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The induction of triploidy in *Oreochromis aureus* by heat shock

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Summary. Triploid fish were obtained using heat-shock treatment. The optimal conditions for the heat shock (39.5 ± 0.2 °C for 3.5–4 min) as well as the exact zygote age (3 min) at which this heat shock was applied were studied. Results showed that this treatment gives rise to 100% of triploid fish with a satisfactory survival rate of 61% beyond the yolk sac resorption. The genital papillae of this triploid fish were underdeveloped in comparison to normal diploid fish. However, no morphological or growth-rate differences between diploid and triploid fish could be observed up to the age of 6 months. Triploidy was assessed by the karyotyping of embryo cells or adult PHA-stimulated lymphocytes, or by erythrocyte measurements. The occurrence of a heat-shock sensitive event at the zygotic age of 6 min is discussed.

Key words: *Oreochromis* – *Tilapia* – Polyploidy – Triploidy – Heat-shock

Introduction

The extensive interest in induction of polyploidy in plants, animals and particularly in fish, arises from the potential contribution of this research to three main fields.

1 Efficiency of growth

It has been reported that triploidy in fish leads to sterility (Swarup 1959a; Purdom 1972; Thorgaard 1979; Allen and Stanley 1979; Gervai et al. 1980). This sterility has considerable impact on food conversion rate and on rapidity of growth because the energy which is normally needed for gametogenesis in a fertile fish might be used in triploids to increase the quantity of edible tissue. In that case, the commercial culture of triploid fish might be more profitable than that of normal fish (Rafstie et al. 1977; Allen and Stanley 1979; Thorgaard 1979; Wolters et al. 1982). Moreover, it has been suggested that in some species (e.g., Tilapias, Salmonids) the rate of mortality normally observed during the season of reproduction is considerably reduced in triploids (Refstie et al. 1977; Allen and Stanley 1979).

2 Cytogenetical research

Chromosomal manipulations and investigation of the effects of extra sets (one or more) of chromosomes on the individual's morphology and functioning have had a considerable influence on the development of cytogenetic research: influence of extra sets of chromosomes on growth rate, sex determination and fertility (Swarup 1959b; Purdom 1972; Gervai 1980; Beck and Biggers 1983). In addition, research in polyploidy enables the investigation of quantitatively expressed genes (relationship between number of copies of gene and amount of product) (Purdom 1972; Stanley and Allen 1984), and of regulatory genes, as well as learning about processes which occur in the cell during the development of the zygote.

3 Gynogenesis

The technique used to induce triploidy in a diploid zygote has been used successfully for diploidization of an activated haploid gamete and production of gynogenetic individuals (Nagy et al. 1978; Chourrout 1980; Chourrout and Quillet 1982; Refstie 1983).

Polyploidy has been observed in nature following hybridization of a polyploid parent with another polyploid or diploid parent (Thorgaard 1979; Beck and Biggers 1983). Artificial induction of polyploidy is based on interfering with cell cytokinesis by (1) prevention of extraction of the first or second polar body in meiosis I or II and (2) suppression of the first cleavage. Such interventions have been carried out by chemical (mitotic inhibitors not interfering in DNA synthesis) and physical treatments. Cytochalasin B has been used to induce polyploidy in salmonides (Refstie et al. 1977; Allen and Stanley 1979), in American oysters (Stanley et al. 1984) and in mice (Snow 1973). Nocadazole and cytochalasin D have been used in mammalian cells (Zieve 1984). Edwards (1958a, b) used colchicin in his experiments in mice. Concerning physical

treatments, pressure shocks given to the fertilized eggs at a critical time following insemination were reported to be efficient in amphibians (Rinschmidt 1979) and in fish (Benfey and Sutterlin 1984; Chourrout 1984). Heat or cold temperature-shocks are actually the most common techniques for inducing polyploidy in fish. Heat shocks were used by Swarup (1959a); Chourrout (1982); Refstie (1983); Utter et al. (1983); Chourrout and Itskovich (1983) and Benfey and Sutterlin (1984), whereas cold shock treatment was successfully used by Swarup (1959a); Purdom (1972); Valenti (1975); Nagy et al. (1978) and Gervai et al. (1980).

