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AS Holub

RT Smith

GA Wyngaard

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## EMBRYONIC DEVELOPMENT AND A QUANTITATIVE MODEL OF PROGRAMMED DNA ELIMINATION IN *MESOCYCLOPS EDAX* (S. A. FORBES, 1891) (COPEPODA: CYCLOPOIDA)

Michelle K. Clower<sup>1</sup>, Ashton S. Holub<sup>2</sup>, Rebecca T. Smith<sup>3</sup>, and Grace A. Wyngaard<sup>2,\*</sup>

<sup>1</sup> North Carolina Coastal Federation, 128 Grenville St., Manteo, NC 27954, USA

<sup>2</sup> Department of Biology, MSC 7801, James Madison University, Harrisonburg, VA 22807, USA

<sup>3</sup> Eastern Shore Laboratory, Virginia Institute of Marine Science, Wachapreague, VA 23480, USA

### ABSTRACT

The highly programmed fragmentation of chromosomes and elimination of large amounts of nuclear DNA from the presomatic cell lineages (i.e., chromatin diminution), occurs in the embryos of the freshwater zooplankton *Mesocyclops edax* (S. A. Forbes, 1891) (Crustacea: Copepoda). The somatic genome is reorganized and reduced to a size five times smaller even though the germline genome remains intact. We present the first comprehensive, quantitative model of DNA content throughout embryogenesis in a copepod that possesses embryonic DNA elimination. We used densitometric image analysis to measure the DNA content of polar bodies, germline and somatic nuclei, and excised DNA “droplets.” We report: 1) variable DNA contents of polar bodies, some of which do not contain the amount corresponding to the haploid germline genome size; 2) presence of pronuclei in newly laid embryo sacs; 3) gonomic chromosomes in the second to fourth cleavage divisions and in the primordial germ cell and primordial endoderm cell during the fifth cleavage division; 4) timing of early embryonic cell stages, elimination of DNA, and divisions of the primordial germ cell and primordial endoderm cell at 22°C; and 5) persistence of a portion of the excised DNA “droplets” throughout embryogenesis. DNA elimination is a trait that spans multiple embryonic stages and a knowledge of the timing and variability of the associated cytological events with DNA elimination will promote the study of the molecular mechanisms involved in this trait. We propose the “genome yolk hypothesis” as a functional explanation for the persistence of the eliminated DNA that might serve as a resource during postdiminution cleavage divisions.

**KEY WORDS:** chromatin diminution, densitometric image analysis, genome size, polar body, primordial germ cell

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### INTRODUCTION

Some species of cyclopoid copepods excise massive amounts of DNA from their somatic genome during ontogeny. The result is a somatic genome that is dramatically reduced in size and reorganized, but the germline genome is unaltered. This phenomenon was first discovered over 100 years ago in parasitic nematodes (Boveri, 1887) and called “chromatin diminution,” because at that time only a reduction in the amount of chromatin, and consequently chromosome length, in the somatic cells could be observed. Soon thereafter a similar phenomenon was found in embryos of a copepod (Häcker, 1894; Amma, 1911) and since then occasional discoveries of chromatin diminution have been reported in additional species (e.g., Beermann, 1977; Einsle, 1994, 1996a, b; reviewed by Wyngaard, 2000).

We now know that DNA sequences are eliminated in phylogenetically distant taxa, including protozoan ciliates, nematodes, copepods, Japanese hagfishes, and the sea lamprey (Kubota et al., 1993; Prescott, 2000; Wang et al., 2012; Smith et al., 2013; Sun et al., 2014; Wang and Davis, 2014). The term chromatin diminution has often been the term of choice to describe this phenomenon in multicellular eukaryotes (reviewed by Zufall et al., 2005; Streit, 2012), but we

prefer “DNA elimination,” which is more descriptive of the process and which has been used extensively in reference to ciliates, and is increasingly being used in reference to other taxa (e.g., Wang et al., 2014).

