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Metamorphosis in *Xenopus laevis* is not associated with large-scale nuclear DNA content variation

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Summary

Amphibian metamorphosis is a complex process that has been speculated to involve DNA amplification and chromatin rearrangement. While recent studies have concentrated on chromatin rearrangement, only a few studies have dealt with variation in the amount of DNA during amphibian metamorphosis. In this study, nuclei were isolated from *Xenopus laevis* at various developmental stages. The nuclei were examined in both an unfixed state and a fixed state. The nuclei were stained with propidium iodide and analyzed by flow cytometry to determine their fluorescence intensity. The unfixed nuclei had higher fluorescence variation compared with the fixed nuclei. This increase in variation appeared due to the presence of nuclei of variable fluorescence intensity within the unfixed nuclei. Upon optimum fixation, which has been speculated to result in more homogeneous chromatin conformation and to reduce staining artifacts, the nuclei were observed to have less fluorescence intensity variation. The differential fluorescence observed in this study is consistent with the hypothesis that large-scale intra-individual DNA variation is not associated with amphibian metamorphosis.

Key words: nuclei, flow cytometry, development, metamorphosis, *Xenopus*.

Introduction

Xenopus laevis metamorphosis has been reported to be associated with variation in cellular DNA content. Fritz et al. (1990) reported that as *X. laevis* tadpoles developed from stage 35 to stage 57, their nuclear fluorescence as measured by flow cytometry increased significantly from stage 46–56 and then started to decrease. Although the ultimate conclusion was that the nuclear DNA content was varying in these cells due to gene amplification during development, the authors also indicated that chromatin compaction could also be responsible for the observed increase. While the authors favored the conclusion of increasing DNA content they could not exclude the possibility of chromatin compaction being involved.

Fritz et al. (1990) used the fluorochrome 4-6-diamidino-2-phenylindole (DAPI) in their experiments. DAPI is a dye that stains DNA and, more specifically, A–T-rich DNA (Shapiro, 1995). Fritz et al. (1990) mentioned that reports have indicated that DAPI has minor affinity to heterochromatin. Their experience suggested that the increase in fluorescence intensity observed using this dye was due to increasing DNA content. Other researchers have suggested that DAPI is less affected by chromatin condensation than other DNA dyes (Shapiro, 1995). Rayburn et al. (1989) observed that in maize nuclear DNA amounts obtained by flow cytometry using the dye DAPI gave similar results to nuclei stained with Schiff's reagent and analyzed by microdensitometry. Important to these findings is that the maize lines used in the study differed substantially in the amount of heterochromatin present. Thus, the data suggested that DAPI was not affected by heterochromatin. However, other studies have indicated that DAPI binding to DNA is affected by chromatin structure (Darzynkiewicz, 1990). Given the conflicting reports regarding the mechanism of DAPI, any conclusions on DNA content *versus* chromatin condensation using this dye is suspect.

One dye that is known to be affected by chromatin differences is propidium iodide (PI). PI is a base-pair-intercalating dye. Rayburn et al. (1992) observed that PI fluorescence did not reflect reported DNA content differences among maize lines differing with respect to the amount of heterochromatin each contained. The authors hypothesized that compacted heterochromatin was not as accessible to the PI dye. Other investigators have observed that during apoptosis, chromatin condensation occurs, which results in reduced PI fluorescence (O'Brien et al., 1998). It has now become an acceptable practice to take advantage of the reduced PI fluorescence due to chromatin compaction to quantify apoptosis by observing the presence of hypodiploid nuclei using flow cytometry (Hess et al., 2001; Schlosser et al., 2003; Souza-Fagundes et al., 2003).

The purpose of this study was to use PI, which is well documented to be affected by chromatin changes, to stain nuclei of developing *X. laevis* tadpoles. Nuclei from the same staged animals were stained in a 'native' state directly after

nuclear isolation and in an altered state (fixed). Any changes observed in the fluorescence intensity of the stained nuclei between the two treatments would indicate that large-scale DNA variation is not associated with metamorphosis.