In this paper the optimization of the conditions for inducing triploidy in *O. aureus* is studied.

Materials and methods

Fish

The Ein Hamifratz line 6.8 of *O. aureus* was used in this study (Avtalion et al. 1982; Mires et al. 1982). The fish were divided into families of 5–6 females and 2 males each. The families were kept in aquaria of 230 liters, each equipped with a circulating closed system, as described by Koiller and Avtalion (1985). The water temperature was 26–28 °C during the spawning season and 23–24 °C in winter. The fish were fed with pellets containing 40% protein.

Artificial fertilization

Artificial fertilization of the eggs was carried out in Petri dishes using modified Eagle's medium (MEM) (Yeheskel and Avtalion, in preparation) as follows: immediately before spawning, the females were gently caught and the eggs were stripped out into a small Petri dish containing 3 ml of MEM, adjusted to pH 6.5–6.8. This medium, containing 5% carp serum, was diluted 1:20 with saline immediately before the experiment. Sperm were stripped out of the male in a similar way and were examined by microscopy for their motility (Wester and Foote 1972) and concentration (about 10^9 cell/ml). When the sperm quality was found satisfactory, the sperm were diluted, depending on the initial concentration, with concentrated MEM pH 7.4, containing 5% carp serum. The eggs were divided into groups of 50–100 each (depending on the total number of eggs available for the experiment). The fertilization was carried out by adding 1 ml of diluted sperm and 2 ml of tap water to each batch of eggs. Following gentle shaking, the inseminated eggs were transferred, 10 min later, to incubation in a Zuger-bottle system (Rothbard and Hulata 1980). In this system the circulation rate in each Zuger-bottle may be controlled separately, and the water flow is mechanically filtrated through active charcoal and sterilized when flowing through a U.V. irradiator (Don et al., in preparation). The exact time when the sperm were added to fertilize the eggs was considered as zero time, which allows determining the age of the zygotes at heat shock treatment. The temperature of the water was kept constant at 25–26 °C. After yolk sac absorption, the embryos were transferred into aquaria and tanks and allowed to grow.

Heat shock treatment

The heat shock was applied in an incubator system equipped with 4 Zuger-bottles, of 250 ml each, and with circulating water adjusted to desired temperature and flow rate. The inseminated eggs were exposed to different heat shock temperatures at different time spans after insemination (see Fig. 2 in "Results"). The duration of each shock was optimized to 3.5–4 min (Fig. 4). Every experiment was carried out in duplicate for the eggs of a single female and repeated at least three times, with eggs of other females.

Survival rates

The survival rates were checked at 4 different developmental stages: morula (6–8 h after fertilization), pigmentation (55–60 h after fertilization), hatching (72–80 h after fertilization) and survival beyond yolk sac absorption (12–13 days after fertilization). In each experiment, the survival rate was calculated as the relative percentage of survivals (×) at a defined developmental stage (z), using the following formula:

$$X_z = \frac{n \times 100}{i \times c/100} = \frac{n \times 10^4}{i \times c}$$

where c is the absolute percent of fertile eggs (% of morula in the non heat-shocked control) i is the initial number of the eggs and n is the number of eggs which survived up to a given developmental stage.

Identification of ploidy

Here we used two different karyotyping techniques to obtain chromosomes at the metaphase stage. In the first technique the chromosomes were obtained from embryos 1–4 days after hatching. From each experiment we took a random sample of 10 embryos. The living embryos were incubated into a colchicin solution, optimized to a concentration of 0.01%–0.015%, for 2–4 h. Then the yolk sacs and heads were discarded and the remaining parts were fragmented into small pieces. The fragments were washed in MEM pH 7.5 and incubated in hypotonic solutions (5 min in distilled water and 25 min in tilapia serum diluted 1:4 in distilled water). Fragments were then incubated in an acetic acid-methanol (1:4) fixative solution for 20 min. Then they were strained through a 50-mesh stainless steel net. The cell suspension was centrifuged for 5 min at 1,200 RPM and then dripped on a preheated slide (about 50 °C). The dried slides were stained with 20% Giemsa stain for 20 min. The second technique was based on Tilapia lymphocyte cultures (Choureki and Avtalion, in preparation). After 4–5 days of culture, colchicin was added to a final concentration of 10 μg/ml, for 6–8 h. Then the cells were harvested, washed three times with 0.2 M PBS pH 7.2, incubated 10 min in hypotonic solution and fixed for 5 min as detailed above. After fixation, the suspension was centrifuged and treated as in the first technique. This technique has the advantage of keeping the fish alive.

