学位論文

田澤一朗

目 次

主論文

Investigations of abnormal development using anurans

（無尾両生類を用いた発生異常の研究）

田澤一朗

公表論文

(1) Effects of hypergravity on oocyte maturation in *Xenopus laevis*

Ichiro Tazawa, Akihiko Kashiwagi, Keiko Kashiwagi, and Nobuaki Furuno

Biol. Sci. Space, 19 (4) (2005) 242-244.

(2) Skin pigmentary variants in *Rana nigromaculata*

Ichiro Tazawa, Hitoshi Okumoto, and Akihiko Kashiwagi

J. Clin. Biochem. Nutr., 38 (3) (2006) 195-203.

参考論文

(1) Expression of helix-loop-helix type negative regulators of differentiation during limb regeneration in urodeles and anurans

Keiko Shimizu-Nishikawa, Ichiro Tazawa, Kohji Uchiyama, and Katsutoshi Yoshizato

Dev. Growth Differ., 41 (1999) 731–743.

(2) Effects of the hypergravity on oocyte maturation of *Xenopus laevis*

Nobuaki Furuno, Ichiro Tazawa, Keiko Kashiwagi, Tomomi Kawakami, Satoshi Yoshitome, Minoru Watanabe, Masamichi Yamashita, and Akihiko Kashiwagi

Space Utiliz. Res., 22 (2006) 228-230.

主 論 文

Investigations of abnormal development using anurans

(無尾両生類を用いた発生異常の研究)

Ichiro Tazawa

Part 1

Effects of hypergravity on oocyte maturation in *Xenopus laevis*

Abstract - Developing oocytes arrest at the prophase of meiosis I, while the hormone progesterone restarts the process, leading to full maturation as fertile eggs. In this study we investigated the effects of hypergravity on this phase of maturation using oocytes of *Xenopus*. No differences were found between $2G$ and $5G$ treated groups and untreated controls regarding time to resumption of maturation, as judged by the appearance of a white spot in the animal hemisphere. However the white spot itself was larger and brighter in treated oocytes at time of appearance, and became even more pronounced as maturation proceeded. These findings suggest that although hypergravity does not affect the resumption of maturation, it might adversely affect the subsequent process and the final results.

Introduction

The study of the effects of space environments on organisms is important for future space travel and colonization on other worlds. The African clawed toad, *Xenopus laevis*, is a suitable model organism for space environment studies because a large number of eggs are obtainable at the same time, fertilization occurs *in vitro,* and embryonic development is easily observed. We have recently investigated the effects of hypergravity on development of *Xenopus* embryos and found that hypergravity induced developmental retardation and various morphological abnormalities including microcephaly and microphthalmia. Such abnormalities seemed to be caused by the suppression of certain gene functions and the induction of abnormal apoptosis in the brain and eyes (Kashiwagi *et al.*, 2003).

Full-grown oocytes of *Xenopus* arrest at the G2/M border of meiosis I. Resumption of meiosis is triggered by the hormone progesterone (PG), and oocytes arrest once again at metaphase of meiosis II. This process is called oocyte maturation. Oocyte maturation is an essential step in the production of fertile eggs (Masui and Clarke, 1979). The effects of hype- and microgravities on oocyte maturation however have received little attention. Only one paper reported that mouse oocyte maturation is not influenced by clinostat rotation (Wolgemuth and Grills, 1984). In this study, we investigate the effects of hypergravity on oocyte maturation in *Xenopus*.

Materials and Methods

Preparation of oocytes

Adult *Xenopus laevis* frogs were purchased from Kato-S-Kagaku and maintained at the Hiroshima University Institute for Amphibian Biology. Ovaries were removed from female *Xenopus laevis*, washed in 1× MBS (Gurdon, 1968; Sive, *et al.*, 2000) and dissected with scissors. Dissected ovaries were incubated with collagenase (2 mg/ml) in 1× MBS for 2 hours on a rotary shaker. After collagenase treatment, oocytes were washed several times with 1× MBS. Oocyte staging was done according to Dumont (1972). Stage VI oocytes were collected. Oocyte maturation was induced by adding progesterone to the medium (1× MBS) to a final concentration of 5 μg/ml.

Hypergravity treatment

After the addition of progesterone, oocytes were placed in 24 well tissue culture plates (Iwaki Co. Ltd) containing 1× MBS. Plates were then placed in 12.5 cm × 12.5 cm × 20.0 cm polystyrene containers and subjected to either 2*G* or 5*G* hypergravity treatment in a swing-bucket type centrifuge at 21℃, with controls receiving no treatment.

Morphological examination

Oocytes on which a white spot appeared were harvested and fixed in Smith's solution. After fixation, oocytes were washed several times with water and stored in 70% alcohol. Images were obtained using MZFLIII (Leica) and digital camera (Olympus DP70).

White spot areas were measured using NIH image J program (http://rsb.info.nih.gov/nih-image/Default.html).

Statistics

Multiple-group comparisons were assessed using analysis of variance (ANOVA) followed by Fisher LSD test. All the results are expressed as mean ± S. E. M. P values below 0.05 are considered significant.

Results and discussion

Effects of hypergravity on resumption of oocyte maturation and white spot configuration

To examine whether hypergravity affects the resumption of oocyte maturation, treated and control oocytes were checked at regular intervals for appearance of the white spot and times and numbers were recorded. The results are shown in Fig. 1. By hour 4 the white spot had appeared in approximately half of all oocytes, and by hour 6 this was virtually 100%. No significant difference was seen between 2*G* and 5*G* treated oocytes and controls, suggesting that hypergravity has no effects on the resumption of oocyte maturation.

As to the visible features of the white spot, Figure 2 (0h) shows the spot to be brighter in treated oocytes compared to controls, 5*G* being the brightest of all.

Figure 3 (0h) shows a dose-related increase in size, 2*G* having an area 1.4 times and 5*G* 2.5 times that of controls. Furthermore, in some treated oocytes the white spot appeared slightly off the animal pole (data not shown).

Effects of hypergravity on progression of oocyte maturation

Normally oocytes complete maturation and reach metaphase of meiosis II approximately 4 hours after appearance of the white spot (Furuno *et al.*, 1994). In order to see if hypergravity might have any visible effects on the normal progression of maturation we harvested oocytes 1, 2, 3 and 4 hours after the appearance of the white spot (0h) and

documented any changes in brightness and size. While control oocytes remained essentially unchanged throughout this period, Figure 2 shows how the white spots of treated oocytes became progressively brighter, and Figure 3 shows that they progressively increased in size: the area of 2*G* white spots being 1.9 times and 5*G* white spots being 3.5 times that of controls at 4 hours.

Although hypergravity did not affect the resumption of oocyte maturation in *Xenopus*, the above findings indicate that hypergravity might have an effect on subsequent progression and final outcome. Indeed preliminary cytological examinations suggest that although treated oocytes complete meiosis I, they fail to properly form spindle after exclusion of the polar body. Over 50% of the oocytes thus examined showed a sort of monopolar spindle.

References

Dumont, J. N. (1972) Oogenesis in *Xenopus laevis*. I. Stages of oocyte development in laboratory maintained animals. *J. Morph.*, **136**, 153-180.

Furuno, N., Nishizawa, M., Okazaki, K., Tanaka, H., Iwashita, J., Nakajo, N., Ogawa, Y. and Sagata, N. (1994) Suppression of DNA replication via Mos function during meiotic divisions in *Xenopus* oocytes. *EMBO J.*, **13**: 2399-2410.

Gurdon, J. B. (1968) Changes on somatic cell nuclei inserted into growing and maturing amphibian oocytes. *J. Embryol. Exp. Morphol.*, **20**, 401-414.

