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Original Article

Differential germ cell proliferation in the salamander *Pleurodeles waltl:* controls by sexual genotype and by thermal epigenetic factor before differentiation of sexual phenotype of gonads

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ABSTRACT In the acceptance that, during early gonadogenesis, variations of germ cell (GC) proliferation express interactions between germ and somatic cells, early events occurring before histological differentiation of gonadal sex has been detected and timed through GC counts on larvae of *Pleurodeles waltl* (urodele amphibia) issued from male ZZ or female ZW monosexual offspring. Gonads differentiate in accordance with sexual genotype in ZZ and ZW larvae at room temperature and in ZZ larvae at 32°C whereas they are sex-reversed at 32°C in ZW larvae, becoming phenotypic neomales. At both the rearing temperatures, in genital ridges, GCs do not proliferate during a period called P_0 period ending earlier in ZZ than in ZW larvae. The time when proliferation starts depends on sexual genotypes and determines a $^{ZZ}P_0$ period shorter than $^{ZW}P_0$ period. After P_0 period, at room temperature, a moderate increase in GC number determining a P_1 period is observed in both ZZ and ZW larvae, whereas a strong proliferation, determining a P_2 period, occurs on a differential pattern in ZZ and ZW larvae; thus, before sexual differentiation of gonads, ZW females have more GCs than ZZ males. At 32°C, GC proliferation is moderate during P_1 period and does not accelerate during P_2 period in ZW larvae differentiating neotestes; they have a lower GC number than ZZ larvae reared at 32°C. Thus, during P_2 period, at both room temperature and at 32°C, GC number correlates with future phenotype of gonads. Results suggest that differential molecular events arise during early gonadogenesis and that testes may differentiate in different ways according to whether phenotype conforms to genotype or sex reversion occurs.

KEY WORDS: *amphibian, gonadal development, gonocyte count, mitotic index, sex-reversal*

Introduction

Mechanisms involved in sexual differentiation have for many years been the subject of numerous investigations, particulary in vertebrate animals (Ponse, 1949; Foote, 1964; Bull, 1983 for reviews). Different and various ways have been used to elucidate this fundamental process. The studies have concerned roles of steroid hormones (Burns, 1961; Jost, 1979; Adkins-Regan, 1987 for review), of anti Müllerian hormone (Josso, 1981; Vigier *et al.*, 1981, 1989), effects of castration (Jost, 1947; Wolff and Wolff, 1951; Gallien, 1965), results of embryonic grafts (Humphrey, 1945; Houillon and Dournon, 1986; Taketo and Merchant-Larios, 1986; Taketo-Hosotani and Sinclair-Thompson, 1987). Some experiments have dealt with sex-reversal (Chang and Witschi, 1956; Gallien, 1967; Witschi, 1971; Collenot, 1973; Pieau, 1974a; Ferguson and Joanen, 1982) or cell interactions through *in vitro* cultures (Wolff, 1952; Vigier *et al.*, 1987). Other reports inquired about roles of H-Y antigen (Ohno, 1979; Muller and Urban, 1981; Goldberg, 1988 for review), and about testis-determining factor, through the study of regulatory genes such as ZFY, ZFX and ZFA in mammals (Page *et al.*, 1987; Mardon *et al.*, 1988; Koopman *et al.*, 1989; Palmer *et al.*, 1989; Schneider-Gädicke *et al.*, 1989).

In heterothermal vertebrates, besides genotypic sex determination, environmental factors such as temperature may act to induce the phenotypical sex of individuals (Pieau, 1974b; Pieau and

Abbreviations used in this paper: GCs, germ cells; M.I., mitotic index; PGCs, primordial germ cells; Sd, standard offspring; St, stage.

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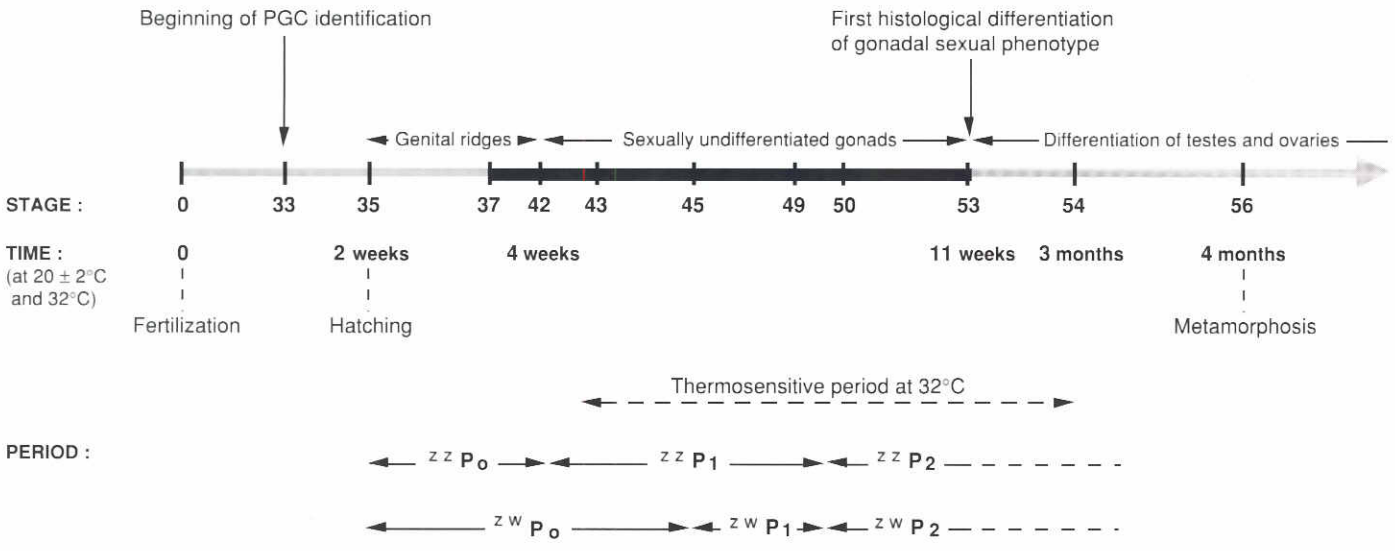

Fig. 1. Periods of GC proliferation in ZZ and ZW developing individuals of *Pleurodeles waltl*.

Dorizzi, 1981; Deeming and Ferguson, 1988). In amphibians, phenotypic inversion of gonads by rearing temperature constitutes an original way for studying gonadogenesis (Witschi, 1929; Gallien, 1974; Dournon *et al.*, 1990). In the salamander *Pleurodeles waltl*, both sex inversion and sex differentiation have been tackled. It was demonstrated that, at room temperature (20° ± 2° C), sexual differentiation conforms strictly to sexual genotype (Dournon *et al.*, 1988), whereas at 32°C, larvae of female ZW genotype become fertile phenotypic males (Dournon and Houillon, 1984). The thermosensitive period for obtaining the sex inversion of all the ZW individuals was established at 32°C (Dournon and Houillon, 1985).

