell numbers. At 8 hours AEL, male gonads, with 14.2 germ cells on average per gonad, have only 2 germ cells more than female gonads with 12.2 germ cells.

The question arose whether the misplaced germ cells that can be seen in some embryos at 8 hours AEL and later are cells that are specifically eliminated in females. Our results show that this is not the case. At 8 hours and later, a similar number of misplaced germ cells are found in female and male embryos. Therefore, we can conclude that at 8 hours AEL, female embryos possess 4 germ cells less than male embryos.

In both sexes, a significant number of germ cells do not reach the gonads: they are eliminated. Sonnenblick (1941) reported that many germ cells remain behind in the gut. Hay et al. (1988) counted a total of 29 germ cells on average per embryo at

a stage that corresponds to 8 hours AEL (26 at the two correct positions where the gonads are about to be formed and 3 misplaced germ cells). This is 14 germ cells less than what they counted before migration through the gut. According to these authors, germ cells are eliminated just prior to and just after exiting the midgut pocket. Our results now confirm that many germ cells are eliminated between the pocket exit stage (stage 10 of Campos-Ortega and Hartenstein, 1985) and 8 hours AEL (stage 11).

The observed sex-specific difference in germ cell number could be due to a control mechanism that eliminates some germ

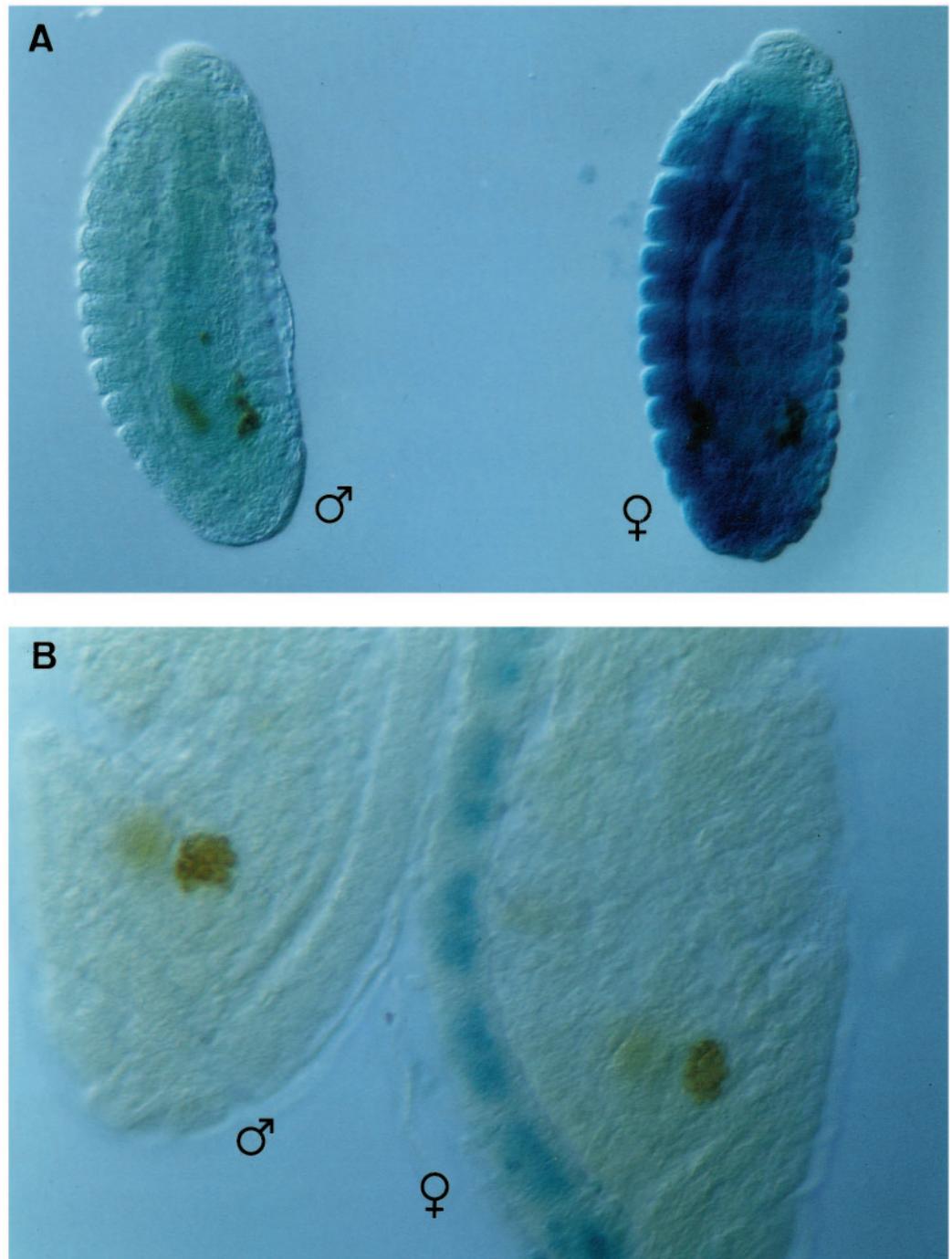


Fig. 3. Germ cells of sexed embryos of later stages. (A) 8 hours old male and female; (B) 14 hours old male and female. Note that females show a blue *fitz* pattern.