DNA elimination in copepods is a highly programmed process, occurring during one or more specific embryonic cleavage divisions and excising specific amounts of DNA (Beermann, 1977). Depending upon the species, the DNA is eliminated during the fourth to seventh cleavage divisions and approx. 1–74 pg of DNA per nucleus, equivalent to 45–99% of the germline genome (Beermann, 1977; Rasch and Wyngaard, 2006a; Wyngaard et al., 2011). The only case of population-level variation in the presence or absence of DNA elimination is the report of its presence in a southern German population of *Cyclops insignis* Claus, 1857 (Einsle, 1996b) and the absence in northern German and Russian populations of the same species (Grishanin et al., 2004; Semeshin et al., 2011). The copepods are distinctive among the taxa that have DNA elimination because most copepods excise far more DNA than the approx. 1 pg excised in other taxa (Beermann, 1977; Kloc and Zagrodzinska, 2001; Wyngaard et al., 2011; Smith et al., 2013). Spectacular images of large amounts of eliminated

\* Corresponding author; e-mail: wyngaaga@jmu.edu

DNA remaining at the metaphase plate immediately after the DNA elimination are easily viewed at the cytological level (Wyngaard and Gregory, 2001). The excised DNA scheduled for elimination is located at the terminal ends, procentric and terminal ends, or interdispersed throughout the prediminished chromosomes according to species (Beermann, 1977). Much of the DNA scheduled for elimination in copepods is densely stained heterochromatin that can be recognized as C-bands (Standiford, 1989). Cytological studies in copepods are useful for defining the timing of various cytological events leading up to the elimination, as well as measuring the amount of excised DNA.

The most pressing questions about DNA elimination in copepods concerns the identity of the eliminated sequences and the possible function or adaptive significance of this trait. Molecular studies describing the eliminated sequences in the copepod *Mesocyclops edax* (S. A. Forbes, 1891) revealed that most of the eliminated sequences are comprised of transposable elements (TEs) and complex repeat sequences of unknown origin (Drouin, 2006; McKinnon and Drouin, 2013; Sun et al., 2014). PCR studies of the eliminated DNA in *Cyclops kolensis* Lilljeborg, 1901 also suggest that repeated sequences, including rRNA genes, are among the eliminated DNA (Degtyarev et al., 2004; Grishanin et al., 2006; Zagoskin et al., 2010). Sun et al. (2014) hypothesized that many of the eliminated TEs are active, potentially deleterious, and that the DNA elimination serves as a host-defense mechanism, consistent with other studies that have ascribed a role of host defense to the DNA elimination process in ciliates (e.g., Chalker and Yao, 2011; Noto et al., 2015). Additionally, DNA elimination in ciliates rearranges the genome and produces a new combinatorial assembly of DNA segments that might promote the evolution of new genes and speciation (Chen et al., 2015; Gao et al., 2015). The elimination of genes in nematodes has been ascribed to roles in gene silencing and sex determination (Wang et al., 2012; Streit et al., 2016), and in the lamprey, to gene silencing (Smith et al., 2013).

Altogether, these taxa have in common a mechanism that maintains genome stability. Whether DNA elimination in these taxa has a common origin or is independently derived is unknown and will require detailed knowledge at the molecular level.

The relative proportions of genes, transposable elements, and other repeats comprising the eliminated DNA vary dramatically across the major taxa that undergo DNA elimination, suggesting that the origins, molecular mechanisms, and functional roles could also differ. This argues for developing a model copepod system in which the details of the molecular biology driving this trait can be studied and experimentally manipulated. A necessary step toward this goal is obtaining a detailed description of the timing of cytological events associated with DNA elimination. Knowledge of such a time course will enable studies that target particular molecular processes at specific points in time, as is often needed in transcriptomic and other genetic studies. Only then can the critical experiments be designed to elucidate the molecular mechanisms and functional roles of DNA elimination in copepods.

We constructed a comprehensive timetable of the sequence of cytological events from presence of pronuclei in the newly laid egg sac until hatching of the embryo into a nauplius larva. We chose *Mesocyclops edax* because it is a widely distributed freshwater, zooplanktonic copepod whose biology is well studied (Wyngaard and Chinnappa, 1982; Wyngaard, 1986) and whose genomic DNA (Sun et al., 2014) is the best described among copepods that undergo DNA elimination. *Mesocyclops edax* has a diploid germline genome size of 15 pg of DNA, and during embryogenesis excises 12 pg DNA per nucleus, or 80% of its germline genome, resulting in a somatic genome that contains 3 pg DNA per nucleus (Rasch et al., 2008). The quantitative behavior of DNA throughout embryogenesis was documented by measuring DNA contents in the germline and somatic genomes, polar bodies, and excised DNA. This detailed account of the cytological events accompanying DNA elimination should aid the mechanistic studies of the molecular processes that so dramatically reduce and reorganize the somatic genome.