Germ cell proliferation in developing gonads has also been investigated to study the pattern of sexual differentiation of gonads (Van Limborgh, 1958, 1961; Mittwoch *et al.*, 1969). The present work is based on such an investigation.

In the salamander *Pleurodeles waltl*, it has been previously observed that at the beginning of larval development, before the thermosensitive period and when genital ridges are rising, primordial germ cells (PGCs) do not proliferate during a two week-long period. During this period, called the P_0 period, it was also demonstrated that the PGC number per larva is independent of ZZ and ZW sexual genotypes, and also that each offspring is characterized by an average PGC count per larva. The average counts were then used to distribute fifteen different offspring into three groups: group I (96.9 ± 3.8 PGCs), group II (51.0 ± 1.4 PGCs), group III (31.1 ± 2.3 PGCs) plus a hybrid group (67.7 ± 0.2 PGCs) resulting from groups I and II (Dournon *et al.*, 1989). When the thermosensitive period ends, histological differentiation of gonad sex begins and discrimination between future testes and future ovaries becomes possible. Houillon (1956) had yet noticed that at room temperature, future testes contained, at this time, fewer GCs than future ovaries.

In the present work, modifications of GC proliferation are considered as being the expression of interactions between germ and somatic cells involved in sexual differentiation. Our report is based on the general assumption that following sexual determination, somatic cells of the gonad interact with GCs to induce the sexual differentiation of GCs themselves. GC proliferation was studied and compared for ZZ genotypic and for ZW genotypic individuals before histological differentiation of the sexual phenotype of gonads, both when at room temperature gonadal sex differentiation conforms strictly to sexual genotypes, and when at 32°C sexual differentiation is inverted. With this aim, monosexual offspring were obtained from selected parents and were reared at two appropriate temperatures.

Results and interpretation

Gonadogenesis

In heterothermal animals, the development rate varies according to the rearing temperature; moreover, in *Pleurodeles waltl*, animals reared at 30° or 32°C are dwarfs (see Materials and Methods). Whatever the rearing temperature, 20° ± 2°C or 32°C, the gonadal organogenesis observed in transverse sections always correlates with the morphogenesis of posterior limbs. So determination of developmental stages (Gallien and Durocher, 1957) was based on morphogenesis of posterior limbs.

In cross-section, at stage 42, GCs hang down into the coelomic cavity and together with some somatic cells form the genital ridge (Figs. 2a and 3a). At stage 45 more somatic cells constitute a pedicle from which GCs are suspended (Figs. 2b and 3b). At stage 50, the gonadal anlage separates into two parts: the fat body and the gonad itself (Figs. 2c and 3c). Just before stage 53, the histological differentiation of the gonadal sex occurs. The developing testis is spearhead in shape and delimited by a layer of cells comprising small and round nuclei. Inside, surrounded by other somatic cells, are a very few large germinal cells with light and polylobed or round nuclei (Fig. 2d). The ovary, at the same stage, is more massive with numerous germ cells located outside. Germ cell nuclei are round and light. Some somatic cells are observed on the surface, others are gathered near the hilum but they are scarce inside the gonad (Fig. 2e). Subsequently, in ovary, oocytes enter

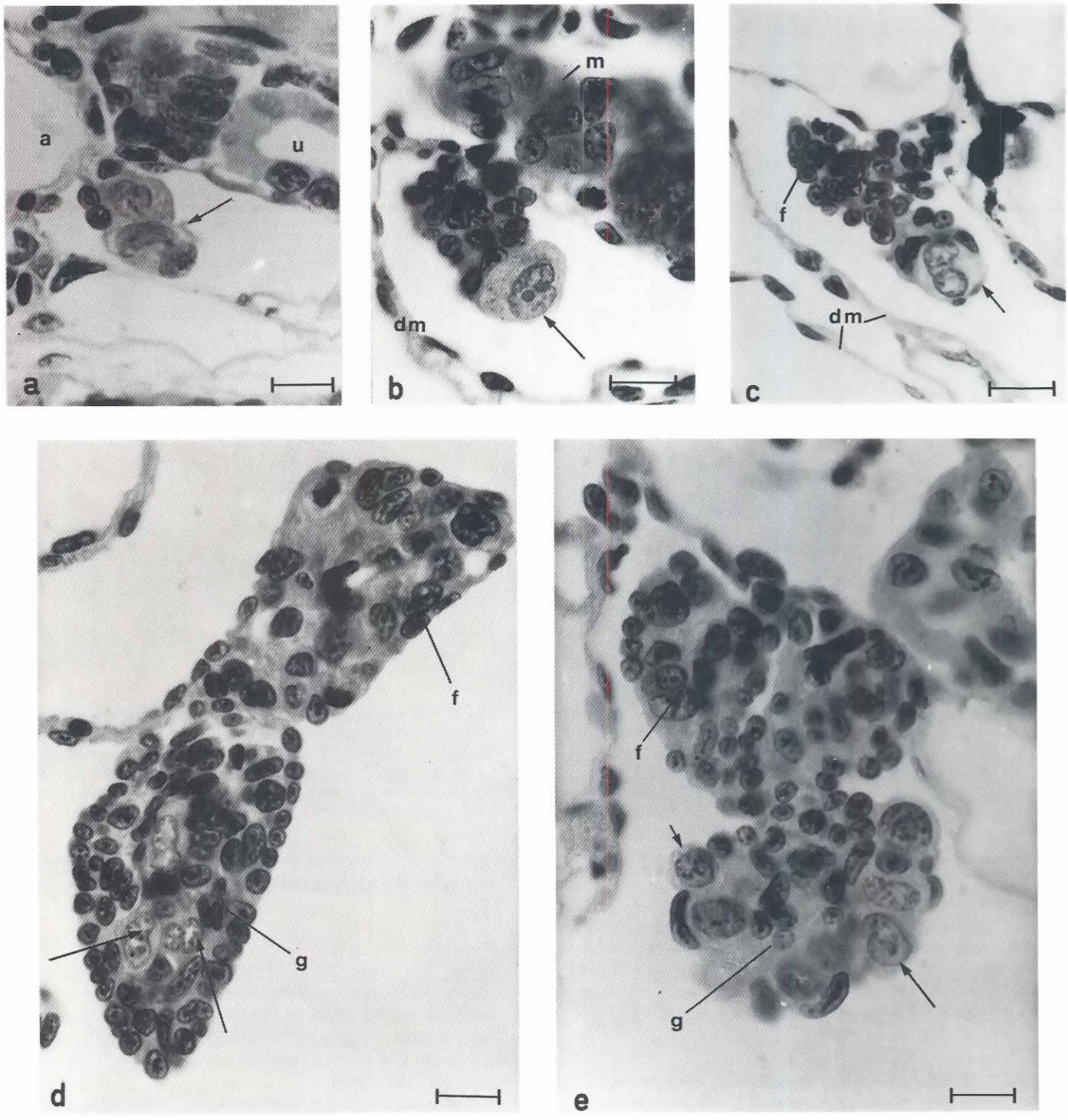

Fig. 2. Transverse sections of *Pleurodeles waltl* larvae reared at 20° ± 2° C. (a) *Genital ridge at stage 42.* **(b)** *Sexually undifferentiated gonad at stage 45.* **(c)** *Sexually undifferentiated gonad at stage 50.* **(d)** *Testis at stage 53.* **(e)** *Ovary at stage 53. (Arrow: germ cell; a: aorta; dm: dorsal mesentery; f: fat body; g: gonad; m: mesonephros;u: primary ureter; scale bar: 20 μm).*

meiosis at metamorphosis, whereas in testis, spermatocytes begin meiosis much later. At 32°C, the differentiating gonad is always a testis since ZW individuals are sex reversed (Fig. 3d). The neotestes of dwarf neomales are always smaller (both in length and diameter) than testes of normal males reared at room temperature; this reduced size is discernible in Figs. 2d and 3d.