Kashiwagi, A., Hanada, H., Kawakami, S., Kubo, H., Shinkai, T., Fujii, H., Kashiwagi, K. (2003) Effects of high gravity on amphibian development. *Biol. Sci. Space,* **17**, 215-216.

Masui, Y. and Clarke, H. J. (1979) Oocyte maturation. *International Review of Cytology,* **57**, 186-282. ed by Bourne, G. H. and Danielli, J. F. Academic Press Inc.

Sive, H. L., Grainger, R. M. and Harland, R. M. (2000) Early development of *Xenopus laevis* – A laboratory Manual. Cold Sprig Harbor Laboratory Press. 283-284.

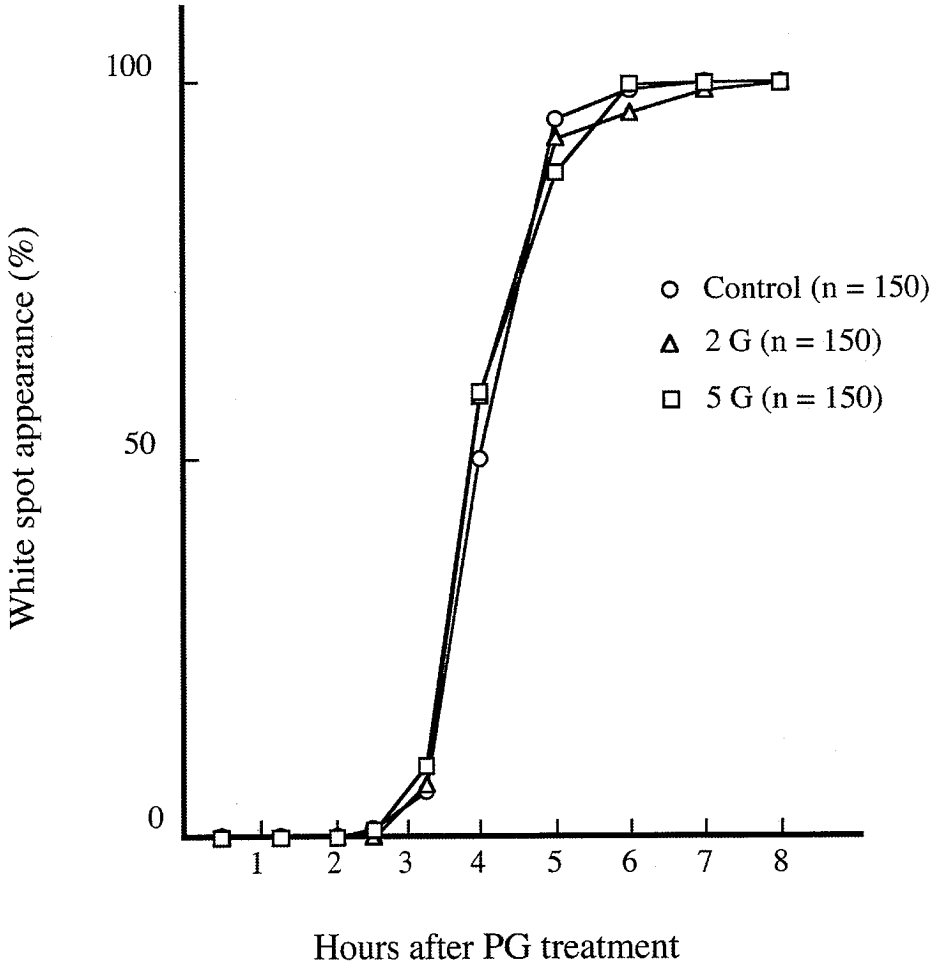
Wolgemuth, D. J. and Grills, G. S. (1984) Effects of clinostat rotation on mouse meiotic maturation in vitro. *Physiologist,* **27** (6 suppl), S99-100.

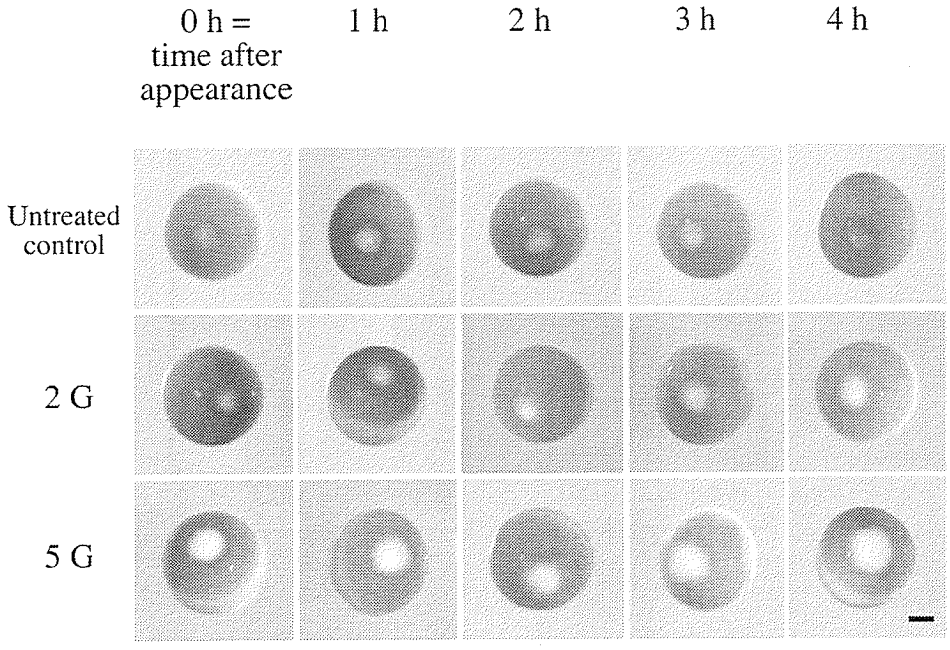
Figure legends

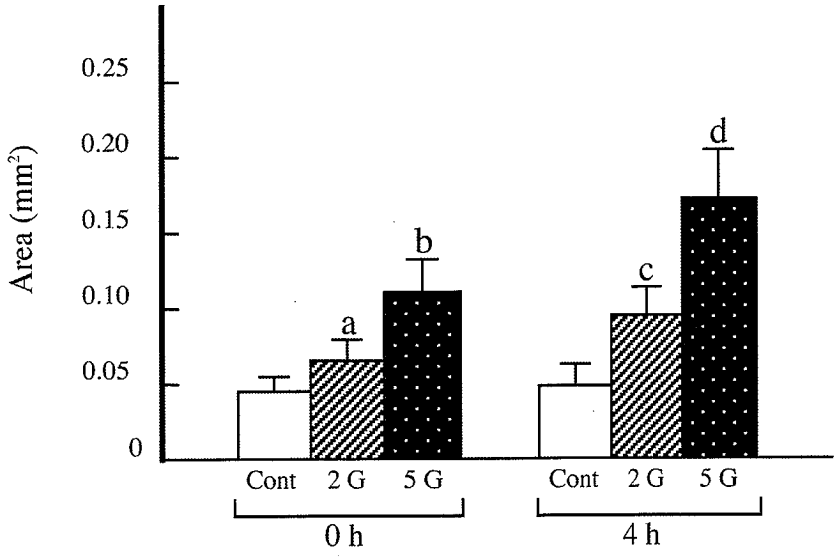
Figure 1. Time to appearance of white spot. 2*G*, 5*G* and control groups contained 150 oocytes each. ○ = control, △ = 2G, □ = 5G.

Figure 2. Relative brightness of white spot. Ten sample oocytes were collected from each group at 0, 1, 2, 3 and 4 hours after white spot appearance and were fixed and photographed. Note that the white spot in 2*G* and 5*G* becomes gradually larger and brighter as opposed to controls. 0h = appearance of white spot. 1h = one hour later. 2h = two hours later. 3h = three hours later. 4h = four hours later. Bar, 200 μm.