Results

Viability

The mean percentage of viability of untreated controls, in their four developmental stages is presented in Fig. 1. The highest mortality rate (16%) occurs between the morula (M) and pigmentation (P) stages. As to the embryos who have reached the hatching stage (H), in most cases, they survive beyond yolk sac absoption (V).

Heat shock treatment

Three parameters related to heat-shock-treatment were studied: a) the interval of time between fertilization

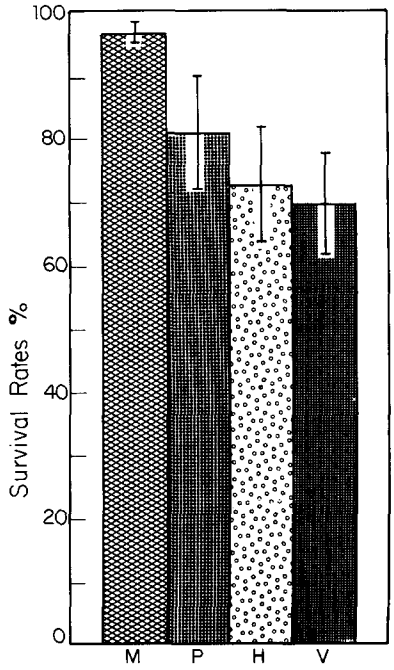

Fig. 1. Viability at different embryo stages of artificially inseminated eggs in the absence of heat-shock treatment. *M* morula; *P* pigmentation; *H* hatching; *V* viability beyond yolk sac resorption

and heat shock (zygote age); b) the heat shock temperature level; c) the heat-shock duration. The influence of the first two variables on the viability of embryos is shown in Fig. 2. Embryos of different ages (2–9 min) were subject to heat shock and different temperature levels were tested: (38.5 ± 0.2 °C, 39.5 ± 0.2 °C, 40.5 ± 0.2 °C, and 41.5 ± 0.2 °C – Fig. 2a–d, respectively). Results show that heat shocks at 38.5 °C had almost no effect on the viability of morulas which remain nearly equal to 100%, independently of the zygote age. Other stages (pigmentation, hatching and viability following yolk resorption) show almost the same patterns as in the control embryos. However, a slight depression in the curves (higher mortality rate) can be observed at the zygote age of 6 min. No polyploidy was observed in embryos of all ages heat-shocked at 38.5 °C. On the other hand, while the formation of morulas was not affected in heat-shocks carried out at 39.5 °C (Fig. 2b), two strong depressions in the curve can be observed for stages P, H and V. The first depression occurs at the zygote age of 2 min and the second at 6 min. When we examined the chromosome numbers of embryos from all surviving zygote ages, we observed that a high percentage (nearly 100%) of triploidy (66 chromosomes) occurs only in embryos which have been heat-