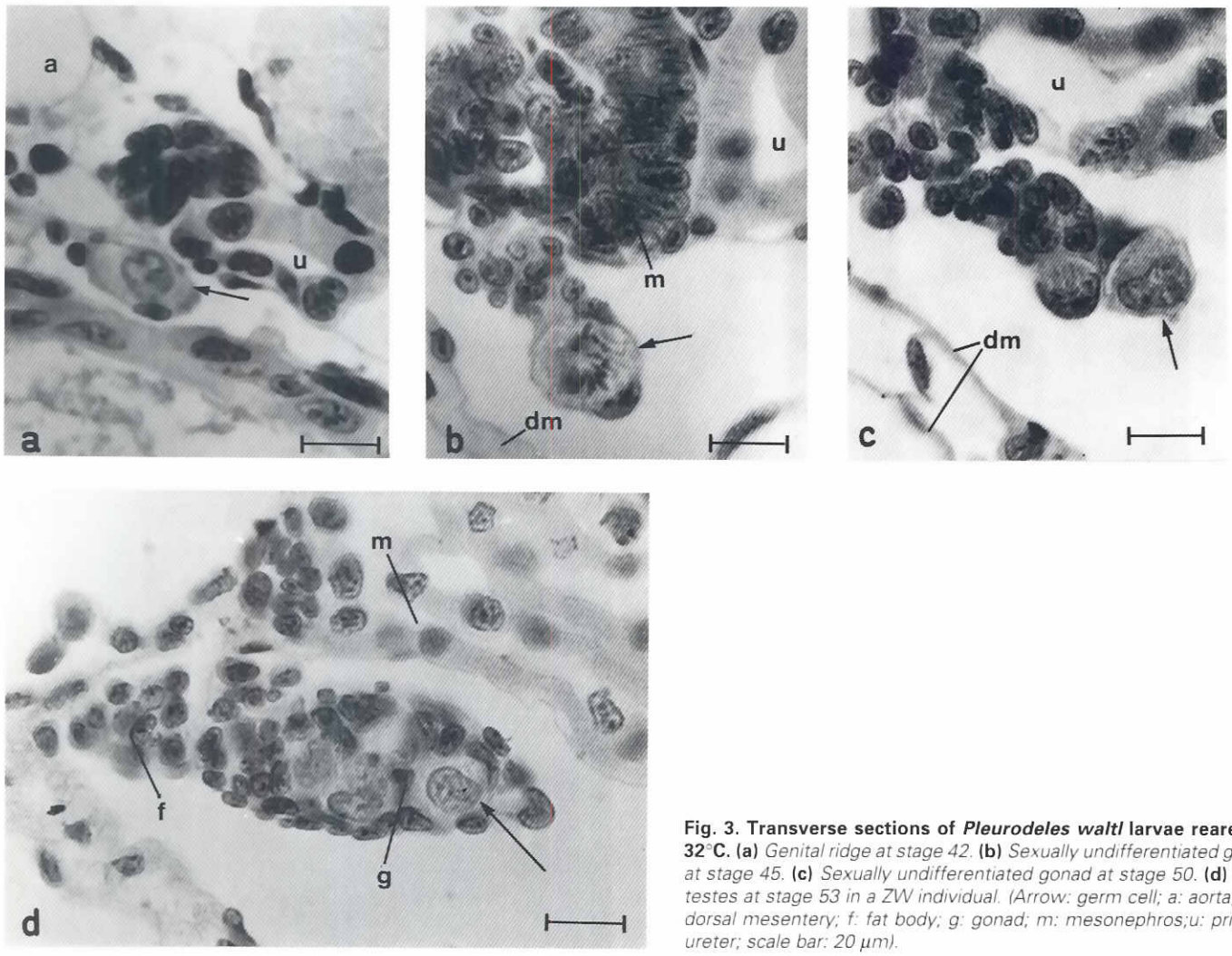

Fig. 3. Transverse sections of *Pleurodeles waltl* larvae reared at 32°C. (a) *Genital ridge at stage 42.* **(b)** *Sexually undifferentiated gonad at stage 45.* **(c)** *Sexually undifferentiated gonad at stage 50.* **(d)** *Neotestes at stage 53 in a ZW individual. (Arrow: germ cell; a: aorta; dm: dorsal mesentery; f: fat body; g: gonad; m: mesonephros;u: primary ureter; scale bar: 20 μm).*

Effect on the sex differentiation of gonads of rearing at 32°C

ZZ animals

A batch of thirty ZZ genotypic animals was reared at 32°C from stage 40 up to metamorphosis. After that, animals were reared at room temperature (20° ± 2°C). The thirty animals differentiated into phenotypic males (100% males). At 32°C, temperature does not modify the sexual differentiation of the male gonad.

ZW animals

At 32°C, it was previously demonstrated that ZW individuals differentiated into phenotypic males (100% thermoneomales) (Dournon and Houillon, 1985).

Germ cell proliferation according to sexual genotype at room temperature

Differential proliferation between ZZ and ZW offspring

In ZZ 85 and ZW 82 monosexual offspring belonging to the hybrid group, GC proliferation develops through three distinct phases: the first is the non-proliferation P_0 period, the second, P_1 period, is characterized by a moderate GC proliferation and the third, P_2 period, is clearly marked by a strong acceleration of GC proliferation (Table 1, Fig 1).

P_0-P_1 periods

During this spell, GC proliferation has been simultaneously studied by mitotic index calculation and by GC numeration (Table 1).

For ZZ larvae, mitotic index (M.I.) is nil from stage 37 up to stage 40 but increases steadily during and after stage 42 (M.I. = 4%). For ZW larvae, M.I. is nil until stage 43 and remains very low at stage 45 (M.I. = 0.4%) (Fig.6).

The GC average number does not increase from stage 37 up to stage 42 for ZZ larvae or up to stage 45 for ZW larvae (Fig. 4a). So, GC number variations confirm M.I. variations.