Figure 3. Relative area of white spot. Ten sample oocytes were collected from each group at 4 hours after white spot appearance. These were photographed and white spot size was measured. [a]Significantly less ($P < 0.01$) than 5*G* value. [b]Significantly greater ($P < 0.01$) than control value. [c]Significantly greater ($P < 0.01$) than control value. [d]Significantly greater ($P < 0.01$) than 2*G* value.


100
50
0
White spot appearance (%)
1
2
3
4
5
6
7
8
Control (n = 150)
2 G (n = 150)
5 G (n = 150)
Hours after PG treatment


0 h =
time after
appearance
1 h
2 h
3 h
4 h
Untreated
control
2 G
5 G


Area (mm²)
0.25
0.20
0.15
0.10
0.05
0
a
b
c
d
Cont
2 G
5 G
Cont
2 G
5 G
0 h
4 h

Part 2

Skin pigmentary variants in *Rana nigromaculata*

Abstract – Because there is mounting evidence to suggest that oxidative stress is involved in the pathophysiology of albinism, albino amphibians are useful tools for studies on imbalances in the oxidant-antioxidant system. In the course of maintaining albino mutant frog strains it was found that crosses between albino males and heterozygous females of *Rana nigromaculata* sometimes produce offspring displaying pigmentary mosaicism. After hatching hypopigmented portions appear on the left or right side of the body, and this is accompanied by such abnormalities as poor viability, asymmetrical curvature of the body toward the hypopigmented side, and limb deformity. Histological examination of mosaics showed the cells of various tissues (except kidney) to be smaller on the hypopigmented side and larger on the pigmented side compared to corresponding cells in wild type offspring. Cytogenetic analysis of cultured skin cells revealed that wild type and albino individuals were diploidal with 26 chromosomes, the same as normal *R. nigromaculata*. In mosaics on the other hand, cells of hypopigmented portions were almost exclusively haploidal with 13 chromosomes, while pigmented portions were a mixture of roughly 75% triploidal, 39 chromosome cells and roughly 25% haploidal, 13 chromosome cells.

Introduction

Albinism is a congenital disorder that causes little or no melanin pigment production in the skin, hair and eyes. Accumulating evidence suggests that oxidative stress and damage are involved in the pathophysiology of albinism [*1,2*]. Measurement of lipid peroxide accumulation provides one means of assessing the involvement of ROS (reactive oxygen species) in biological processes such as aging. Lipid peroxidation of the depigmented eye pigment epithelium in albino rabbits occurs at a higher rate than in normally pigmented rabbits [*3,4*]. Thiobarbituric acid reactive substance (TBARS) concentration is used as an indicator of the susceptibility of tissues to lipid peroxidation. Corsaro et al. [*5*] reported that TBARS levels are greater in albino *Xenopus* liver than in pigmented liver, and that the addition of isolated purified melanin to albino liver leads to a significant decrease in TBARS level, suggesting that melanin pigment acts as an antioxidant for ROS detoxification. Albino frogs are a suitable model organism for studies on the involvement of oxidative stress in albinism.

There have been many reports of worldwide declines and extinctions in amphibian populations [*6-9*], which possibly stem from a variety of reasons, including ultraviolet (UV) radiation exposure. Little et al. [*10*] exposed toad tadpoles to ultraviolet-B (UVB) radiation and found that *Bufo boreas* tadpoles are more tolerant than *Bufo woodhousii* tadpoles. They surmise that the higher resistance of *B. boreas* tadpoles to UVB is due to the presence of greater amounts of melanin in the skin. Kvam and Dahle [*11*] demonstrated that

the depigmented cells of albino mice are more susceptible to ultraviolet-A (UVA) radiation-induced lipid peroxidation and glutathione depletion than the pigmented cells of normal mice, resulting in membrane damage in melanin forming melanocytes. In this context, albino amphibians are excellent tool for in vivo studies on the effects of UV exposure.

Several investigations have shown that breakdown in the oxidant-antioxidant balance plays a role in the development of an acquired pigment loss disorder, or vitiligo [*12-17*].

Different types of somatic chromosomal mosaicism are known to cause various pigmentary disorders [*18-20*]. Donnai et al. [*18*] reported two patients with cutaneous manifestations of hypomelanosis of Ito [*21*], resulting form a mixture of diploid/triploid cells. Trisomy 7 mosaicism is seen in patients with Blaschkolinear dysplasia syndrome (= hypomelanosis of Ito), appearing as swirls of pigmentation/depigmentation on the trunk and linear streaks on the limbs in a Blaschko linear type distribution [*22,23*]. This syndrome is accompanied by asymmetric body abnormalities, developmental delay, growth retardation and a variety of other anomalies.

The Amphibian Inbred Strain Maintenance Team at Hiroshima University Institute for Amphibian Biology maintains approximately 20 thousand frogs of various inbred amphibian strains, including 60 different types of color mutants and 60 wild species. In the course of maintaining albino strains of the black-spotted pond frog *Rana nigromaculata*,

tadpoles with Blaschkolinear dysplasia like syndrome were found among the offspring resulting from crosses between albino males and heterozygous females. In this paper we report the morphological and cytogenetic features of skin pigmentary variants in *R. nigromaculata*, which should be of use in future studies on albinism.

Materials and Methods

Animals

The frogs used for mating belong to 15 inbred lines of *Rana nigromaculata.* Twelve of the lines, Ex, Fc, Go, Hr, Km, Ns, Sn I, Sn II, Tj, Ty, Ym I, and Ym II have already been reported [*24,25*]. They are categorized into 5 complement genetic groups: group 1 (Ex, Fe, Hr, Sn I, Sn II, Tj, and Ym II), 2 (Go), 3 (Ym I), 4 (Km), and 5 (Ns). The other three lines, Bf, Sj, and Ts are newly reported here. All three were originally collected in Hiroshima Prefecture, Japan: Bf from Fuchu City prior to 1991, Sj from Saijo Town, Hibagun prior to 1988, and Ts from Toyosaka Town, Kamogun in 1991. Albinism in all 15 lines is recessively inherited. Frogs collected in the vicinity of Hiroshima having homozygous wild genes were also mated. Artificial insemination was done at room temperature from April to June in our laboratory in Higashihiroshima City, Hiroshima Prefecture, Japan. Ovulation was induced by injecting 3–12 year old females with 2 to 3 acetone-dehydrated pituitaries from *Rana catesbeiana* or *R. nigromaculata* suspended in 500 μl of 70% human physiological saline. Males of the same line were anesthetized with vaporized diethyl ether to remove a single testis. Eggs were released onto glass slides and inseminated artificially with a sperm suspension of homogenized testis in dechlorinated tap water. Twenty minutes after insemination eggs were transferred to dechlorinated tap water at room temperature and allowed to develop. Tadpoles were fed mashed boiled spinach and frogs were fed living crickets (*Gryllus bimaculatus*). Developmental stage was determined according to Shumway [*26*] and Taylor and Kollros [*27*].

Histological examination

Tadpoles and frogs were fixed in Bouin's fixative solution for 6–12 hours, dehydrated through a graded series of ethanol and Hemo-De (Falma, Tokyo, Japan), embedded in Paraplast plus (Sigma-Aldrich, St Louis, MO, USA), and sectioned at 7–10 μm. De-paraffinized and rehydrated sections were stained with Mayer's Hematoxylin solution (Sigma-Aldrich) and Eosin Y solution (Sigma-Aldrich).