Materials and methods

Xenopus laevis Daudin tadpoles of various stages were obtained from Xenopus1 (Dexter, MI, USA) and NASCO (Fort Atkinson, WI, USA). Tadpoles were housed in 10 gallon tanks under controlled conditions once received in the laboratory. All animal care and husbandry procedures were performed under the NRC guide for the care and use of animals, 1996 revision, under the oversight of the University of Illinois campus Animal Care and Use Committee. All staging was done according to the Normal Table of *X. laevis* (Nieuwkoop and Faber, 1956).

The DNA content analysis protocol is a modification of the Gold et al. (1991) fish cell protocol for flow cytometry. Tadpoles were homogenized in 10 ml of a 1× phosphate-buffered saline (PBS; Sigma, St Louis, MO, USA) and then filtered through a 53 μm mesh. Four 1 ml aliquots of nuclei suspension per tadpole were placed in microfuge tubes and microcentrifuged for 8 s at 12,000 ***g***. The supernatants were aspirated and nuclear pellets were combined for each tadpole by resuspending all four pellets in a total volume of 800 μl PBS. Microcentrifugation at 12,000 ***g*** was repeated, supernatant aspirated and the resulting pellet resuspended in 800 μl of PBS. The nuclei were then centrifuged in a microfuge for 8 s at 12,000 ***g***, the supernatant aspirated and the nuclear pellet resuspended in a propidium iodide (PI) stain solution [0.8 g sodium chloride (137 mmol l^{-1}), 0.056 g PIPES, 0.028 g disodium EDTA (0.75 mmol l^{-1}), 4 mg deoxyribonuclease-free ribonuclease and 5 mg propidium iodide in 100 ml distilled water brought to pH 7.50 with NaOH]. The RNase treatment and staining are both at 4°C due to the fact that at higher temperatures degradation of the nuclei occurs interfering with the analysis. After incubating 1 h at 4°C in the dark, the samples were filtered with a 53 μm mesh. Cell-cycle analysis was done on an EPICS XL flow cytometer (Coulter Electronics, Hileah, FL, USA) using an excitation wavelength of 488 nm. 50,000 nuclei were analyzed per sample. The flow rate resulting in the best most-consistent results was determined by dividing the nuclei of several animals into three subsamples and analyzing each sample at a different flow rate (Table 1). The full peak coefficients of variation (CV) of the G1 peaks were measured and compared. An ANOVA (analysis of variance) and LSD (least significant difference) were run using SAS (version 8.2, SAS Institute Inc, Cary, NC, USA).

Stage nuclei samples for fixation were isolated as described above. Nuclei samples were then fixed in 50% ice cold ethanol for 20 min at –20°C. Nuclei suspensions were microcentrifuged for 8 s at 12,000 ***g*** and the supernatants removed. The nuclear pellet was then washed in PBS and resuspended in a PI stain solution volume to result in a final concentration of 6×10^5 nuclei ml^{-1} of stain. The fixed samples were then placed on ice in the dark for 1 h and filtered through a 53 μm mesh. 10,000 nuclei were analyzed per fixed sample at a flow rate of 200 nuclei per second. The CVs of the G1 peaks were measured and compared. An ANOVA and LSD were run using SAS.

Table 1. *The impact of flow rate on the coefficient of variation (CV) of the nuclear peak*

Average flow rate*	Number of replications†	Average CV	LSD grouping‡
340	13	5.52	A
190	13	5.27	B
100	13	5.19	B

*Nuclei per second.

†13 total animals were used. Each animal had a subsample analyzed at each of the flow rates.

‡The flow rates with the same letter are not significantly different at P=0.05.