These first results demonstrate that Po period duration at room temperature is correlated with sexual genotype of larvae. For ZZ larvae, $^{ZZ}P_0$ period ends just before stage 42. This corresponds to the 14-day-long P_0 period previously described (Dournon *et al.*, 1989). For ZW larvae, the $^{ZW}P_0$ period is 20 days long and ends after stage 45.

The beginning of P_1 period is characterized in ZZ larvae by a peak

TABLE 1

GC COUNTS AND MITOTIC INDICES FROM STAGE 37 TO STAGE 53 IN LARVAE ISSUED FROM STANDARD OFFSPRING AND FROM MONOSEXUAL ZZ OR ZW OFFSPRING REARED AT 20° ± 2°C OR AT 32°C

	REARING TEMPERATURE: 20° ± 2°C							REARING TEMPERATURE: 32°C					
OFF. REF.	STAGE	NUMBER OF EXAMINED LARVAE	TOTAL GC NUMBER (all larvae)	AVERAGE GC NUMBER ± S.E. (per larva)	MITOSIS NUMBER	MITOTIC INDEX	OFF. REF.	STAGE	NUMBER OF EXAMINED LARVAE	TOTAL GC NUMBER (all larvae)	AVERAGE GC NUMBER ± S.E. (per larva)	MITOSIS NUMBER	MITOTIC INDEX
	41	4	184	46.0 ± 5.3									
	43	4	161	40.3 ± 5.1									
	45	3	161	53.7 ± 13.2									
Sd	47	3	205	68.3 ± 23.9									
79	49	4	461	115.3 ± 19.5									
	51	4	764	191.0 ± 29.6									
	53	4	2018	504.5 ± 53.4									
	37	3	204	68.0 ± 9.1	1	0.490							
	40	4	322	80.5 ± 9.5	0	0		40	3	172	57.3 ± 2.2	0	0
	42	4	258	64.5 ± 7.7	2	0.775		42	4	233	58.3 ± 5.0	4	1.717
ZZ	43	4	455	113.8 ± 12.4	18	3.956	**ZZ**	43	4	232	58.0 ± 6.9	6	2.586
85	45	4	464	116.0 ± 13.1	14	3.017	**86**	45					
	47	4	664	166.0 ± 21.9	10	1.506		47	4	423	105.8 ± 15.3	12	2.837
	49	4	744	186.0 ± 30.7	18	2.419		49	4	705	176.3 ± 41.3	7	0.993
	51	4	1264	316.0 ± 25.8	24	1.898		51	4	959	239.8 ± 17.0	18	1.877
	53	4	1959	489.8 ± 56.4	37	1.888		53	4	1904	476.0 ± 95.5	39	2.048
	38	5	137	27.4 ± 2.1									
	41	3	94	31.3 ± 7.0									
	43	4	104	26.0 ± 5.5									
ZW	45	2	38	19.0 ± 12.0									
79	46	4	102	25.5 ± 4.9									
	48	4	228	57.0 ± 10.7									
	50	4	212	53.0 ± 27.5									
	52	4	534	133.5 ± 22.2									
	38	11	697	63.4 ± 3.9	0	0							
	41	6	415	69.2 ± 4.1	0	0							
	42							42	5	373	74.6 ± 9.9	0	0
	43	5	318	63.6 ± 7.8	0	0		43					
ZW	45	4	262	65.5 ± 8.5	1	0.382	**ZW**	45	4	296	74.0 ± 13.9	1	0.338
82	47	5	498	99.6 ± 22.7	8	1.606	**82**	47					
	48							48	5	590	118.0 ± 12.3	10	1.695
	49	5	512	102.4 ± 22.7	11	2.148		49					
	51	5	1056	211.2 ± 18.0	40	3.788		51					
	52							52	5	865	173.0 ± 16.1	36	4.162
	53	4	2536	634.0 ± 95.2	78	3.076		53	5	1043	208.6 ± 31.8	8	0.767

OFF. REF: reference of offspring

in the mitotic activity. In these ZZ larvae, this induces after stage 42 a sudden increase in GC number immediately followed by a phase in which the GC number first levels off and then moderately increases until stages 49-50 are reached. In ZW larvae, both M.I. and GC number rise moderately from the beginning of P_1 period (stage 45) up to stages 49-50 (Figs. 6 and 4a). For this reason and on account of the different lengths of the $^{ZZ}P_0$ and $^{ZW}P_0$ periods, there are more GCs in ZZ than in ZW larvae at the end of the P_1 period (about stages 49-50).

P_2 period

After stages 49-50, GC proliferation accelerates swiftly both in ZZ and ZW larvae. However, in future testes, GC proliferation accelerates less than in future ovaries.The proliferations are significantly different. At stage 53, the GC number is smaller in ZZ future males than in ZW future females (489.8 ± 56.4 and 634.0 ± 95.2 GCs), although proliferation begins earlier in ZZ larvae (Fig. 4a). The period of differential proliferation was named P_2 period.

At room temperature, GC proliferations of ZZ and ZW individuals which will differentiate into a phenotype in conformity with their sexual genotype, differ on two points: the P_0 period duration and the differential GC proliferation during the P_1 and P_2 periods.

Influence of PGC number during P_0 period on the ulterior GC proliferation

Two offspring ZW 79 and ZW 82 are characterized by different PGC numbers during P_0, respectively 28.9 ± 2.7 PGCs (group III) and 65.4 ± 2.9 PGCs (hybrid group). Their $^{ZW}P_0$ period durations are identical and during P_1 period no significant differences are detected between the GC proliferations of these two ZW offspring (Fig. 4b).

At room temperature and for the same sexual genotype, GCs proliferate, up to stage 50, in the same manner whatever the initial PGC number.