Cytogenetic examination

Tadpole tail tips were prepared for analysis using a water-pretreatment squash method as described in Nishioka [*28*]. Taylor and Kollros stage I–X tadpole tail tips were amputated. After three to five days small regenerate buds had formed on tail stumps, and these and neighboring stump tissues were removed, transferred to a culture medium (60% Leibovitz L-15, 10% fetal calf serum heat-inactivated at 56°C for 30 minutes, 100 units/ml penicillin, 100 μg/ml streptomycin) containing 0.005% colchicine, incubated at 20°C for 12–18 hours, immersed in deionized water for 90 minutes to swell the cells, transferred to a slide glass, stained with orcein solution (1% orcein, 45% acetic acid) at room temperature for 60 minutes, heated with hot wet gauze for 20–30 seconds, and squashed under a cover glass. The edges of the cover glass were sealed with Malinol (Muto Pure Chemicals, Tokyo, Japan). Chromosome morphology was determined according to Levan et al. [*29*] and Green and Sessions [*30*]. Skin patches from specific portions of the body were prepared for

analysis using the following method. Pigmented and hypopigmented skins were peeled separately from the trunk with tweezers and scissors, washed in the culture medium described above, chopped into approximately 1 mm square patches and transferred to IWAKI 35 mm, non-treated hydrophobic petri dishes (1000-035, Asahi Techno Glass, Funabashi, Japan) containing 500 μl of fresh culture medium. Skin patches were incubated at 20°C for one day, followed by a change of medium and further incubation for 2–3 days more. Some of the patches showed wound healing-like epidermal expansion during this culture. Mediums were replaced with fresh medium containing 0.005% colchicine and treatment continued at 20°C for 12–18 hours. Growing skins were peeled off with a tracing spatula, stained and chromosome analysis was performed as with tail tips. Tissue ploidy was confirmed by nucleolar count [*31*].

Results

Pigmentary mosaicism and its frequency

In most cases wild type females produce wild type eggs and albino females produce albino eggs, but on rare occasions albino females will produce a few wild type eggs, as previously described by Nishioka and Ueda [*25*]. Such eggs are not dealt with in the present study. Individual phenotypes, albino or wild, began to be expressed at the beginning of feeding or later (Taylor and Kollros stage I). At this time a number of tadpoles began to display unilateral pigmentary mosaicism. The boundary between pigmented and hypopigmented skin was relatively clear on the back at first (Figure 1A), but became diffused as the tadpole developed. The tail on the other hand, showed a mottled pigmentation pattern from the very beginning. Pigmentary mosaicism carried over metamorphosis, however some froglets displayed a small patch of hypopigmented skin around one eye (Figure 1B). Frequency of the pigmentary mosaic in offspring depended on the genotype of the parents (Table 1). It is interesting to note that homozygous albino females produced no pigmentary mosaics whether they were mated with heterozygous or wild type homozygous males.

Other abnormalities

The bodies of the pigmentary mosaic tadpoles and froglets manifested body asymmetry. They often bended to the hypopigmented side (Figures 1A and B). The pigmentary mosaic tadpoles and froglets moved always curving to the same direction as the

bending. The froglet limbs on the pigmented sides were as large as or larger than those on the hypopigmented sides. Moreover, malformed limbs were found in rare cases, when the forms of the limbs in left and right were not mirror images (Figure 2). Viability of the pigmentary mosaics was low. Seventeen of the 21 pigmentary mosaic tadpoles could not survive to metamorphose, while 30 wild type and 30 albino tadpoles all survived till metamorphosis.

Histological findings

Results of the histological examination are documented in Figure 3. Cells of the hypopigmented portion of mosaics (B, D, G, I, and L) were found to be smaller than corresponding wild type cells (A, C, F, and K), while cells of the pigmented portion (B, E, H, J, and M) were larger in back muscle (A and B), tail muscle (not shown), cartilage (C–E), retina (F–H), spinal cord (K–M), and epidermis (not shown). Compared to the wild type retina (F), retinas in the hypopigmented portion generally lacked pigmentation (G), with some showing spots of pigmentation (I). Retinas in the pigmentary portion were generally pigmented (H), with some showing unpigmented spots (J). Although the kidney itself was abnormally smaller on the hypopigmented side (Q), there was no apparent difference in cell size between the hypopigmented portion (O) and the pigmented portion (P). Cells in both the portions were larger than those in the normal wild type kidney (N).

Cytogenetic findings

Cell and nucleus size in vertebrates generally depends on ploidy [*32,33*], and therefore the above histological findings suggest that the pigmentary mosaicism occurring in our *R. nigromaculata* strains is in fact a feature of chromosomal mosaicism.

Examination of the tail tips of 24 wild type and 10 albino offspring of the pigmentary mosaics' parents revealed that the vast majority of cells in both groups had 26 chromosomes (Figure 4A), which is the normal number for *R. nigromaculata* [*34*]. The karyotype for wild type cells was diploid (Figure 5A).

Analysis of 22 mosaic tadpoles revealed 2 individuals with 13 and 26 chromosome cells (Figure 5B). Karyotype analysis was not performed on these individuals, nor were 13/26 mosaics included in the histological examination. The remaining 20 individuals had predominantly 13 and 39 chromosome cells at a ratio of approximately half and half. (Figure 4C). Thirteen chromosome cells were haploid (Figure 5B), and were the predominant cell of the hypopigmented portion, and comprised roughly 25% of the pigmented portion (Figures 6A and B). Thirty-nine chromosome cells were triploid (Figure 5C), and were found almost exclusively in the pigmented portion, comprising roughly 75% (Figure 6B). Two individuals with wild type pigmentation but with bodies curved in the manner of pigmentary mosaics were also examined and found to have 13 and 39 chromosome cells (Figure 4D), with karyotypes identical to 13/39 mosaics.

Discussion

The findings of the present investigation indicate that as in humans, frog skin pigmentary disorder is induced by somatic chromosomal mosaicism.

Jenkins et al. [*35*] reported an 8-year-old boy with hypomelanosis of Ito, who had trisomy 7 mosaicism and multiple congenital abnormalities, including a prominent forehead with facial asymmetry, bifid uvula, poor dentition, developmental delay and difficult behavior. An older 18 year- old-young man with Blaschkolinear skin hypopigmentation was reported by Magenis et al. [*22*]. This patient had trisomy 7 mosaicism and showed difficult behavior, growth and developmental delay, partial seizure disorder, multiple ear infections, body asymmetry and full-scale intelligence quotient (IQ) of 45-48, and other defects. A 6-year-old boy reported by Kayser et al. [*23*] also demonstrated Blaschkolinear skin pigmentary variation and trisomy 7 mosaicism. He had developmental delay, mixed receptive-expressive language disorder, phonological disorder, and developmental coordination disorder. At age 5 his the Wechsler Preschool and Primary Scales of Intelligence-Revised (WPPSI-R) performance IQ was 106, his verbal IQ was 69, and he had a full scale IQ of 86.

In the present investigation of *R. nigromaculata* tadpole and froglet mosaics, hypopigmented areas first appeared just after the beginning of feeding. Mosaic tadpoles and froglets displayed poor viability, developmental delay, asymmetrical curvature of the body

toward the hypopigmented side, and limb deformity. Cytogenetic analysis of mosaic individuals revealed that several organs, including cartilage, retina and spinal cord, consisted of a mixture of haploid/triploid cells, although haploid cells were present in large numbers in the hypopigmented side of the body, whereas a great number of triploid cells were found in the pigmented side.

Triploidy can be easily induced in amphibians by artificially means. Many triploid tadpoles are viable and attain sexual maturity [*36-40*].

Several investigations in experimental haploidy have been conducted using amphibians because their eggs can be easily be induced to develop with a single set of chromosomes. This means that manifestation of recessive genetic mutations is usually not masked by normal alleles in haploids as they are in diploids, leading to very poor viability in haploid individuals. Haploid embryos and tadpoles succumb to a variety of abnormalities referred to as "haploid syndrome". Of numerous haploids so far produced, only three *Rana rugosa* have successfully been raised to sexual maturity [*41*]. Previous studies have shown that the system for hydrogen peroxide detoxification develops abnormally in haploids, resulting in considerable cell damage, leading to death [*42-44*]. These findings seem to throw light on the reasons for the abnormal development and poor viability seen in the hypopigmented haploid/triploid mosaic tadpoles and frogs in the present study.