For fixation comparisons, nuclei samples from six tadpoles were divided into subsamples and either fixed in 25%, 50% or 75% ethanol for 20 min at –20°C or left unfixed. Nuclei from each animal were counted, stained at a concentration of 6×10^5 nuclei ml^{-1} and analyzed as previously described in the fixation method. 10,000 nuclei were analyzed per sample at

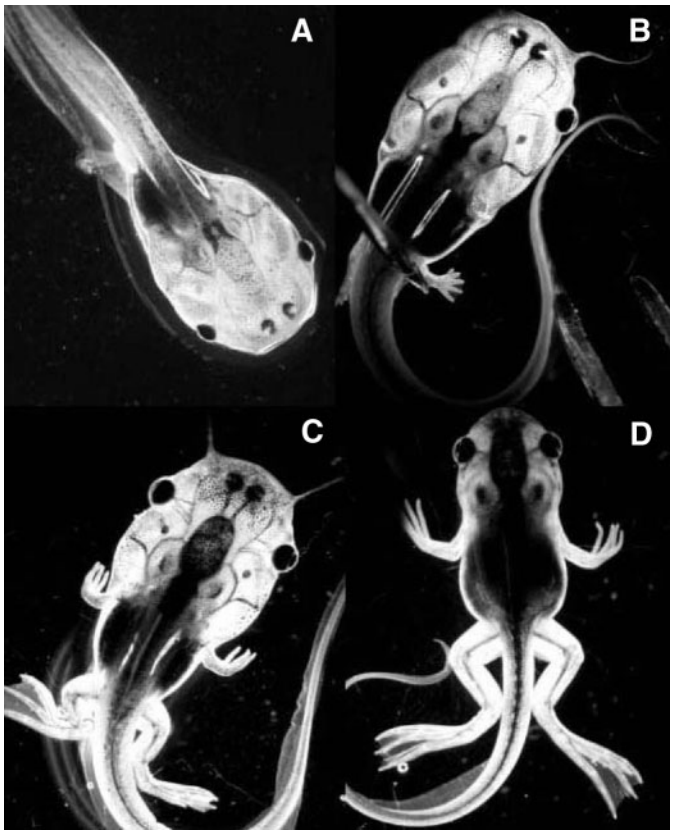

Fig. 1. Various developmental stages of *X. laevis* larvae. (A) Early two-leg tadpole, (B) two-leg tadpole, (C) four-leg tadpole, (D) four-leg metamorph with tail.

200 nuclei per second. The CVs of the G1 peaks were measured and compared. An ANOVA and LSD were run using SAS.

Results

The stages tested ranged from stage 47 to 66 and were represented by developmental clusters identified based on the number of legs and degree of metamorphosis (Fig. 1A–D). Stage 47 was the earliest stage analyzed due to the technique used to isolate nuclei. To obtain a consistent isolation of intact nuclei, the tadpoles had to weigh at least ~250 mg. Animals weighing <250 mg usually gave inconsistent nuclear isolations and, therefore, were of no use to this analysis. The optimum flow rate of the samples was approximately 200 nuclei per second. The rate allowed for the most efficient rate of data acquisition with no significant effect of data quality as measured by CV (Table 1). To ensure that this staining protocol did not result in artifacts, comparisons with chicken red blood cells as an internal standard [defined as having 2.5 pg $2C^{-1}$ (Tiersch and Wachtel, 1991)] were run. The genome size observed for the *X. laevis* was 6.3 pg $2C^{-1}$, within the reported range of *X. laevis* (Birstein et al., 1993).