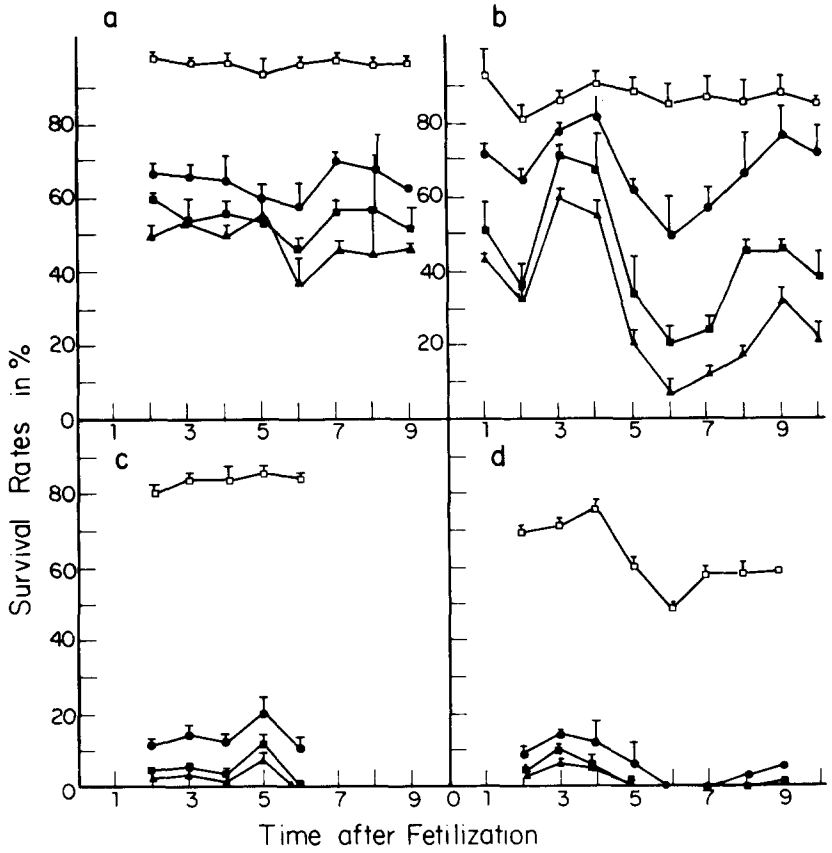

Fig. 2. Viability at different embryo stages following heat-shock at 38.5° (**a**); 39.5° (**b**); 40.5° (**c**) and 41.5° (**d**). □ morula; ○ pigmentation; ■ hatching; ▲ viability beyond yolk sac resorption

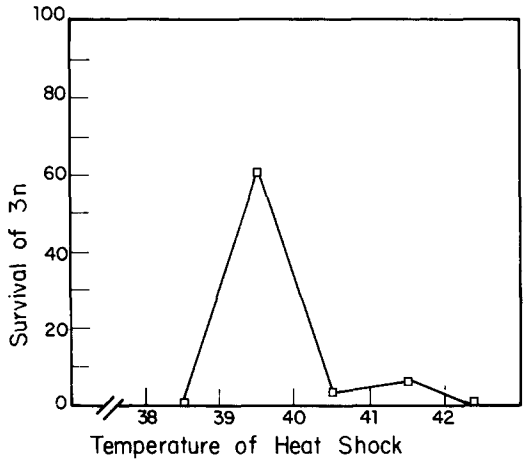

Fig. 3. Influence of heat-shock temperature, applied at the zygotic age of 3 min, on the rate of survival of triploid embryos beyond the yolk-sac resorption stages

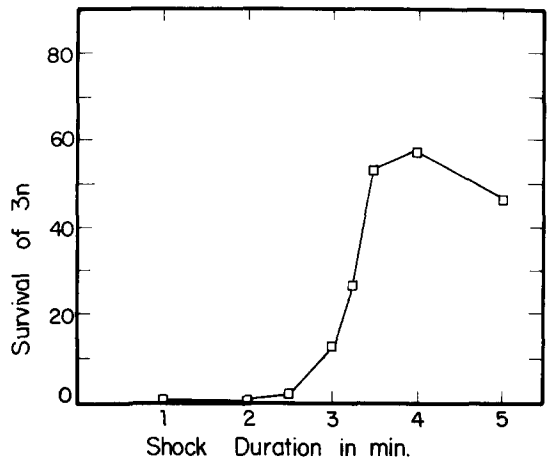

Fig. 4. Influence of heat-shock duration, given at optimal temperature (39.5 °C) and age (3 min), on triploid viability

shocked at zygote age of 3 min. The other embryos, shocked at ages different from 3 min, were 100% diploids (Fig. 6). The heat-shock duration, starting at the end of the third minute after fertilization, was optimized to 3.5–4 min for treatments carried out at 39.5 °C (Fig. 4).