There are two possible explanations for this difference in tissue size. One is that haploid cells themselves are smaller than triploid cells. The other explanation involves a difference in proliferative ability. In spite of differences in cell size, the body size of haploid and other polyploid amphibians is not appreciably different from that of normal diploid individuals [*32*]. To account for this a compensating mechanism involving cell numbers is postulated. In our haploid/triploid mosaics then, this mechanism seems to have broken down. It is hoped that further investigation of mosaicism in amphibians will help shed light on the causes of somatic chromosomal mosaicism and accompanying skin pigmentation disorders in humans. To our knowledge however, there is no report of differences in body size due to gene number.

Cell deletion in tail regression of anuran tadpoles depends on programmed cell death, or apoptosis [*45*]. From the results of previous studies of spontaneous and thyroid hormone (TH)-induced tail regression [*46-50*], we proposed the following mechanism for tail apoptosis: an increase in TH → NO generation → cytochrome oxidation inhibition by NO → O_2^- generation → H_2O_2 accumulation after catalase activity inhibition by NO → lipid peroxidation of lysosomal membranes → caspase-3 activation by lysosomal enzyme release → DNA fragmentation → apoptosis. In the Introduction, we indicated that a oxidant-antioxidant imbalance is involved in pathogenesis of mammalian albinism. Because it seems highly likely that a similar phenomenon occurs in amphibian albinism, inbred albino

frog strains are thought to be excellent material for investigating the effect of oxidative stress on tadpole tail apoptosis.

Albinism or hypopigmentation is accompanied by a high level of erythrocyte SOD (superoxide dismutase) activity, a marked reduction of catalase activity and the accumulation of excess H_2O_2, and NO generation as seen in patients with vitiligo [*51-53*], and the occurrence of a lipoperoxidative process in albino rabbits [*3,4*]. Previously we reported that cells in haploid frogs are more susceptible to oxidative stress compared to cells in diploid and triploid frogs [*42-44*]. In the present study, an abnormal oxidant-antioxidant system in the haploidal portions of mosaic *R. nigromaculata* may operate to cause abnormal apoptosis, resulting in poor proliferation of cells and tissues, which leads to asymmetrical body curvature toward the hypopigmented side and limb deformity.

At the Hiroshima University Institute for Amphibian Biology all specimens are maintained under laboratory conditions with daily artificial illumination. Data on the effect of UV radiation-mediated oxidative stress on development, growth and survival rate in amphibians are not available however. At present, our laboratory is conducting investigations aimed at clarifying this problem using albino *R. nigromaculata.*

Acknowledgments

The authors are grateful to Dr. M. Nishioka, Professor Emeritus of Hiroshima University, for her constant guidance.

References

[1] Kumar, D., Bansal, A., Thomas, P., Sairam, M., Sharma, S.K., Mongia, S.S., Singh, R., and Selvamurthy, W.: Biochemical and immunological changes on oral glutamate feeding in male albino rats. *Int. J. Biometeorol.*, **42**, 201-204, 1999.

[2] Peters, S. and Schraermeyer, U.: Characteristics and functions of melanin in pigment epithelium. *Ophthalmologe*, **98**, 1181-1185, 2001.

[3] Dontsov, A.E., Sakina, N.L., and Ostrovskii, M.A.: Comparative study of lipid peroxidation in the eye pigment epithelium of pigmented and albino animals. *Biokhimiia*, **45**, 923-928, 1980.

[4] Ostrovsky, M.A., Sakina, N.L., and Dontsov, A.E.: An antioxidative role of ocular screening pigments. *Vision Res.*, **27**, 893-899, 1987.

[5] Corsaro, C., Scalia, M., Blanco, A.R., Aiello, I., and Sichel, G.: Melanins in physiological conditions protect against lipoperoxidation. A study on albino and pigmented *Xenopus*. *Pigment Cell Res.*, **8**, 279-282, 1995.

[6] Blaustein, A.R. and Wake, D.B.: The puzzle of declining amphibian populations. *Sci. Am.*, **272**, 56-61, 1995.

[7] Barinaga, M.: Where have all the froggies gone? *Science*, **247**, 1033-1034, 1990.

[8] Houlahan, J.E., Findlay, C.S., Schmidt, B.R., Meyer, A.H., and Kuzmin, S.L.: Quantitative evidence for global amphibian population declines. *Nature*, **404**, 752-755, 2000.

[9] Stokstad, E.: Global survey documents puzzling decline of amphibians. *Science*, **306**, 391, 2004.

[10] Little, E.E., Calfee, R.D., Fabacher, D.L., Carey, C., Blazer, V.S., and Middleton, E.M.: Effects of ultraviolet radiation on toad early life stages. *Environ. Sci. Pollut. Res. Int.,* **10**, 167-172, 2003.

[11] Kvam, E. and Dahle, J.: Pigmented melanocytes are protected against ultraviolet-A-induced membrane damage. *J. Invest. Dermatol.,* **121**, 564-569, 2003.

[12] Schallreuter, K.U., Moore, J., Wood, J.M., Beazley, W.D., Gaze, D.C., Tobin, D.J., Marshall, H.S., Panske, A., Panzig, E., and Hibberts, N.A.: In vivo and in vitro evidence for hydrogen peroxide (H_2O_2) accumulation in the epidermis of patients with vitiligo and its successful removal by a UVB-activated pseudocatalase. *J. Invest. Dermatol. Symp. Proc.*, **4** , 91-96, 1999.

[13] Schallreuter, K.U., Elwary, S.M.A., Gibbons, N.C.J., Rokos, H., and Wood, J.M.: Activation/deactivation of acetylcholinesterase by H_2O_2: more evidence for oxidative stress in vitiligo. *Biochem. Biophys. Res. Commun.*, **315**, 502-508, 2004.

[14] Aronoff, S.: Catalase: kinetics of photooxidation. *Science*, **150**, 72-73, 1965.

[15] Yildirim, M., Bavsal, V., Inalos, H.S., Kesici, D., and Delibas, N.: The role of oxidants and antioxidants in generalized vitiligo. *J. Dermatol.,* **30**, 104-108, 2003.

[16] Koca, R., Armutcu, F., Altinvazar, H.C., and Gurel, A.: Oxidant-antioxidant enzymes and lipid peroxidation in generalized vitiligo. *Clin. Exp. Dermatol.*, **29**, 406-409, 2004.

[17] Hazneci, E., Karabulut, A.B., Ozturk, C., Batcioglu, K., Dogan, G., Karaca, S., and Esrefoglu, M.: A comparative study of superoxide dismutase, catalase, and glutathione peroxidase activities and nitrate levels in vitiligo patients. *Int. J. Dermatol.*, **44**, 636-640, 2005.

[18] Donnai, D., Read, A.P., McKeown, C., and Andrews, T.: Hypomelanosis of Ito: a manifestation of mosaicism or chimerism. *J. Med. Genet.*, **25**, 809-818, 1988.

[19] Moss, C., Larkins, S., Stacey, M., Blight, A., Farndon, P.A., and Davison, E.V.: Epidermal mosaicism and Blaschko's lines. *J. Med. Genet.*, **30**, 752-755, 1993.

[20] Sybert, V.P.: Hypomelanosis of Ito: a description, not a diagnosis. *J. Invest. Dermatol.*, **103 (Suppl),** 141S-143S, 1994.

[21] Ito, M.: Studies on melanin. XI. Incontinentia pigmenti achromians. A singular case of nevus depigmentosus systematicus bilaterus. *Tohoku J. Exp. Med.*, **55 (Suppl)**, 57-59, 1952.