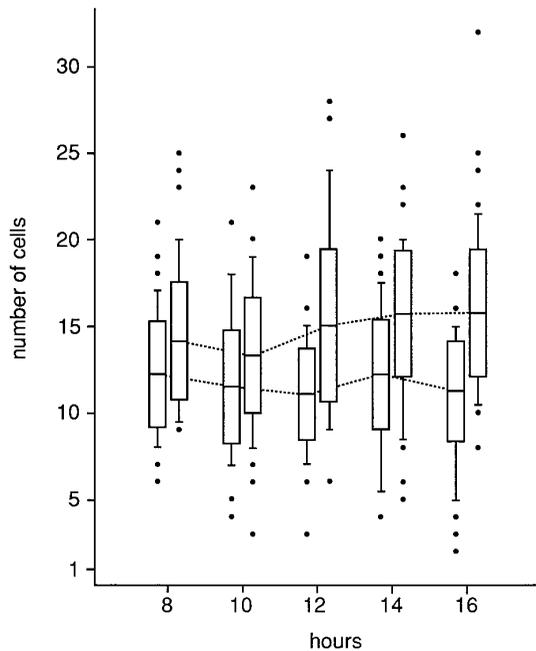


Fig. 4. Number of germ cells counted in female (left) and male (right) embryos that were 8 hours, 10 hours, 12 hours, 14 hours or 16 hours old. The mean values of females and males of different ages are connected by dotted lines (see also legend to Fig. 1).

cells in a sex-specific way. Alternatively, male but not female germ cells might start mitotic divisions as soon as the germ cells have exited the midgut pocket. We favor the former hypothesis, because we only see a minimal increase in the number of germ cells contained in male gonads, during the 8 hours that follow the first apparent sexual dimorphism in germ cell number. Similarly to our results, Sonnenblick found that germ cells start dividing mitotically after 16 hours AEL. The same conclusion was reached by Technau and Campos-Ortega (1986), who individually transplanted 33 marked germ cells from embryos beginning gastrulation into hosts and found in each of the 33 cases one germ cell when analysing older host embryos in which gonads had long been formed, and not a single case in which a germ cell had divided.

Autonomous or non-autonomous control events?

In first instar larvae, inductive signals determine the sex-specific characteristics of germ cells. XX animals that develop as males because they lack *tra* activity possess gonads with male characteristics at this early stage (Seidel, 1963; Steinmann-Zwicky, 1994a). Similarly, germ cells of young XX and XY larvae that will develop as intersexes because they lack *dsx*, display characteristics that are neither typically male nor typically female (Steinmann-Zwicky, 1994a). Since neither *tra* nor *dsx* are required within germ cells (Marsh and Wieschaus, 1978; Schüpbach, 1982), these genes must affect the sex-specific characteristics of germ cells of first instar larvae by acting in somatic tissue.

The very early sex-specific difference in germ cell number might already result from a somatic sex-determining signal. Since male embryos had on average more germ cells than female embryos at 8 hours AEL, before the gonad is formed,

the difference in germ cell number cannot be caused by an inductive signal emitted by the somatic cells of the gonad. Germ cells might rather be influenced by cells of the midgut primordium, while attempting to migrate towards their final position. Transplanting pole cells from older into younger embryos and vice versa, it was shown that the migration of the germ cells and specifically the timing of the exit from the midgut pocket is controlled by somatic tissue, probably by the tissue of the gut primordium itself (Jaglarz and Howard, 1994). Thus, gut tissue induces germ cells to initiate a migrating behavior. Such a control could show sex-specific elements. A limited number of germ cells might be induced to pass the wall of the midgut, this number being larger in males than in females. It is also possible that male midgut cells elicit mitotic behaviour in germ cells.

Alternatively, germ cell-autonomous signals might cause a sex-specific difference in the number of germ cells of embryos. It is difficult, however, to imagine how such a control would function in the complete absence of inductive signals. At least some initial signal must probably be provided by somatic tissues.

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