## MATERIALS AND METHODS

### Field Collection

Adult females of *Mesocyclops edax* were collected 0.5-1 h after sunset from Lake Shenandoah, Rockingham County, VA, USA (38°28'N, 78°83'W) with a 64  $\mu$ m Wisconsin plankton net. Because *M. edax* undergoes diapause in this lake, we collected specimens only between May and September when it is abundant in the plankton. Most of the data presented here are derived from collections made in 2014-2015, and these were supplemented with data collected in 2012-2013.

### Histological Stains and Specimen Preparation

The fluorescent DNA stain DAPI (4'-6-diamidino-2-phenylindole; Thermo Fisher Scientific, Waltham, MA, USA) was used to label the pronuclei prior to their fusion. Whole embryo sacs of *M. edax* were fixed and stored in approx. 350  $\mu$ l 4% formalin for 20-24 h before the formaldehyde solution was replaced with 200  $\mu$ l of 5  $\mu$ g/ml DAPI in distilled water. The embryos were stored in the dark at 4°C for 24 h, then transferred to distilled water in small dishes with cover-glass bottoms of size 1.0 (MatTek, Ashland, MA, USA). Specimens were viewed with an inverted Nikon TE 2000-E laser scanning confocal microscope and excited by a 408 nm laser. Images were made with an air immersion PLAN APO20x (NA = 0.75) objective. Fifty-six Z-stacked slices were taken at intervals of 0.75  $\mu$ m for each embryo and 57 slices for each nauplius. Z-stacks were exported as both Nikon nd2 files and Avi files.

C-banding was performed to visualize regions on the chromosomes containing heterochromatin, most of which are expected to be excised during DNA elimination. Egg sacs at stages prior to DNA elimination were placed in a drop of 45% acetic acid on a glass slide. Eggs were dispersed using a dissecting needle and then heated by flame for a few seconds to soften the egg membranes. A coverslip was placed on the specimens to squash the eggs sacs and then removed by freeze-flipping in liquid nitrogen. Preparations were aged for two weeks and then stained with Giemsa following precisely the protocol of Standiford (1989), except that the mitotic inhibitor 8-hydroxyquinoline was not used.

A 1% aceto-orcein stain was used to count the nuclei and observe mitotic figures when determining the duration of individual embryonic stages and the number of nuclei in a newly hatched nauplius. The 1% aceto-orcein stain was prepared by pouring 55 ml of boiling glacial acetic acid over 1 g of synthetic orcein and diluting that solution to 45% with distilled water before filtering through Whatman no. 2 filter paper. Whole embryos were fixed in 3:1 ethanol:acetic acid for at least 3 min before transferring to the 1% aceto-orcein stain for 10 min and squashing under a coverslip. The number of nuclei was counted for 1 to 32-cell embryos as the nuclei in a single field of view at 63 $\times$  magnification on a Nikon stereomicroscope. Later embryonic stages and the nauplius I larvae were photographed at 300-600 $\times$  in multiple views, and their nuclei were counted on a compound Nikon Optiphot compound microscope.

We used Feulgen Image Analysis Densitometry (FIAD) to quantify the amounts of DNA in polar bodies, excised DNA droplets, and nuclei of germline and somatic cells. Preparation of the Schiff reagent (basic fuchsin, Sigma-Aldrich, St. Louis, MO, USA) and the protocol for the Feulgen reaction were according to Hardie et al. (2002) with the exceptions that tissues were hydrolyzed in 5 N HCl at  $23 \pm 0.5^\circ\text{C}$  in a Convicon CMP 6010 incubator for 20 min and then rinsed in 0.1 N HCl at room temperature for 30 s. Only non-overlapping nuclei were selected for measurement. Interphase nuclei were selected for measurements of adult somatic tissues to avoid errors caused by compaction.

Embryos and nauplius I larvae, along with their mothers, were fixed for staining according to Rasch (2004) and Rasch et al. (2008). Histological preparations were apportioned among three staining batches. Slides with films of blood cells (5.2 pg DNA per nucleus) of domestic chicken *Gallus gallus domesticus* (Linnaeus, 1758) (2.5 pg DNA per nucleus) and rainbow trout *Oncorhynchus mykiss* (Walbaum, 1792) served as external standards. Adult female *M. edax* also served as an internal standard because their somatic DNA contents (3.0 pg DNA per nucleus) do not vary significantly among widely separated geographical populations (Rasch and Wyngaard, 2006b).