The other temperatures (40.5 and 41.5 °C) tested were found to be highly lethal to all zygotic ages. None of the 6-min zygotes could survive in all developmental stages, except at the stage of morula. However, this stage was also affected at the extreme temperature of 41.5 °. Results showed that, even at these temperatures all the surviving 3-min zygotes were triploids (Fig. 2c and Fig. 3). This experiment points out that at extreme temperatures the development of morula is also sensitive at the 6-min zygote age (Fig. 2d).

Table 1. Measurements, ±SD, of cell and nucleus length (CL, NL) and width (CW, NW), and their combined parameters (given in μ, μ^2 or μ^3). The statistical significance of the differences between 2n and 3n erythrocytes was determined by the student's *t*-test. The cell and nucleus volume (Vc, Vn) calculations, based on the assumption that both the cell and the nucleus are perfect ellipsoids, were made as follows; $V = 3\,\Pi\,ab^2/4$; where a = CL/2 or NL/2; b = CW/2 or NW/2

Trait	2n	3n	Student's *t*-test *P*
CL	11.78± 1.7	15.97± 2.31	<0.004
CW	8.33± 0.84	9.16± 0.8	<0.776
NL	5.38± 0.46	7.67± 0.55	<0.395
NW	2.97± 0.33	3.12± 0.50	<0.065
CL×CW	98.27±14.21	146.58± 28.91	<0.001
CL+CW	20.12± 1.48	25.13± 2.54	<0.01
NL×NW	16.03± 2.30	23.95± 3.91	<0.015
NL+NW	8.36± 0.57	10.80± 0.69	<0.375
CL/CW	1.42± 0.19	1.7 ± 0.27	<0.087
NL/NW	1.83± 0.27	2.52± 0.45	<0.017
CL/NL	2.19± 0.18	2.09± 0.33	<0.009
CW/NW	2.82± 0.34	2.99± 0.49	<0.107
Vc	241.00±53.90	399.83±111.60	<0.002
Vn	14.9 ± 3.44	22.49± 7.05	<0.001
Vc/Vn	17.81± 5.00	19.11± 6.37	<0.0257

Identification of triploid fish

Different techniques have been used in this paper to identify diploid and triploid fish. The growth rates as well as morphology of diploid and triploid fish were found to be quite similar during the period between hatching and the age of 6 months (not shown). On the other hand, cell size measurements, karyotyping and thymidine uptake of lymphocyte culture showed highly significant differences between diploid and triploid fish. Erythrocytes from triploid fish were reported to be different in size from those of diploids (Benfey and Sutterlin 1984). In this work we measured the width and the length of cell and nucleus, and calculated the nucleus volume, the cell volume and other combined parameters, as detailed in Table 1. The results showed that the nucleus volume, the cell volume and the product CL×CW are the most significant parameters for the characterization of triploids (Table 1 and Fig. 5). The karyotyping results showed consistantly 44 chromosomes (2n) in diploids and 66 chromosomes (3n) in triploids (Chourrout and Itskovich 1983) (Fig. 6). Using thymidine uptake (per 10^6 cells) of cultured triploid and diploid lymphocytes, we found that in the triploid culture, the thymidine incorporation rate was 1.5 times higher than that of the diploids (not shown).