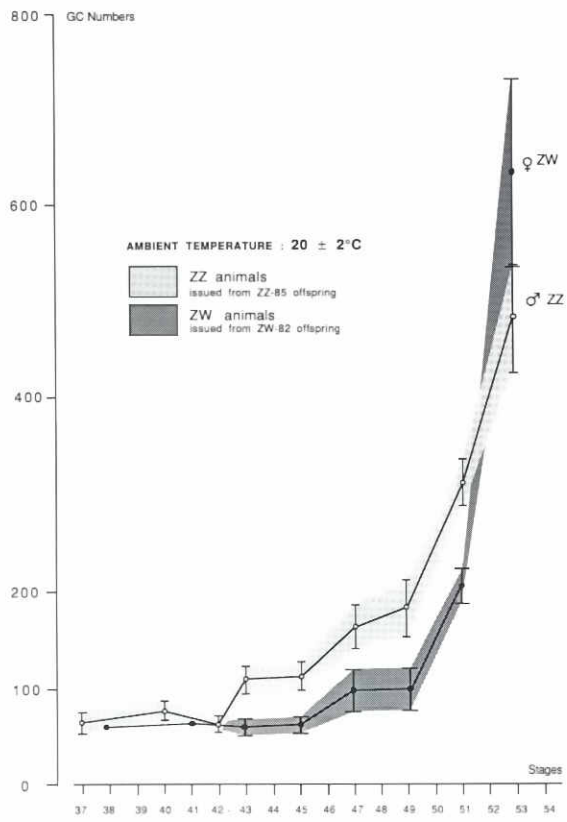

Figure 4 a

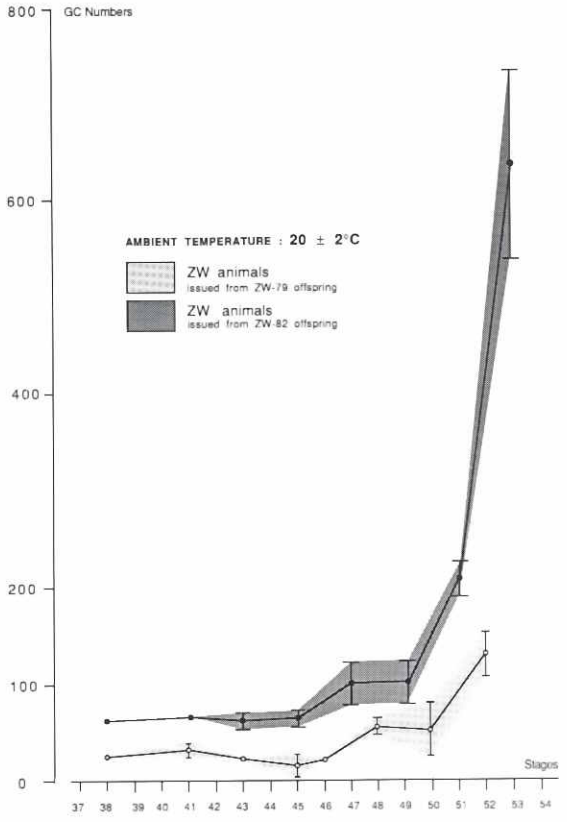

Figure 4 b

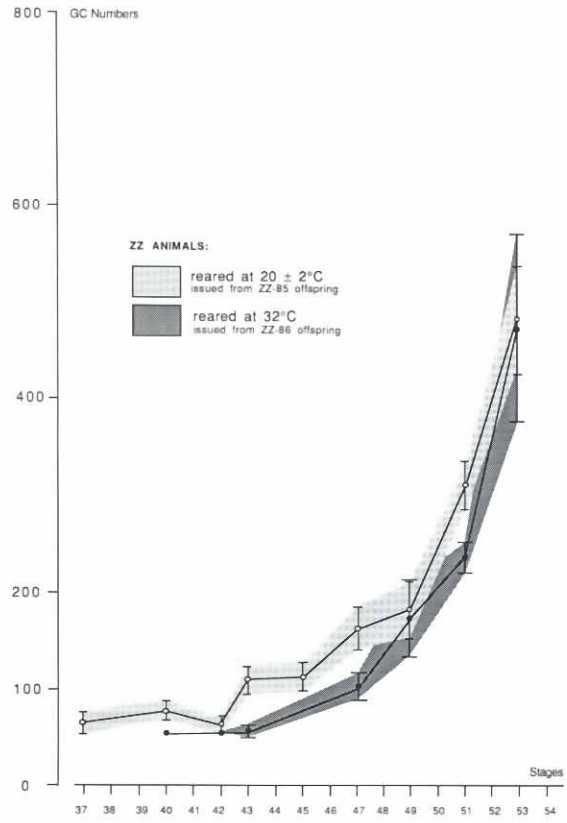

Figure 4 c

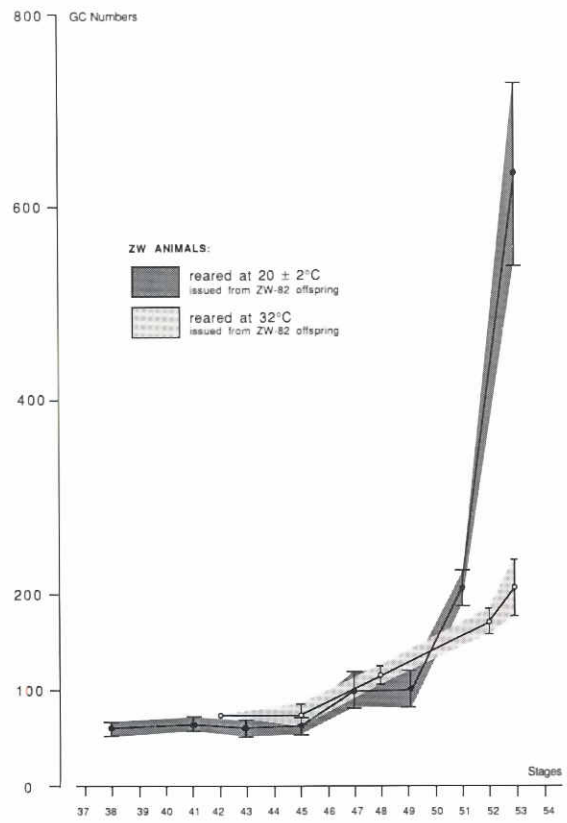

Figure 4 d

Fig. 4. Relationships between GC numbers and developmental stages: (a) *at room temperature (20° ± 2°C) in ZZ and ZW animals.* **(b)** *At room temperature (20° ± 2°C) in two ZW offspring belonging to two different groups: ZW 79 from group III (28.9 ± 2.7 PGCs) and ZW 82 from the hybrid group (65.4 ± 2.9 PGCs).* **(c)** *In ZZ animals reared at room temperature or at 32°C.* **(d)** *In ZW animals reared at room temperature or at 32°C. S.E. are drawn only for values higher than 6.*

Fig. 5 gathers the results concerning GC proliferations of standard offspring (Sd 1-79) belonging to group II and of ZZ 85 and ZW 82 offspring both of the hybrid group. The standard offspring presents a significantly lower initial PGC number than the two monosexual offspring; if this difference is taken into account and if a translation is effected then, the standard GC proliferation corresponds to the resultant from GC proliferation of ZZ type and from GC proliferation of ZW type. All these offspring consequently confirm that GC proliferation after P_0 period is independent of the PGC number during P_0 period, *i.e.* independent of the groups.

These results indicate that GC proliferations of individuals coming from different groups are not different so long as the larvae have the same sexual genotype.

Effects of the rearing temperature on germ cell proliferation

Two comparisons have been made: the one between two ZZ offspring (ZZ 85 and ZZ 86) reared at room temperature and at 32°C and the other, between two batches from the ZW 82 offspring, reared respectively at room temperature and at 32°C.

Germ cell proliferation in ZZ larvae

P_0-P_1 periods

At 32°C, M.I. of the ZZ offspring is nil at stage 40. At stage 42 and later on, M.I. rises suddenly and then decreases as at room temperature (Table 1, Fig.6). GC number rises in ZZ larvae from stage 43 at 32°C. This differs little from the starting of GC proliferation found at 20°C. A similar GC number characterizes ZZ offspring at stage 43 and at stages 49-50 whatever the rearing temperature: however, it may be noticed that from the end of P_0 period up to stage 47, GC proliferation at 32°C is weaker than GC proliferation observed at 20°C, whereas from stage 47 up to stages 49-50, GC proliferation is stronger than at 20°C (Fig. 4c). Thus a rise in rearing temperature modifies the progress of GC proliferation in ZZ larvae during P_1 period.