#### Image Analysis

Prior to measurement of DNA contents in *M. edax*, we verified that the average adult soma of a haphazardly chosen slide of an adult *M. edax* contained 3.0 pg DNA per nucleus based on the standard 2.5 pg DNA per nucleus in the chicken and 5.2 pg DNA per nucleus in the trout. This verification was performed for all three staining batches. Subsequent measurements of DNA contents on each slide used adult somatic nuclei as an internal standard.

Integrated optical densities (IOD) of DNA contents of individual nuclei, polar bodies and excised DNA "droplets" were measured by scanning microdensitometric software (Version 11.2.6; Bioquant Image Analysis, Nashville, TN, USA). Data were processed at 560 nm with the green channel at 185-190 intensity units. Specimens were measured in immersion oil (refractive index 1.5150, Type B) with a 63X, N/A 1.25 objective and Zeiss Axioscope A1 equipped with a Qimaging CCD camera. The lower and upper 5% of the range of the integrated optical density standard measurements were excluded from the measurements of the nuclear DNA contents of the standards when we computed the mean IOD. The sample sizes of polar bodies and DNA droplets per embryo were too small to allow this correction. Nor did we exclude any values at the extremes of the ranges from the representative pre- and postdominated embryos derived from only two females (Fig. 2). Measurements of about 100 chicken and adult soma nuclei each had coefficients of variation of 4-10%. The amounts of pg of DNA were computed according to:

$$pg_u = (pg_s / IOD_s) \times IOD_u$$

where  $pg_u$  is the unknown amount of pg DNA in an embryo, polar body or excised DNA "droplet,"  $pg_s$  the amount of pg DNA of the standard nuclei,  $IOD_s$  the average IOD value of the standard, and  $IOD_u$  the IOD value of the unknown value in the embryo, polar body, or excised DNA "droplet." Nuclear DNA contents can be converted from pg of DNA to Gb of DNA using the conversion factor of 1 pg DNA =  $0.978 \times 10^9$  bp (Doležel et al., 2003).

#### Embryonic Cell Stage Durations

Females and embryos were maintained in a Convicon CMP 6010 (Convicon, Winnipeg, MB, Canada) incubator at  $22 \pm 0.5^\circ\text{C}$  with a 12:12 hour light and dark cycle. A total of 26 clutches of *M. edax* embryos were observed to study the timing of the cell stages prior to DNA elimination (first to fifth cleavage divisions). Females with dark oviducts were isolated and observed at 63X with a stereomicroscope every 10 min to determine the extrusion of reproductive material into their embryo sacs. The 1-cell stage was identifiable as a dark mass resembling the shape of an erythrocyte with a "dimple" and lacking any visible division furrows. Two- to 16-cell stages were identified by the presence of grooves between cells.

To determine the cell stage and total number of cleavage divisions during embryogenesis, 16-cell to approx. 1024-cell stage embryos were fixed, stained, and squashed with orcein or Schiff reagent (as described above) and their nuclei counted. After the fifth cleavage division (34-cell stage), the total number of nuclei could only be estimated. Cleavage divisions after the 16-cell stage are asynchronous, the numbers of cells do not increase

in a geometric progression, and so the recorded numbers of nuclei are approximations.

The timing of the excision of DNA was calculated as the time from extrusion of reproductive material into the embryo sacs until the appearance of anaphase figures with large masses of eliminated DNA at the metaphase plate. Orcein stained squashes of individual embryos were observed at 10-15 min intervals. The duration of embryogenesis was measured as the time from the extrusion of reproductive material into the embryo sacs until the embryos hatched into nauplius I larvae. Durations of cell stages are presented as median values because the distribution of durations could not be verified to be normally distributed in sample sizes less than 20 clutches.

## RESULTS

DNA elimination in *Mesocyclops edax* is a process that takes place over multiple embryonic stages, from the preparation of the molecular machinery that excises large fragments of chromosomes to the DNA elimination and eventual degradation of the excised "droplets" of DNA. These events are highly programmed. They result in quantitative behaviors of nuclear DNA in the germline, presomatic, and somatic cell lineages at specific time points that are reproducible and that deviate considerably from the conventional germline-soma differentiation of most eukaryotes (Table 1, Fig. 1).