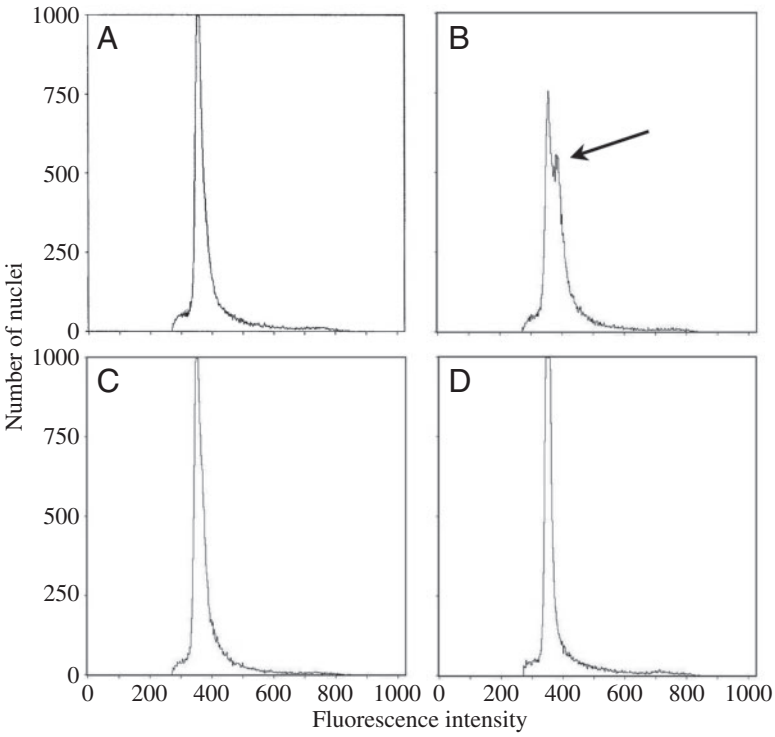

Fig. 2. Representative nuclear histogram from unfixed nuclei at various developmental stages of *X. laevis* larvae. (A) Early two-leg tadpole, (B) two-leg tadpole, arrow denotes hyperdiploid nuclei, (C) four-leg tadpole, (D) four-leg metamorph with tail.

For our analysis, stages 48–51 were clustered in a group denoted as the early two-leg stage (Fig. 1A). The histograms for these animals were observed to have a single peak and to be very consistent from animal to animal (Fig. 2A). At stages 52–55 (two-leg stage; Fig. 1B) the single peak began to broaden. In many cases it no longer appeared as a single peak. In a few tadpoles, a second, distinct hyperdiploid peak appears at a higher DNA amount than the previous peak (Fig. 2B). As the tadpole continues through stages 56–57 (late two-leg stage), more individuals are observed to have the distinct hyperdiploid peak. At stages 58–62 (four-leg tadpole; Fig. 1C) the number of individuals with the hyperdiploid peak decreases and in many of the tadpoles the resolution of the two peaks becomes less evident (Fig. 2C, Table 2).

As metamorphosis continues, the peaks begin to narrow with the hyperdiploid peak disappearing. At stage 63 (Fig. 1D), the peak symmetry improves (Fig. 2D) and the CV decreases (Table 2). When stage 65 is reached, the peak appears at its best with a very narrow and symmetrical peak and the lowest recorded CV (Table 2). No sign of the hyperdiploid peak is apparent. This nice distinct peak remains until stage 66. As the tadpole completes metamorphosis and develops into a small juvenile frog, the technique used to isolate intact nuclei is no longer effective due to mechanical limitations. Three very distinct LSD groupings were observed among the various stages with respect to CV (Table 2). It should be noted that while presented as discrete units, the stage groupings during metamorphosis are continuous data. Therefore, various types

Table 2. *The coefficients of variation (CV) of the nuclear peak from native nuclei isolated from tadpoles sampled at different stages of development*

Stage	Number of tadpoles	Average CV	LSD grouping*
No legs (stage 47)	22	7.74±1.63	A
Early two legs (stages 48–51)	23	6.98±1.83	A,B
Two legs (stages 52–55)	33	6.73±1.11	B
Late two legs (stages 56–57)	10	7.29±0.87	A,B
Four legs (stages 58–62)	14	7.68±1.46	A
Four-leg metamorph with tail (stages 63–64)	15	5.10±0.84	C
Four-leg metamorph no tail (stages 65–66)	43	5.03±1.04	C

*CVs with same letter are not significantly different at P=0.05.