P_2 period

At stage 53, the same GC average numbers characterize testes differentiated at 20°C or at 32°C (489.8 ±56.4 GCs for ZZ 85 and 476.0 ± 95.5 GCs for ZZ 86) (Fig. 4c). However, it may be considered that during P_2 period, GC proliferation at 32°C is relatively more important in reduced neotestes of dwarf individuals than at room temperature in gonads of normal animals and so that the development of gonads occurs independently of the growth of the individuals.

Germ cell proliferation in ZW larvae

P_0-P_1 periods

At both 20° ± 2°C and at 32°C, in the two batches of ZW offspring, M.I. are the same for the two rearing temperatures: they are weak until stage 45 (Fig. 6). GC proliferation is stopped up to stage 45. Thus $^{ZW}P_0$ period has the same duration at both temperatures. After stage 45 up to stage 50, GC proliferation is the same in ZW larvae reared at 20°C or at 32° (Fig. 4d).

The above results make it possible to conclude that during P_1 period, GC proliferation is independent of the rearing temperature.

P_2 period

After stage 50, an intense acceleration of the GC proliferation occurs at 20°C, whereas it remains very moderate and gradual at 32°C. No acceleration occurs and proliferation goes at the same rate during P_2 period as during P_1 period. So, at stage 53, in reference with ZW genotype, the heat-treated dwarf ZW neomales have in their testes an average number of GCs (208.6 ± 31.8) which is a third of the average GC count found in ovaries of normal ZW females reared at room temperature (634.0 ± 95.2 GCs); in reference to size or to male phenotype, this GC count of the neomale testes is about half the GC count of testes of dwarf ZZ males reared at 32°C or even of testes of normal ZZ males reared at room temperature, respectively 476.0 ± 95.5 and 498.8 ± 56.4 GCs (Fig. 7). We never observed dead cells during the periods of GC counts.

So this strong decrease in GC number cannot be attributed only to the facts that heat-treated animals are ZW animals or are dwarfs. The low GC number of ZW neomales is clearly related to the inversion phenomenon of sexual differentiation of gonads under the effect of the rearing temperature.

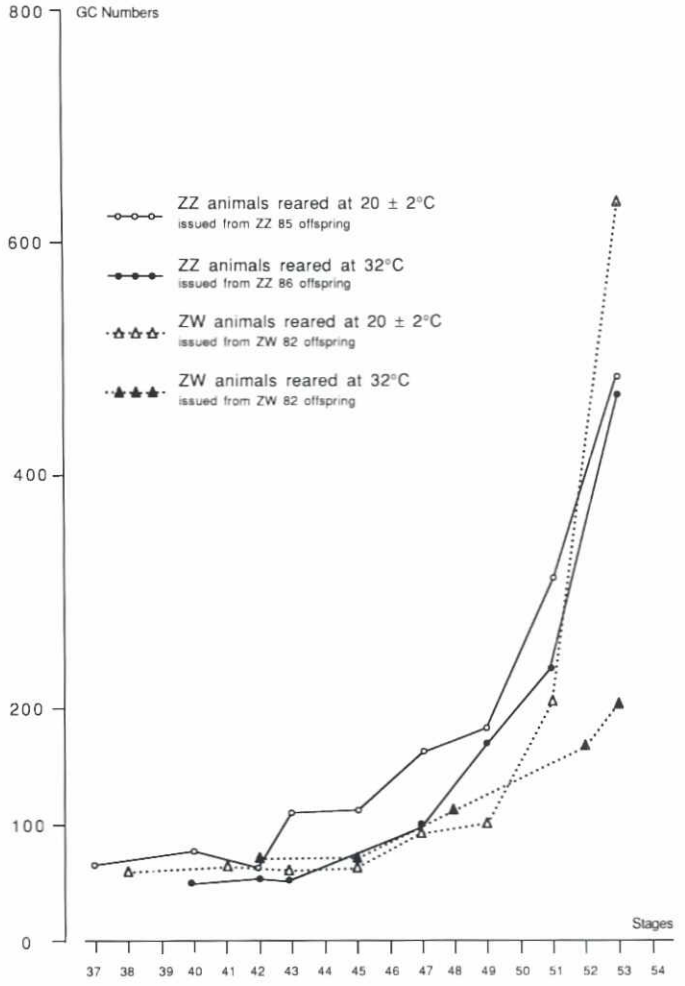

Fig. 5. Relationships between GC numbers and developmental stages in ZZ and ZW animals (group hybrid) and Sd animals (group II) reared at room temperature. *For Sd graph, each point corresponds to the GC average number of three or four unknown genotype larvae (Table 1). The dotted line corresponds to a translation of the Sd graph. This translation has been realized so as to conform and compare the Sd offspring with offspring from hybrid group. S.E. is drawn only for values higher than 6.*

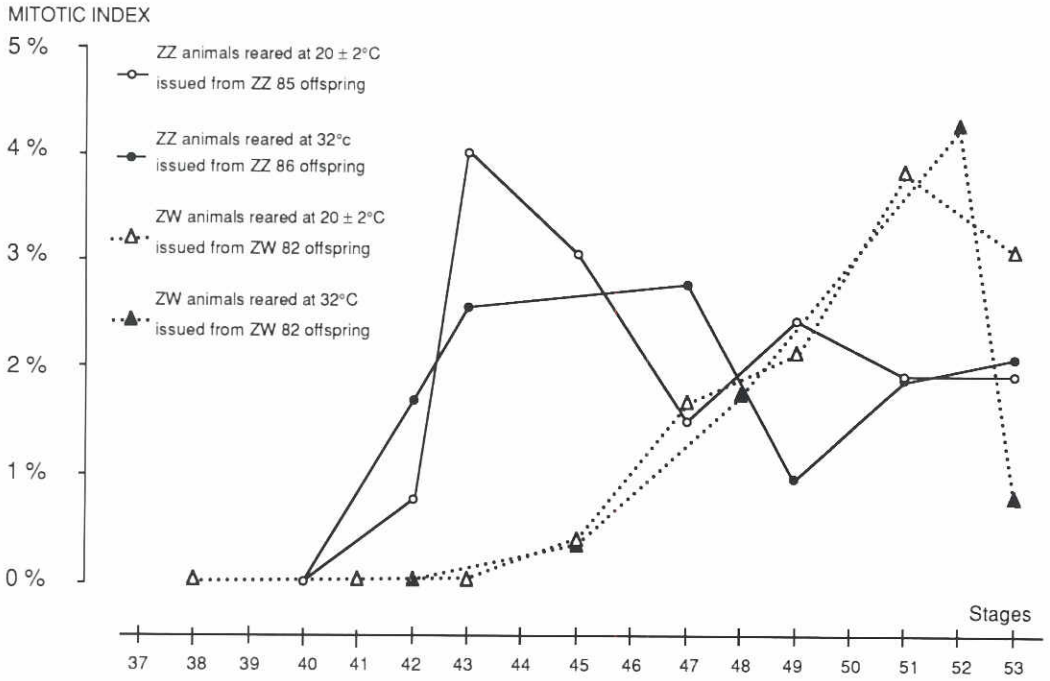

Fig. 6. Comparison of the relations between mitotic indices and developmental stages in ZZ and ZW animals reared at 20° ± 2°C or at 32°C.