#### Early Embryonic Cleavage Divisions Prior to DNA Elimination

Some embryo sacs were filled with reproductive material within a minute, while others required 20 min to fill. This time delay resulted in some sacs containing, for example, 4-cell embryos at the distal end and 2-cell embryos at the proximal end. Maternal and paternal pronuclei were visible within 5 min after extrusion of reproductive material into the egg sacs and appeared as separate, uncondensed, masses located near the cell membrane (Fig. 2A1). A single polar body (not shown in Fig. 2) was visible near the outside of each egg membrane and on a plane different from the Z-stack slices shown in Fig. 2A1. Female and male pronuclei fuse to form a single nucleus (Fig. 2A2), and this sequence indicates that fertilization occurs within the embryo sac. At this stage of development the external morphology of the unsquashed 1-cell embryo begins to resemble that of a "dimpled" red blood cell. Just prior to the first cleavage division, the chromosomes appear more densely stained.

The early 1-cell embryos had chromosomes in the form of long, flexible filaments (Fig. 2B) and contained 14.9 pg DNA per nucleus, which is within measurement error of the expected 2C value of 15 pg DNA per nucleus. Homozygous gonomic divisions at the second cleavage division shown in late anaphase (Fig. 2C) clearly indicate the separation of maternal and paternal sets of chromosomes on the spindle apparatus, which is also suggested by the arrangement of chromosomes in Fig. 2B. These maternal and paternal sets of chromosomes have equal DNA contents (7.5 pg DNA), hence the term "homozygous" to describe this kind of gonomery. The maternal and paternal chromosomes remain as separate entities until the DNA is excised in the 15 presomatic cells during the fifth cleavage division. Gonomic chromosomes are also present during the division of the primordial germ cell during the fifth cleavage division (arrow in Fig. 2I). The C value, which by definition corresponds to the amount of DNA in the haploid germline genome, should be 7.5 pg DNA per nucleus. This is consistent with the 7.5 pg of

Table 1. Timetable of embryonic development in *Mesocyclops edax* at  $22 \pm 0.5^\circ\text{C}$ . Duration of embryonic cell stages prior to DNA elimination, presence of gonomery, polar bodies, swollen heterochromatic regions of DNA to be excised, primordial germ cell (PGC), and endoderm cell (PEC), and excised DNA droplets are included. Five of the six measurements of duration at the 16-cell stage were estimated by subtracting the observed time of entry into the 32-cell stage (as defined by chromosomal figures with eliminated DNA at the metaphase plate) from the sum of the median durations (440 min) of each of the 1 through 8-cell stages.

Cell stage or cleavage interval	Median duration (range) (min)	No. of clutches	Events	Excised DNA	Polar body present
1-cell (including pronuclei)	84 (56-116)	19	Pronuclei fuse just prior to 2 cell stage (Fig. 2A)	No	Yes
2-cell	60 (40-72)	12		No	Yes
4-cell	111 (37-132)	10	Gonomeric chromosomes (Fig. 2C)	No	Yes
8-cell	185 (157-209)	9	Gonomeric chromosomes	No	Yes
16-cell	146 165 (161-180)	1 5	Early interphase (Fig. 2D)	No	Yes
Fifth cleavage division: 17 cells (15 somatic and 1 PGC and 1 PEC)			Dense heterochromatic regions in presomatic nuclei scheduled for excision; a single cell divides to give rise to PGC and PEC (Fig. 2E, F)	Yes	Yes
Fifth cleavage division: 30 diminuted somatic cells and 1 PEC and 1 PGC			15 diminution figures representing 15 presomatic nuclei which have undergone diminution (Fig. 2G, H)	Yes	Yes
Fifth cleavage division: 30 diminuted somatic cells and 2 diminuted PECs and 2 PGCs			Dense heterochromatic regions in PEC scheduled for excision appear swollen (Fig. 2H); gonomeric chromosomes of PGC produce 2 PGCs at the same time the PEC undergoes a delayed diminution to produce 2 cells (see Fig. 2I, J)	Yes	Yes
34-cell and subsequent cell stages			Asynchronous cell division	Yes	Yes, until 512-cell stage

DNA in each maternal and paternal set of gonomeric chromosomes (Fig. 2C).