[22] Magenis, E., Webb, M.J., Spears, B., and Opitz, J.M.: Blaschkolinear malformation syndrome in complex trisomy-7 mosaicism. *Am. J. Med. Genet.*, **87**, 375-383, 1999.

[23] Kayser, M., Henderson, L.B., Kreutzman, J., Schreck, R., and Graham, J.M.Jr.: Blaschkolinear skin pigmentary variation due to trisomy 7 mosaicism. *Am. J. Med. Genet.*, **95**, 281-284, 2000.

[24] Nishioka, M.: Color variants induced by radiation and their inheritance in *Rana nigromaculata. Sci. Rep. Lab. Amphibian Biol., Hiroshima Univ.,* **2**, 25-89, 1977.

[25] Nishioka, M. and Ueda, H.: Genetics and morphology of 13 albino stocks in the *Rana nigromaculata* group. *Sci. Rep. Lab. Amphibian Biol., Hiroshima Univ.,* **7**, 1-121, 1985.

[26] Shumway, W.: Stages in the normal development of *Rana pipiens*. I. External form. *Anat. Rec.,* **78**, 139-147, 1940.

[27] Taylor, A.C. and Kollros, J.J.: Stages in the normal development of *Rana pipiens* larvae. *Anat. Rec.,* **94**, 7-23, 1946.

[28] Nishioka, M.: The karyotypes of the two sibling species of Japanese frogs, with special reference to those of the diploid and triploid hybrids. *Sci. Rep. Lab. Amphibian Biol., Hiroshima Univ.,* **1**, 319-337, 1972.

[29] Levan, A., Fredga, K., and Sandberg, A.A.: Nomenclature for centromeric position on chromosomes. *Hereditas*, **52**, 201-220, 1964.

[30] Green, D.M. and Sessions, S.K.: Nomenclature for chromosomes, *in Amphibian Cytogenetics and Evolution*, eds. By Green, D.M., and Sessions, S.K., Academic Press, San Diego, pp. 431-432, 1991.

[31] Fankhauser, G. and Humphrey, R.R.: The relations between the number of nucleoli and number of chromosome sets in animal cells. *Proc. Natl. Acad. Sci. USA*, **29**, 346-350, 1939.

[32] Fankhauser, G.: The effects of changes in chromosome number on amphibian development. *Q. Rev. Biol.,* **20**, 20-78, 1945.

[33] Henery, C.C. and Kaufman, M.H.: Relationship between cell size and nuclear volume in nucleated red blood cells of developmentally matched diploid and tetraploid mouse embryos. *J. Exp. Zool.*, **261**, 472-478, 1992.

[34] Nishioka, M.: Abnormalities of the offspring of nucleo-cytoplasmic hybrids between *Rana nigromaculata* and *Rana brevipoda. Sci. Rep. Lab. Amphibian Biol., Hiroshima Univ.,* **1**, 1-94, 1972.

[35] Jenkins, D., Martin, K., and Young, I.D.: Hypomelanosis of Ito associated with mosaicism for trisomy 7 and apparent 'pseudomosaicism' at amniocentesis. *Med. Genet.*, **30**, 783-784, 1993.

[36] Kawamura, T. and Nishioka, M.: Aspects of the reproductive biology of Japanese anurans, *in The Reproductive Biology of Amphibians*, eds. By Taylor, D.H., and Guttman, S.I., Plenum Press, New York, pp. 103-139, 1977.

[37] Kawamura, T.: Polyploidy in amphibians. *Zool. Sci.*, **1**, 1-15, 1984.

[38] Okumoto, H.: Studies on meioses in male hybrids and triploids in the *Rana nigromaculata* group. I. Interspecific hybrids between *Rana nigromaculata* and *Rana brevipoda. Sci. Rep. Lab. Amphibian Biol., Hiroshima Univ.*, **4**, 201-216, 1980.

[39] Kawamura, T., Nishioka, M., and Okumoto, H.: Production of autotetraploids and amphidiploids from auto- and allotriploids in *Rana nigromaculata* and *Rana brevipoda. Sci. Rep. Lab. Amphibian Biol., Hiroshima Univ.*, **6**, 47-80, 1983.

[40] Okumoto, H.: Studies on meioses in male hybrids and triploids in the *Rana nigromaculata* group. II. Auto- and allotriploids in *Rana nigromaculata* and *Rana brevipoda*. *Sci. Rep. Lab. Amphibian Biol., Hiroshima Univ.*, **6**, 183-205, 1983.

[41] Kashiwagi, K.: Mature haploids and their reproductive capacity in *Rana rugosa*. *Sci. Rep. Lab. Amphibian Biol., Hiroshima Univ.*, **4**, 217-237, 1980.

[42] Kashiwagi, A. and Kashiwagi, K.: Comparison of liver peroxisomal enzyme activity changes during development of diploid and haploid anurans. *Comp. Biochem. Physiol. B*, **113**, 533-536, 1996.

[43] Kashiwagi, A., Kashiwagi, K., Takase, M., Hanada, H., and Nakamura, M.: Comparison of catalase in diploid and haploid *Rana rugosa* using heat and chemical inactivation techniques. *Comp. Biochem. Physiol. B*, **118**, 499-503, 1997.

[44] Kashiwagi, A., Kashiwagi, K., Takase, M., Hanada, H., Yamashita, M., Naitoh, T., and Nakamura, M.: Inhibitor and temperature effect on catalase in the liver of adult diploid and haploid *Rana rugosa*. *Comp. Biochem. Physiol. B*, **119**, 235-239, 1998.

[45] Kerr, J.F.R., Wyllie, A.H., and Currie, A.R.: Apoptosis: A basic biological phenomenon with wide-ranging implications in tissue kinetics. *Br. J. Cancer*, **26**, 239-257, 1972.

[46] Kashiwagi, A.; Peroxisomal enzyme activity changes in the tail of anuran tadpoles during metamorphosis. *Comp. Biochem. Physiol. B*, **111**, 483-489, 1995.

[47] Hanada, H., Kashiwagi, A., Takehara, Y., Kanno, T., Yabuki, M., Sasaki, J., Inoue, M., and Utsumi, K.: Do reactive oxygen species underlie the mechanism of apoptosis in the tadpole tail? *Free Radic. Biol. Med.*, **23**, 294-301, 1997.

[48] Kashiwagi, A., Hanada, H., Yabuki, M., Kanno, T., Ishisaka, R., Sasaki, J., Inoue, M., and Utsumi, K.: Thyroxine enhancement and the role of reactive oxygen species in tadpole tail apoptosis. *Free Radic. Biol. Med.*, **26**, 1001-1009, 1999.

[49] Kashiwagi, A., Hanada, H., Yabuki, M., and Utsumi, K.: Reactive oxygen species and the mechanism for tail apoptosis in *Rana rugosa* and *Rana japonica*. *Recent Res. Dev. Comp. Biochem. Physiol.*, **1**, 51-66, 2000.

[50] Inoue, M., Sato, E.F., Nishikawa, M., Hiramoto, K., Kashiwagi, A., and Utsumi, K.: Free radical theory of mitochondria-dependent apoptosis and metamorphosis. *Redox Report*, **9**, 237-247, 2004.

[51] Casp, C.B., She, J.X., and McCormack, W.T.: Genetic association of the catalase gene (CAT) with vitiligo susceptibility. *Pigment Cell Res.*, **15**, 62-66, 2002.

[52] Yildirim, M, Baysal, V., Inaloz, H.S., Kesici, D., and Delibas, N.: The role of oxidants and antioxidants in generalized vitiligo. *J. Dermatol.*, **30**, 104-108, 2003.