Therefore during P_2 period, the rise in rearing temperature induces indirectly in ZZ male larvae a probable acceleration of GC proliferation, whereas it induces a strong reduction of GC proliferation in sex-reversed ZW larvae.

Discussion

In the developing gonads, before their sexual differentiation, GC proliferation extends in *Pleurodeles waltl* through three successive periods during which both sexual genotype and rearing temperature act to control GC proliferation itself and to modify the duration of the proliferation period.

For the first period, our previous work indicated that the period extending from stage 35 up to stage 41 was a non-proliferation period (P_0 period) during which PGC number per larva is independent of sexual genotype (Dournon *et al.*, 1989). The present work demonstrates moreover that the extent of P_0 period is a function of ZZ or of ZW sexual genotypes; $^{ZZ}P_0$ period goes up to stage 42 for male genotypic larvae whereas $^{ZW}P_0$ period ends after stage 45 for female genotypic larvae. These $^{ZZ}P_0$ and $^{ZW}P_0$ periods are not proliferative periods. Moreover, M.I. and GC numbers both indicate that the extent of $^{ZZ}P_0$ period and the extent of $^{ZW}P_0$ period are independent from rearing temperature. The end of P_0 period is characterized by the beginning of GC proliferation but the nature of the signal for GC proliferation is unknown.

For the second period, called P_1 period and which ends about stage 50, at 20°C and at 32°C, GC number is higher in ZZ larvae than in ZW larvae. For ZZ animals, a rise in rearing temperature modifies the progress of GC proliferation whereas in ZW larvae GC proliferation appears to be temperature independent.

During P_1 period, the mesonephrotic blastema differentiates. Houillon (1956), in *Pleurodeles waltl*, has indicated that in its presence, GC proliferation occurs and that in its absence, proliferation not only does not occur but GC number decreases swiftly before P_0 period ends. In *Bufo*, an experimental excess of medulla cells stimulates GC proliferation (Gipouloux, 1973). So, a signal for germ cell proliferation at beginning of $^{ZZ}P_1$ and $^{ZW}P_1$ periods could be emitted by the mesonephrotic blastema cells. However it may be observed that the basis of this hypothesis is in contrast with suggestions of Merchant-Larios and Villalpando (1981) indicating that cells of gonadal medulla would come from coelomic epithelium in Anuran. At the present time, we have not studied whether, in the monogenic offspring, the mesonephrotic blastema differentiates earlier in ZZ males than in ZW females.

P_1 period ends about stages 49-50. At this time four phenomena may be related to the transition between P_1 and P_2 periods. First, at about stage 50, the thermosensitive period determined at 32°C for female inversion goes through transition phases. In particular, heat treatment applied up to stage 50 does not induce sexual inversion, whereas a heat treatment applied before and after stage 50 or applied from stage 50 on is effective (Dournon and Houillon, 1985). Second, at that time in the sexually undifferentiated gonads, some GC polylobed nuclei go into a spherical shape which indicates a cellular differentiation of the germ line. Third, morphogenetic potencies of GC nuclei analyzed by germ nuclear graft into oocytes are cut down about stage 50. Experimental larvae reaching the hatching stage are obtained in large numbers when they issue from GC nuclei taken from larvae before stage 50, but are scarce when they issue from GC nuclei originating from larvae after stage 50 (Lesimple *et al.*, 1987). Fourth, at the same stage, sexually undifferentiated gonads begin to divide longitudinally into fat bodies located on the median side and into true gonads, not yet sexually differentiated, located on the distal side. All these phenomena concerning interactions of germ and somatic cells indicate that molecular events preceding histological differentiation of the gonadal sexual phenotype occur at the early beginning of P_1.

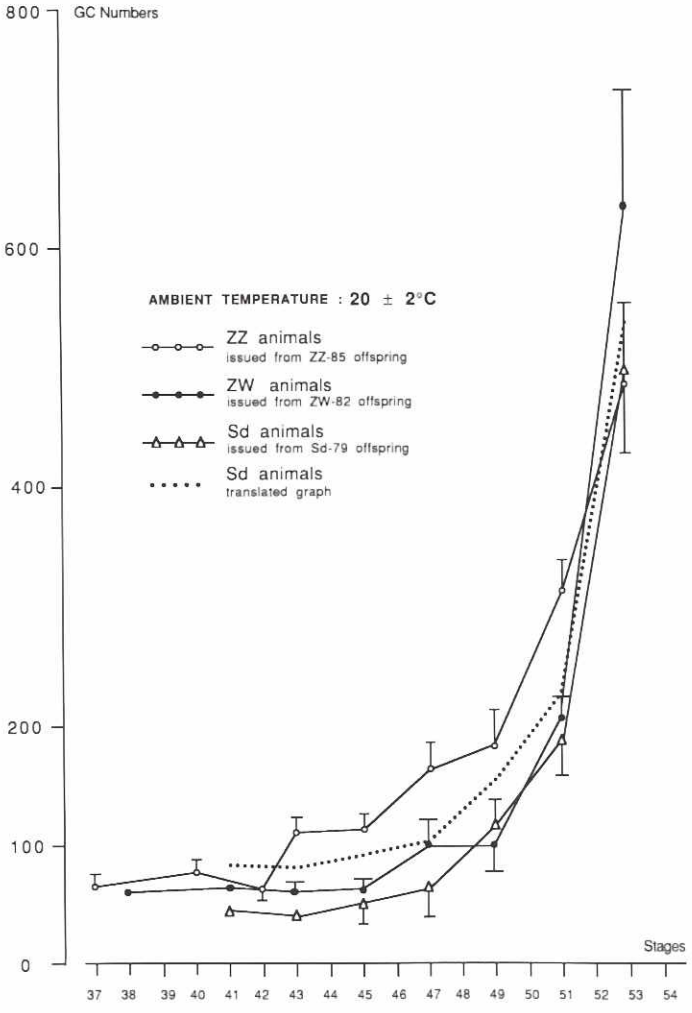

Fig. 7. Comparison of the relations between GC numbers and developmental stages in ZZ and ZW animals reared at 20° ± 2°C or at 32°C.