Prior to the excision of the DNA, embryos at the 1-8 cell stages contained between the 2C (15 pg) and 4C (30 pg) amounts DNA per nucleus, depending upon the time point in the cell cycle at which they were measured. This is illustrated by comparing the DNA contents of a clutch of 4-cell stage embryos (Fig. 3A) with the somatic DNA contents of their adult mother (Fig. 3D), as well as

with postdiminuted embryos produced by two other mothers (Fig. 3C). This exemplar set of measurements of a single female and her clutch of embryos with the embryos of two clutches following DNA elimination illustrates that 12 pg of DNA is excised from each presomatic nucleus. Embryos after DNA elimination contained half anaphase figures with 3 pg DNA and a whole metaphase figure containing 6 pg, precisely the DNA amounts expected at a stage when the diploid amount is 3 pg DNA per nucleus. Other values were

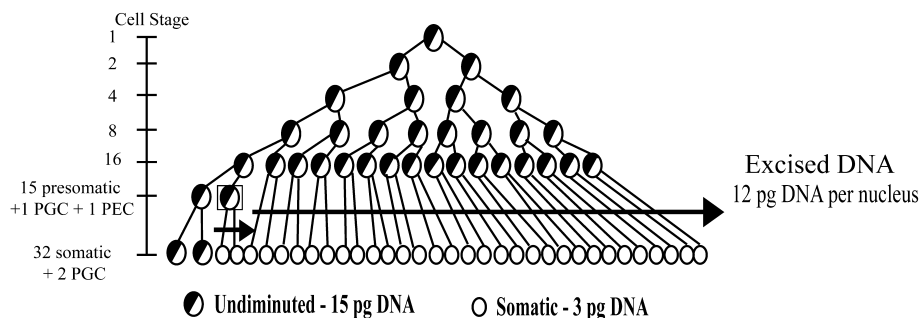


Fig. 1. Quantitative model of embryogenesis from the 1-cell zygote through DNA elimination in *Mesocyclops edax*. Elimination of DNA from 15 presomatic cell lineages occurs early in the fifth cleavage division; the primordial germ cell (PGC) remains undiminuted. A delayed diminution and division in the primordial endoderm cell (PEC, in box) and delayed division of the primordial germ cell (PGC) occurs after the DNA elimination in the 15 presomatic cells.