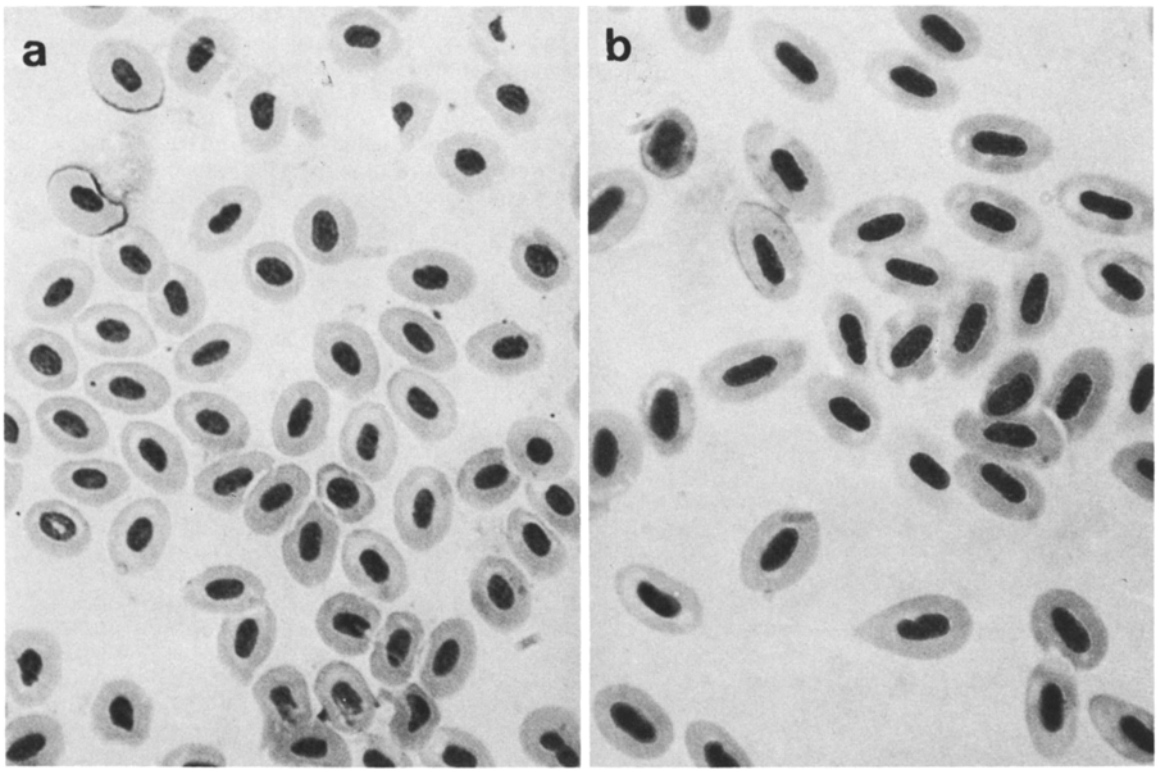

Fig. 5. Giemsa staining of erythrocytes from diploid (**a**) and triploid (**b**) adult tilapias (×400)