According to our initial hypothesis, somatic cells still cytologically undifferentiated but already sexually determined would induce a sexual GC differentiation. An inverse hypothesis is evoked by Shirane (1987) who considers that molecules from PGCs act as a trigger for the expression of genes that control sexual differentiation of somatic cells. This hypothesis does not agree with the fact that GC differentiation conforms always to sexual phenotype of somatic cells in embryonic grafts (Humphrey, 1945; Collenot, 1973; Dournon and Godbillon, in preparation).

During the third period, P_2 period, two different events concerning GC number have been observed. In the first one, at room temperature, GC proliferation is higher in future ZW ovaries than in future ZZ testes. This intense proliferation characterizes ZW future ovaries. At that temperature, GC proliferation is controlled by sexual genotype. In the second one, at 32°C, rearing temperature inhibits the intense GC proliferation of ZW individuals which now differentiate neotestes. This slowed proliferation observed during P_2 period in ZW larvae appears correlated with progressive masculinization of gonad and with inversion phenomena.

The comparison between ZZ larvae reared at 20°C or at 32°C shows that during P_2 period, the rise in temperature would induce a relative acceleration of GC proliferation with regard to the growth of individuals that are normal at 20°C but dwarf at 32°C. In ZZ larvae, the rise in rearing temperature would induce a probable stimulation of GC proliferation. At 32°C, the comparison of GC number in neomale ZW larvae with GC number in male ZZ larvae allows us to infer that the control by sexual genotype of GC proliferation in ZW larvae is perturbed and relayed by an indirect control of GC proliferation by temperature. Moreover, it may be stated that the rise in rearing temperature acts on GC proliferation to induce either its acceleration in ZZ larvae or its restriction in ZW ones. In *Pleurodeles waltl*, the discordance, brought to light by rearing at 32°C, between individual growth and GC proliferation indicates that mechanisms involved in the control of GC proliferation could present a sexual specificity.

At room temperature, after the beginning of histological differentiation of gonadal sex, in some amphibians, *Xenopus laevis* (Ijiri and Egami, 1975; Züst and Dixon, 1977), *Euproctus asper* (Rouy, 1971), in some fishes (Hardisty, 1967) and in birds (Witschi, 1956), the GC number is higher in females. Likewise, in *Pleurodeles*, during and just after differentiation of gonads, the female heterogametic sex is characterized by a greater number of GC than the male homogametic sex. In mammals such as rat (Beaumont and Mandl, 1962, 1963; Charpentier and Magre, 1989) and rabbit (Chretien, 1966), the GC number is higher in males. So it appears that a higher GC number characterizes the heterogametic sex in heterothermal vertebrates as well as in homeothermal vertebrates (Mittwoch, 1971, 1983).

In mammals, a modification of GC number after inversion of sexual differentiation has been observed in gonads of calf freemartins (Jost *et al.*, 1975) but also in immature female gonads of rat cultured *in vitro* with purified bovine anti Müllerian hormone (Vigier *et al.*, 1987); for both cases, the GC number is lower in sex-reversed ovaries than in normal ovaries. So it appears that in *Pleurodeles*, as in mammals, the masculinization of ovaries is characterized by a decrease in GC number.

In normal development, the greatest GC number always characterizes the heterogametic sex. In sexual inversion of the genotypical females, the reduction of GC number in future neotestes is observed in the heterogametic as well as in the homogametic females. From these results, we propose the following interpretation of normal sexual differentiation and sexual inversion of females concerning both heterothermal and homeothermal vertebrates: there would exist distinct molecular ways for testis differentiation, one followed when gonad differentiation conforms to sexual genotype, and others leading to phenotypic inversions.

Materials and Methods

Origin of larvae and rearing conditions

So as to be ensured of sexual genotype of studied larvae, we used monosexual offspring issued from individuals whose sexual genotype had been established through the study of the sex-linked peptidase-1 enzyme (Ferrier *et al.*, 1980, 1983; Dournon *et al.*, 1988) and the genetical study of one of their offspring.

Three types of offspring were used:

- standard offspring (Sd) issued from laboratory breeding. At room temperature, ZZ animals become males (50%) and ZW ones become females (50%);

- monosexual male offspring (ZZ) issued from crosses between ZZ standard males and ZZ neofemales obtained after estradiol treatment during larval stages (Gallien, 1951). In these offspring at room temperature, all the individuals differentiate into ZZ males (100% ZZ males);
- monosexual female offspring (ZW) issued from crosses between ZZ standard males and WW thelygenous females (Dournon and Houillon, 1984; Dournon *et al.*, 1988). All the animals issued from these offspring become ZW females (100% ZW females) at room temperature, whereas at 32°C, the sexual phenotype of ZW individuals is sex-reversed (100% ZW neomales) (Dournon and Houillon, 1985).

Offspring were labeled by type (Sd, ZZ or ZW) and by birth year (Sd 79) if necessary preceded by a serial number (Sd 1-79).

In heterothermal animals, a rise in temperature speeds up the development rate. In *Pleurodeles waltl*, this remains true up to 30°C but, at 32°C, the development rate of this species is about the same as at 20°C. However, at 30°C as well as at 32°C, metamorphosed animals are dwarfs. At stage 53, at 32°C, the total length of a larva is 26 mm, whereas at room temperature, the length is 34 mm.

Studied period of development

GC proliferation, which has been previously investigated before stage 41 (Dournon *et al.*, 1989), was studied in the present work on larvae from stages 37 to 53. Stages are defined from the developmental table of Gallien and Durocher (1957). The analysed period is reported in Fig. 1.

Germ cell counts

Size permits discrimination between PGCs (Ø 30 µm) and somatic cells (Ø 10 µm). In young larvae, a polylobed nucleus and cytoplasmic pigment granules characterize PGCs (Lesimple *et al.*, 1987, 1989). In aging larvae, some GC nuclei become round. With hemalum-eosine, GCs are less colored than somatic cells.

GC numbers were established by counting GC nuclei through 7 µm thick histological serial sections from the cloacal position up to the genital ridge or gonad disappearance in the anterior region.

The margin of error was assessed as 2% from stage 37 to stage 53.

Selection of comparable offspring

In the present work, three parameters have been taken in account: the group characterizing the offspring during P_0 period, the sexual genotype and the rearing temperature. We have worked with monosexual offspring belonging in an unforeseeable manner to different groups. To ensure valid comparisons between GC proliferation of offspring we checked to see whether it was necessary or not to compare offspring issued from the same group. Thus, in a first experiment, two offspring, ZZ 85 and ZW 82 both from the hybrid group, reared both at room temperature, made it possible to compare GC proliferation between offspring of different sexual genotypes. In a second experiment, three other offspring, ZW 79, ZW 82 and a standard offspring, all reared at room temperature but belonging respectively to group III, to the hybrid group and to group II, enabled us to answer the question of whether the PGC number established during P_0 period subsequently influences GC proliferation. Moreover, GC proliferation in the standard offspring was compared to GC proliferation in the two different monosexual offspring ZZ 85 and ZW 82. In the second part of this work, the influence of rearing temperature on GC proliferation has been studied using offspring with different sexual genotypes, ZZ 86 (group III) and ZW 82 (hybrid group).

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