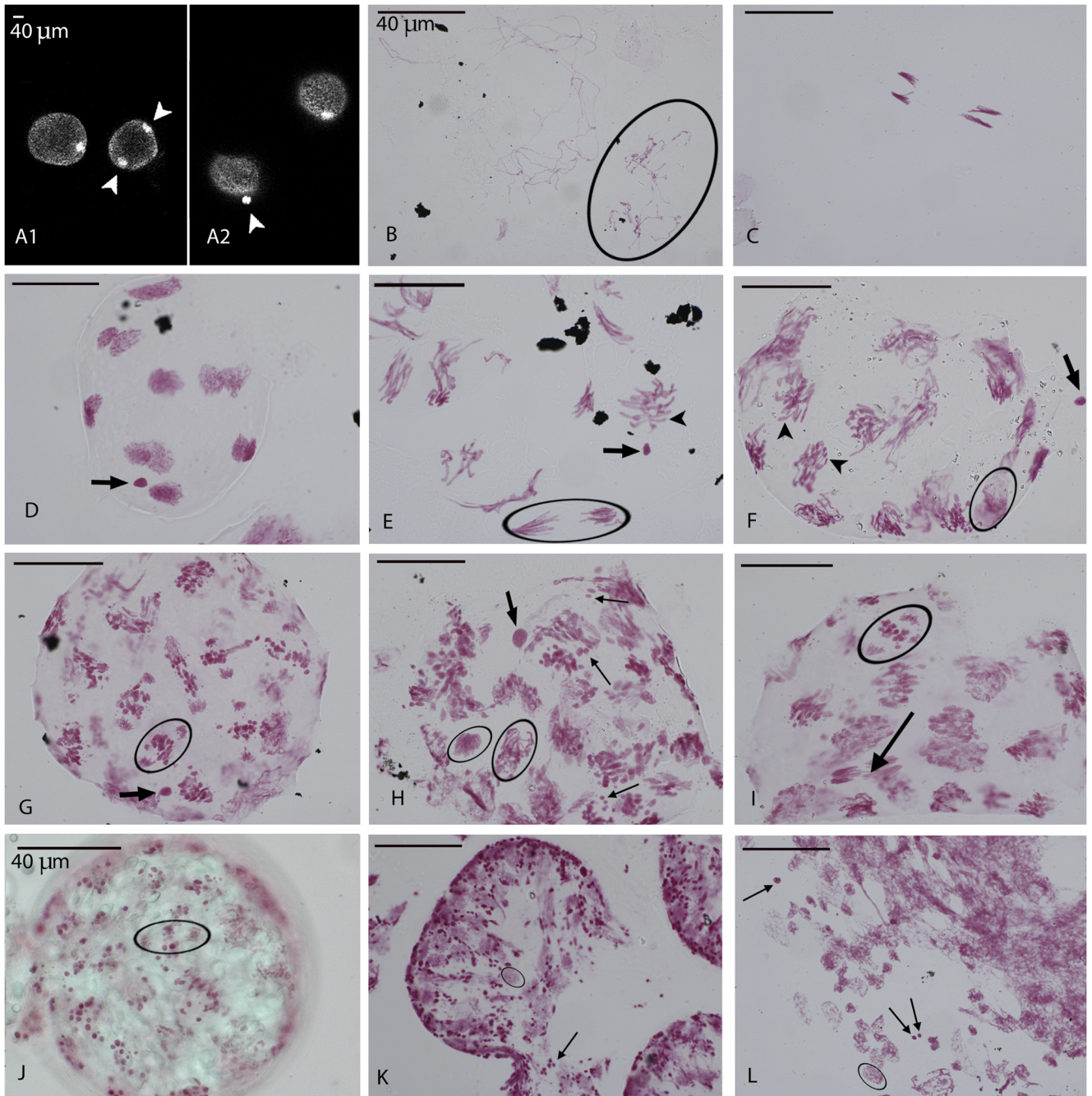


Fig. 2. *Mesocyclops edax* embryos stained with the Feulgen reaction for DNA unless otherwise noted. E-I depict sequential timepoints during the fifth cleavage division in embryos from the same embryo clutch and squash preparation. A1, DAPI-stained eggs, 5 min after egg laying. Male and female pronuclei (arrows) lie at opposite ends of the egg prior to fusion; a single polar body lies near the membrane of the embryo in a different z-stack (not shown); A2, DAPI-stained eggs, two pronuclei (arrow) at a stage immediately following that shown in A1. The pronuclei lie very close to one another just prior to fusion; B, chromosomes of an early 1-cell embryo containing 14.9 pg DNA per nucleus (circle). The chromosomes appear as two distinct sets, which is consistent with the presence of homozygous gonometry; C, a 4-cell embryo showing one of the two homozygous gonometric figures in anaphase; D, early-stage 16-cell embryo immediately after fourth cleavage division with polar body (arrow); E, partial view of 17-cell stage embryo; an asynchronous division of the nucleus gives rise to the PGC and PEC (circled); polar body (arrow); swollen chromosomes of presomatic cells (arrowhead). Black masses are charcoal remaining from staining process; F, 15 presomatic nuclei with dense heterochromatic regions scheduled for excision (arrowheads); the putative PGC and PEC (both in circle) result from the division in E and combined contain 30 pg of DNA; a polar body (arrow); G, fifth cleavage division with 15 presomatic nuclei, one of which is circled, having just undergone DNA elimination; note anaphase figures and the large "masses" of excised DNA remaining at the metaphase plates; H, 32-cell stage with 30 postdiminished nuclei and large and small DNA "droplets" (thin arrows); the undiminished PGC (16.6 pg DNA) (smaller circle), and the undiminished PEC (31.4 pg DNA) (larger circle) display thick heterochromatic regions; thick arrow denotes polar body; I, an intact embryo showing the 15 postdiminished cells, division of the PGC with gonometric chromosomes (arrow) and a chromosomal figure of delayed diminution of the PEC during the late fifth cleavage division (circle); J, embryo stained during the same stage as shown in I; delayed diminution of the PEC (circle); note numerous DNA "droplets"; K, an approx. 64-cell stage embryo with numerous excised DNA "droplets"; somatic nuclei (circle) appear diffuse and more lightly stained than the DNA "droplets" (arrow); L, an approx. 1024-cell stage embryo showing somatic cells (circle) and excised DNA "droplets" (arrows).