Table 3. *The coefficients of variation (CV) of the nuclear peak from fixed nuclei isolated from tadpoles at different stages of development*

Stage	Number of tadpoles	Average CV	LSD grouping*
No legs (stage 47)	8	5.30±0.78	A,B
Early two legs (stages 48–51)	34	5.40±0.83	A,B
Two legs (stages 52–55)	13	5.65±0.88	A
Late two legs (stages 56–57)	20	5.87±0.80	A
Four legs (stages 58–62)	9	5.25±0.74	A,B
Four-leg metamorph with tail (stages 63–64)	6	4.70±0.44	B
Four-leg metamorph no tail (stages 65–66)	4	4.85±0.51	B

*CVs with same letter are not significantly different at P=0.05.

of histograms were observed in each group cluster. The peaks were not necessarily homogeneous within each developmental cluster requiring a large sample size to determine the representative histogram.

Upon using 50% ethanol fixation the CVs of all stages of the developing larvae were improved (Table 3). Only single-peak histograms were obtained (Fig. 3A–D). Owing to the presence of only a single peak, fewer animals were required to establish a profile for each stage cluster. Only two overlapping LSD clusters were observed among the stages (Table 3). As in the unfixed nuclei, the best CVs were observed at the four-leg stages. However, no significant difference was observed from the no-legs stage through the early four-leg stage (Table 3).

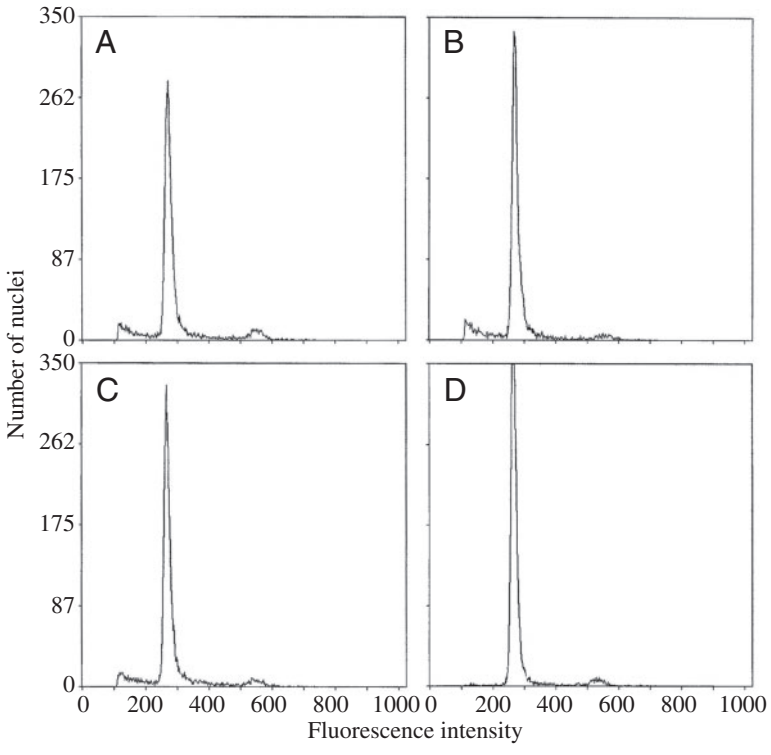

Fig. 3. Representative nuclear histogram from fixed nuclei at various developmental stages of *X. laevis* larvae. (A) Early two-leg tadpole, (B) two-leg tadpole, (C) four-leg tadpole, (D) four-leg metamorph with tail.

Table 4. *The coefficients of variation (CV) of nuclei fixed in various concentrations of ethanol*

Fixation	Number of samples	Average CV	LSD grouping*
No fixation	6	5.90±0.46	A,B
25% EtOH	6	5.53±0.01	B
50% EtOH	6	4.59±0.06	C
75% EtOH	6	6.15±0.29	A

*CVs with same letter are not significantly different at P=0.05.

Four different fixation methods were compared (Table 4). Nuclei from individual animals were divided into four subgroups. Nuclei from all six animals were analyzed in an unfixed state and fixed at 25%, 50% and 75% ethanol. The largest CV was obtained with the 75% ethanol fixation. The 25% ethanol fixation gave similar CVs to the unfixed nuclei. The best CVs were obtained with 50% ethanol.