[53] Hazneci, E., Karabulut A.B., Ozturk, C., Batcioglu, K., Dogan, G., Karaca, S., and Esrefoglu, M.: A comparative study of superoxide dismutase, catalase, and glutathione peroxidase activities and nitrate levels in vitiligo patients. *Int. J. Dermatol.*, **44**, 636-640, 2005.

Figure Legends

Figure 1. Photographs of wild type, albino, and pigmentary mosaic *Rana nigromaculata.* (A) Taylor and Kollros stage I tadpoles. (B) Froglets.

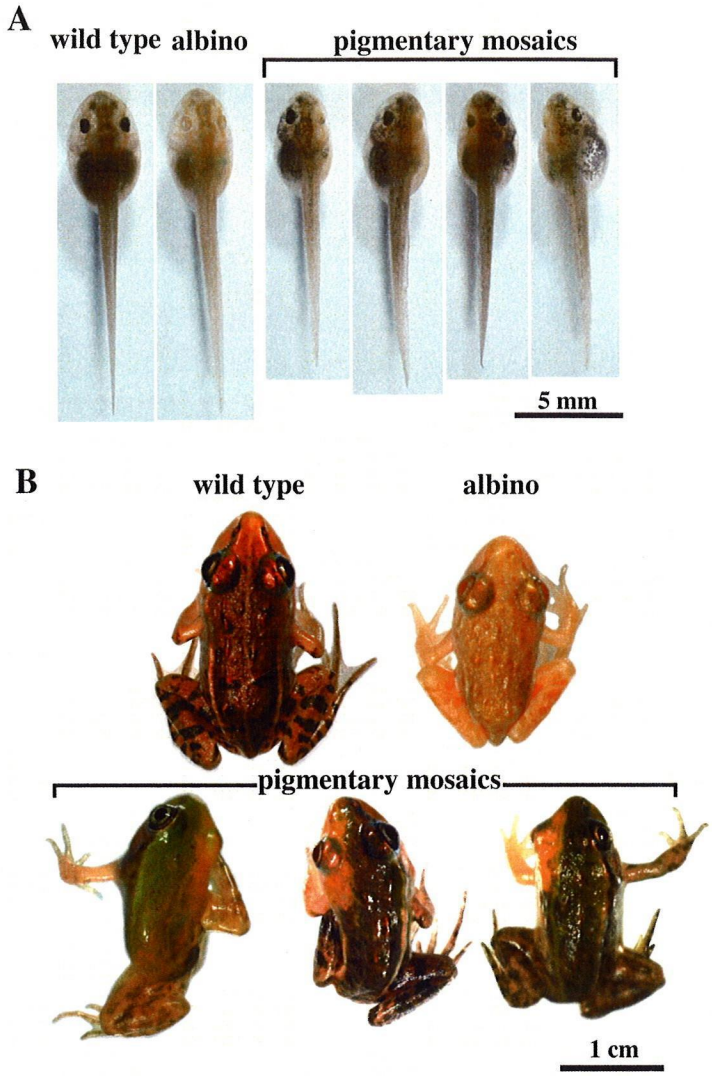
Figure 2. A malformed forelimb of a mosaic froglet. The left limb has the normal four digits, whereas the right has only two.

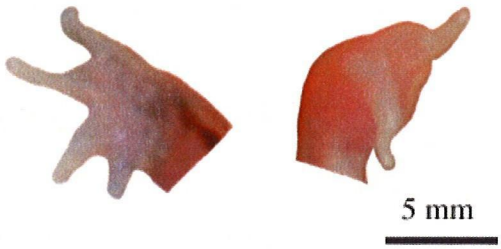
Figure 3. Histological documentation of wild type and mosaic *R. nigromaculata.* (A and B) Taylor and Kollros stage VII and V tadpole, respectively. (C-Q) Froglets.

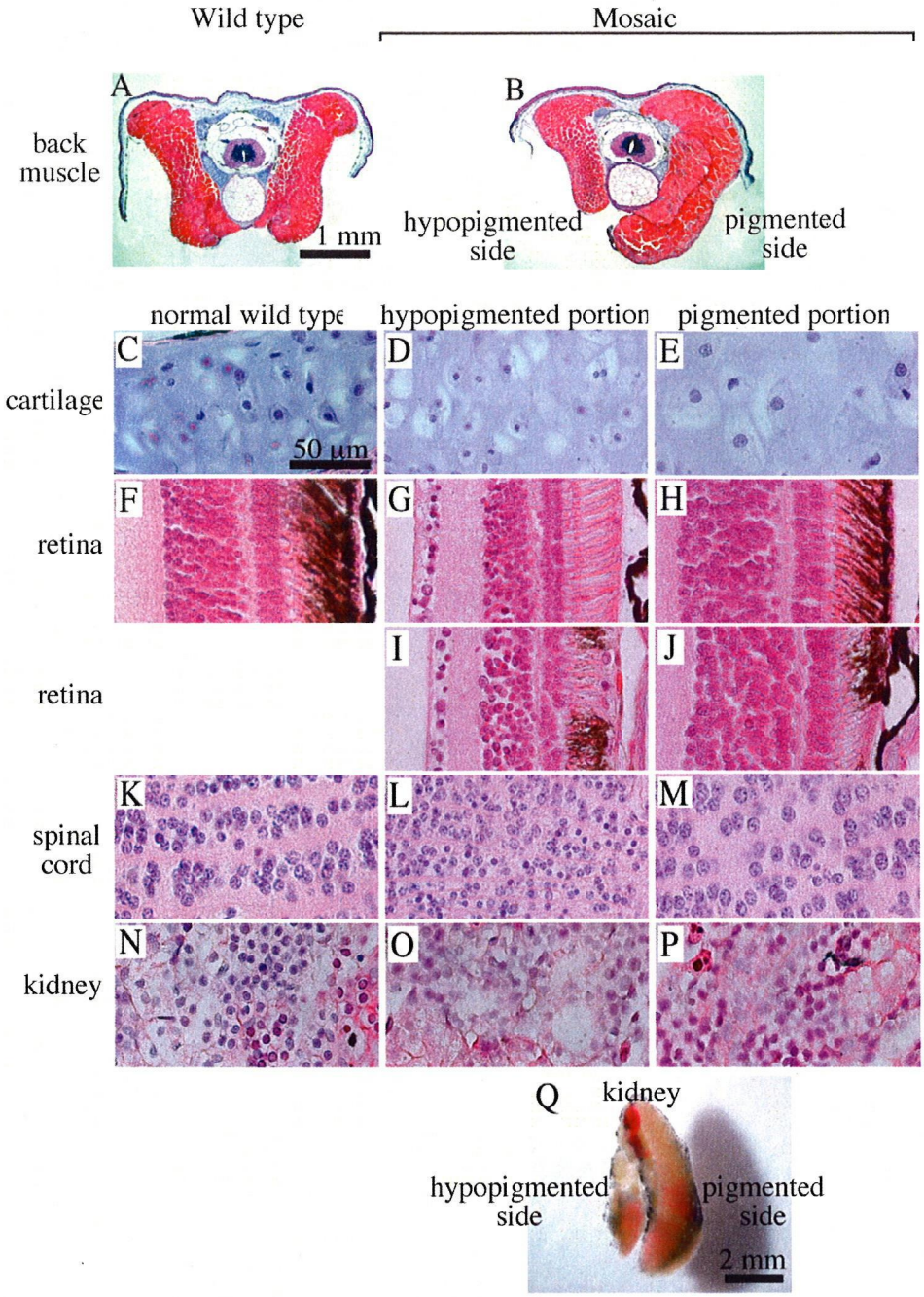
Figure 4. Chromosome counts. (A) Wild type (n = 24) and albino (n = 10) from matings of mosaics' parents. *Significantly larger than any other count ($P < 0.01$). (B) 13/26 mosaics (n = 2). Not examined further in the present study. (C) 13/39 mosaics (n = 20). *Significantly larger than any other count except ** ($P < 0.01$). **Significantly larger than any other count except * ($P < 0.01$). (D) Wild type - curved body (n = 2). Ratio = n chromosome cells ÷ total number of counted cells.