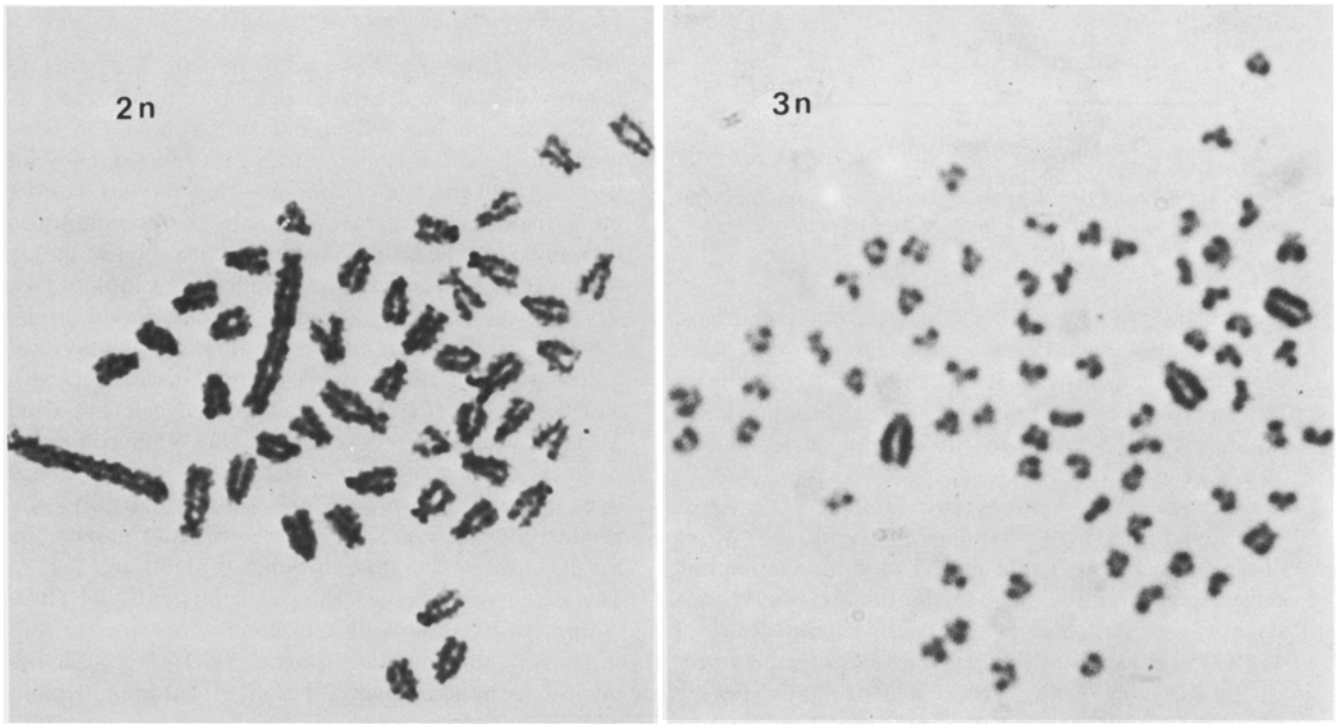

Fig. 6. Early-metaphase karyotype of diploid (2n=44, ×1,000) and metaphase karyotype of triploid (3n=66, ×630) tilapias (direct giemsa staining)

Discussion

The induction of triploidy in *O. aureus* was investigated. The technique used in this study gave 100% triploidy with a reasonable survival percentage of 61%. The level of the heat shock temperature and the age of the embryo were both highly critical for the induction of triploidy. The optimal heat-shock temperature was $39.5° \pm 0.2$ when applied exactly at the beginning of the fourth minute after fertilization for 3.5–4 min. The negative results obtained with the other temperatures (38.5, 40.5 and 41.5) at embryo ages higher or lower than 3 min, indicate that the process we are dealing with, namely the disturbance of the spindle mechanism and prevention of second polar body extrusion, (Edwards 1958a; Purdom 1972) is very short-lived and sensitive. It is noteworthy that these results are apparently in disagreement with the conclusions of Valenti (1975) that triploidy could be obtained following a temperature shock starting at the embryo-age of 15 min. It seems more likely that first cleavage, rather than second polar body extrusion, was involved in the technique used by Valenti. In fact, his calculations of polyploid/diploid ratios, (for nuclear and cell volumes) were nearly equal to 2 and different from the 1.5 ratio shown in this paper (Table 1 and Fig. 5a, b) and by others (Swarup 1959b; Beck and Biggers 1983, Benfey et al. 1984). As shown in Fig. 3, the exact level of the heat-shock temperature needed for the induction of polyploidy was optimised at 39.5 ± 0.2 °C. This result indicates that the temperature susceptibility of the cellular event related to the polar-body extrusion is located within a narrow range of 1 °C. At one degree below this optimal temperature, the survival rate was high, but no triploids could be detected. On the other hand, at 1 °C above the optimal temperature, the survival rate was very low, but still only triploids could be shown.

Fluctuations in viability, following the optimal treatment, at different zygote ages, were observed by different investigators in other species (Swarup 1959a; Nagy et al. 1978; Gervai et al. 1980). In our experiments (Fig. 2b), the dramatic decline in viability rates, mainly of 6-min-old zygotes, points out the occurrence of a heat-sensitive event connected to this developmental stage. Even though the cause of the viability decline is not known, it is clearly not involved with cell division, since following heat shock, the 6-min zygote successfully achieved 7–8 divisions (128–256 cells morula). It seems that this heat sensitive event is associated with some early occurring product(s) which might be similar to the heat-shock proteins mentioned in the literature (Asburner and Bonner 1979; Nevins 1982; Bensaude et al. 1983).

In this study, we followed growth and morphology changes in diploid and triploid populations from fertilization up to the age of 6 months and no significant difference could be observed. Only external sex characters were found underdeveloped in triploids in comparison to the well-developed male and female papillae of the control diploid fish. These findings are consistent with those reported in the literature (Purdom 1972; Thorgaard 1979; Gervai et al. 1980; Wolters 1982; Utter et al. 1983 and Stanley et al. 1984). For instance, the body size of the fish did not increase due to triploidy, even though cell volume became larger in a linear relation with chromosome number (Fig. 5a, b).

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