Discussion

The appearance and disappearance of the hyperdiploid peak coincides with the increasing DNA amount reported by Fritz et al. (1990). Fritz et al. (1990) suggested that this increase in DNA could be due to gene amplification during larvae development. They did however present an alternate hypothesis. Fritz et al. (1990) could not rule out the decondensation of compact chromatin during larvae development as the cause of the observed increase in DNA content. Bonaly et al. (1987) reported that chromatin structure of the nucleus could affect the binding of specific fluorochromes to DNA. One fluorochrome particularly susceptible to chromatin structure is PI, the dye used in the present study. Upon comparing the potential binding of two fluorochromes with nuclei with differing amounts of compact heterochromatin, Rayburn et al. (1992) found that PI fluorescence was influenced by the amount of heterochromatin. They hypothesized that DNA binding of PI was reduced in heterochromatin resulting in an underestimate of total DNA. If chromatin decondensation was occurring in *X. laevis* during larvae development such changes would reflect an apparent increase in total DNA amount.

Vassetzky et al. (2000) reported that chromatin domains do rearrange during *X. laevis* development. The domain rearrangement did not appear random but seemed to be related to the timing of gene expression during development. If such chromatin rearrangement does occur during larvae development the amount of facultative heterochromatin may fluctuate. Such fluctuations could result in different intercalation of the fluorochrome PI within the

nucleus of differentiating tissues. This would result in the DNA amounts appearing different at various stages due to the heterogeneity of nuclei of different organs or tissue. This heterogeneity of nuclei could be reflective of differential timing of metamorphosis in specific tissue/organs. Also, the nuclear heterogeneity could well be due to the presence of alternative mechanisms of metamorphosis not involving chromatin changes (Sachs and Shi, 2000). Regardless of the mechanism involved in nuclear heterogeneity within metamorphosing tadpoles, the data presented indicates that large-scale DNA variation is not involved in metamorphosis.

The results of this study are consistent with DNA content variation within individuals not being associated with metamorphosis. Holtfreter and Cohen (1990) made an interesting observation in hemopoietic frog cells. Studies in their laboratory indicated that the chromatin of erythrocytes had differential accessibility to PI than the chromatin of the leukocytes. Upon fixation with 50% ethanol, the erythrocytes and leukocytes were observed to have the same PI fluorescence. The fixation step resulted in the homogeneity of staining between the erythrocytes and leukocytes. Thus, if the DNA differences observed in the *X. laevis* developing larvae disappeared, one would expect that large-scale nuclear DNA content variation would not be involved in metamorphosis. Upon fixation the differential PI fluorescence is lost. All of the nuclei appear homogeneous with respect to PI fluorescence intensity.

The ethanol fixation used here has never been reported to result in selective degradation of DNA. On the contrary, 50% ethanol fixation has been suggested as the fixation of choice when DNA analysis requires a balance of homogeneity of staining and high resolution of staining (Poulin et al., 1994). Ethanol mechanism of action has not been associated with DNA changes. Reports have indicated that varying the concentration of ethanol in the fixative from 50% results in increased variation in the fluorescence of nuclei stained for DNA content due to fluctuations in chromatin density (Shapiro, 1995). Thus in the present study, if homogeneity of the nuclei distribution is affected by altering the concentration of ethanol in the fixative, evidence for fluctuations in nuclear DNA content being involved in metamorphosis would be weakened. The results presented here clearly demonstrate that deviations from 50% ethanol fixation results in increasing CVs which is indicative of heterogeneity of the nuclei due to changes other than DNA content changes.

With the differential fluorescence of the larvae being associated with development not observed after optimum fixation (which results in homogenous nuclei and reduces artifacts), it appears clear that the differential fluorescence due to the presence of hyperdiploid nuclei is not the result of large-scale total nuclear DNA variability. Small amplification events too small to be detected by flow cytometry could still be occurring. However, such events could not be responsible for the large amount of fluorescence variability observed in the nuclei of metamorphosing individuals.

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