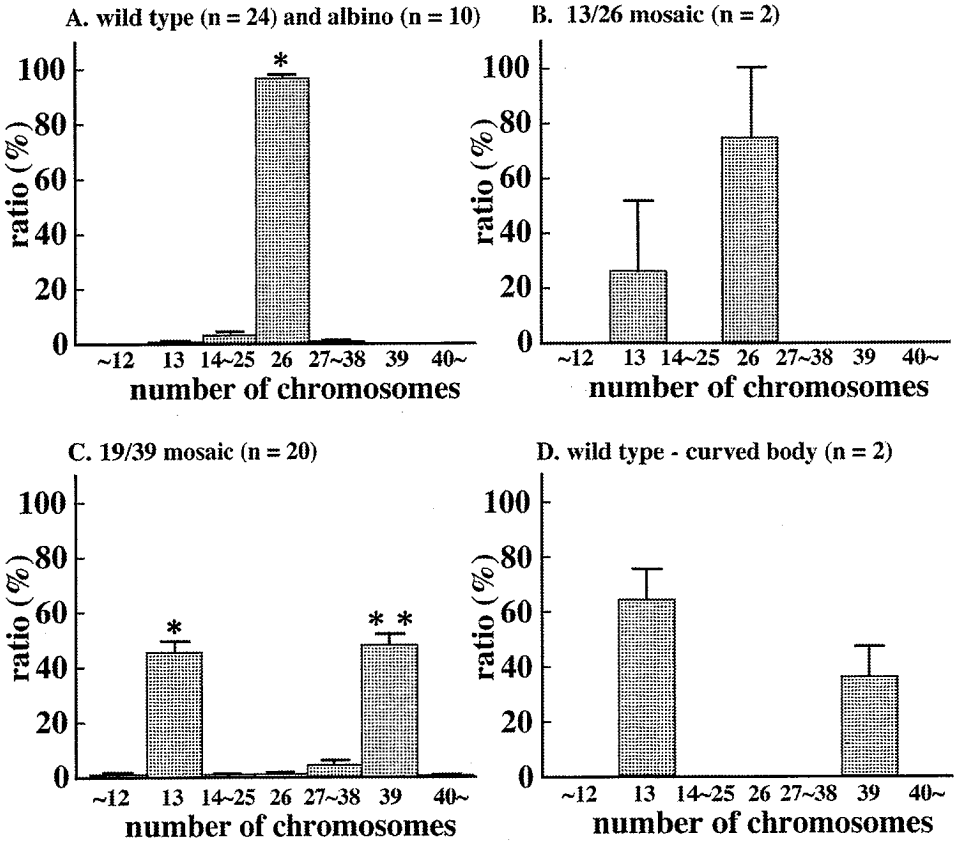
Figure 5. Karyotypes. (A) Wild type, 26 chromosomes. (B) Hypopigmented portion of mosaic, 13 chromosomes, haploid. (C) Pigmented portion of mosaic, 39 chromosomes, triploid. Magnifications of A, B, and C are identical.

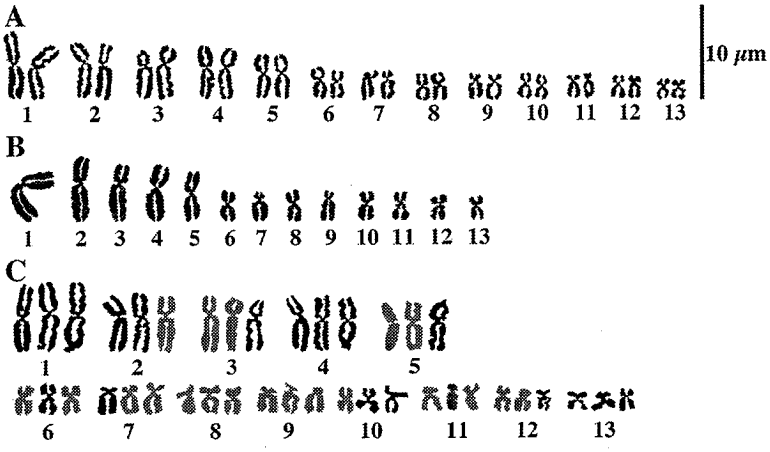
Figure 6. Cell ploidy and distribution. (A) Ploidy of cells in hypopigmented portion. *Significantly greater than other counts ($P < 0.01$). (B) Ploidy of cells in pigmented portion. *Significantly greater than other counts except ** ($P < 0.01$). **Significantly greater than other counts except * ($P < 0.01$). Based on the examination of 6 mosaic individuals (n = 6).


A
wild type albino
pigmentary mosaics
5 mm
B
wild type
albino
pigmentary mosaics
1 cm


5 mm


Wild type
Mosaic
A
B
back muscle
1 mm
hypopigmented side
pigmented side
normal wild type
hypopigmented portion
pigmented portion
C
D
E
cartilage
50 μm
F
G
H
retina
I
J
retina
K
L
M
spinal cord
N
O
P
kidney
Q
kidney
hypopigmented side
pigmented side
2 mm


A. wild type (n = 24) and albino (n = 10)
B. 13/26 mosaic (n = 2)
C. 19/39 mosaic (n = 20)
D. wild type - curved body (n = 2)
ratio (%)
100
80
60
40
20
0
*
**
~12
13
14~25
26
27~38
39
40~
number of chromosomes


A
10 μm
1 2 3 4 5 6 7 8 9 10 11 12 13
B
1 2 3 4 5 6 7 8 9 10 11 12 13
C
1 2 3 4 5
6 7 8 9 10 11 12 13

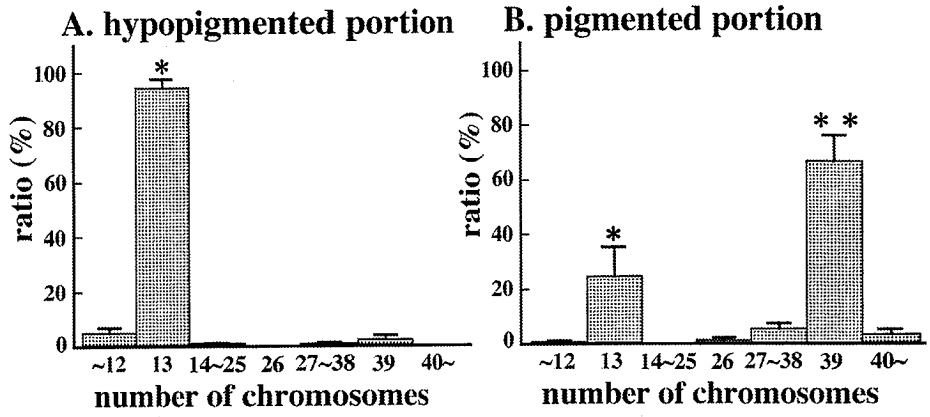

A. hypopigmented portion
B. pigmented portion
ratio (%)
100
80
60
40
20
0
*
* *
~12
13
14~25
26
27~38
39
40~
number of chromosomes

Table 1. Genotype of parents and frequency of pigmentary mosaics in their offspring. (Taylor and Kollros stage I). *w*, wild type. *a*, albino.

parents			offspring	
genotype		pairs	total tadpoles	pigmentary mosaics (%)
♀	♂			
w homo	*w* homo	8	4735	0 (0.000)
w homo	*a* homo	2	832	28 (3.365)
hetero	hetero	18	5606	29 (0.517)
hetero	*a* homo	14	2518	32 (1.271)
a homo	*w* homo	2	1516	0 (0.000)
a homo	hetero	11	2394	0 (0.000)