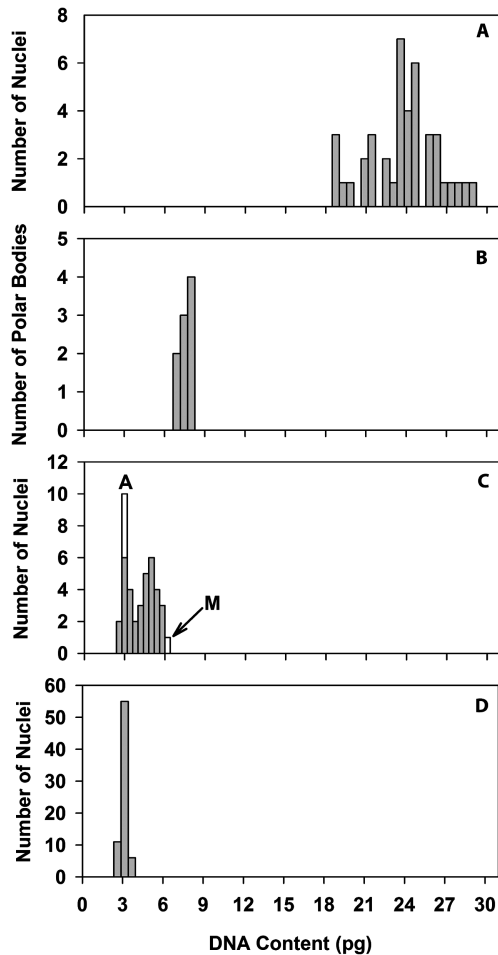


Fig. 3. Nuclear DNA contents of a single representative adult female of *Mesocyclops edax*, her embryos prior to DNA elimination, and their polar bodies are compared with embryos after DNA elimination from two representative females. A, 4-cell embryos prior to DNA elimination; B, polar bodies; C, embryos after DNA elimination at the approx. 128- and approx. 512-cell stages; "A" refers to half anaphases in the white bar and "M" to a metaphase figure in the white bar; D, somatic nuclei in adult female.

between 3 and 6 pg, reflecting different points in the cell cycle; the exceptions are two nuclei containing 2.9 pg, which is within accepted measurement error.

Feulgen-stained polar bodies were recognized by their distinctive morphology (arrows in Fig. 2D-F, H). Some embryos had polar bodies that contained the expected haploid amount of 7.5 pg DNA or amounts approximating this, as was the case illustrated in Fig. 3B. These particular polar bodies were associated with the pre-diminished embryos of a single adult female. Polar bodies from dozens of other embryos, both before and after DNA elimination and from different mothers, had DNA contents that varied from 1.2 to 9.9 pg DNA (Fig. 4).

After the first cleavage division, the stained chromosomes were more condensed and visible (Fig. 2C-J). Densely stained, heterochromatic regions scheduled for excision were visible at the terminal ends of the chromosomes and persisted until the diminution division (Fig. 2E, F, *arrowheads*). The heterochromatic regions scheduled for elimina-

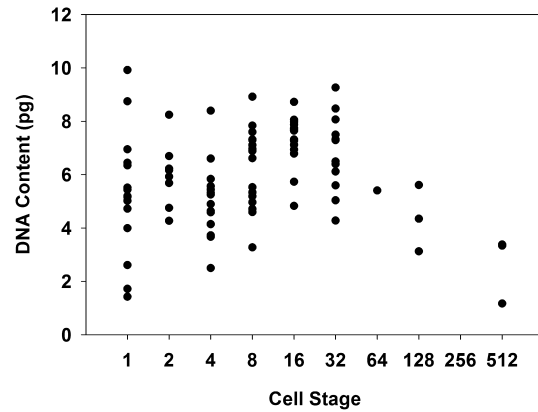


Fig. 4. Nuclear DNA contents in polar bodies of embryos of *Mesocyclops edax*. The number of cells at the approx. 64-cell and subsequent stages are approximated due to asynchronous cell divisions after DNA elimination.

tion were particularly visible in the C-banded chromosomes of an 8-cell stage embryo prior to DNA elimination (Fig. 5).

Median durations among cell stages varied considerably, with the longest duration of 185 min occurring during the 8-cell stage when the molecular machinery for DNA elimination was being assembled (Table 1). Most of the variation in duration of a particular cell stage was caused by variations among clutches from different females.

#### DNA Elimination During the Fifth Cleavage Division

Four distinct division events occurred during the fifth cleavage division (Table 1, Fig. 2E-J). The first is the division of a single cell that produced one primordial germ cell (PGC) and one primordial endoderm cell (PEC) (circle in Figs. 1, 2E). The 15 presomatic nuclei had densely stained heterochromatin in the terminal regions of chromosomes destined for excision (*arrowheads* in Fig. 2E, F; Fig. 5).

During the second event, the DNA elimination in 15 presomatic cells occurred (Table 1, Fig. 2G). The average time from extrusion of meiotic products into the embryo sac and appearance of eliminated DNA at the metaphase plate was 10.3 h at 22°C (Table 2). This estimate of the timing of DNA elimination was obtained by observing egg sacs being laid and removing one egg from the distal portion of each egg sac at 10-15 min intervals between 9.52-10.57 h after egg laying. Each embryo was squashed and stained with orcein to examine for the presence of whole anaphase chro-

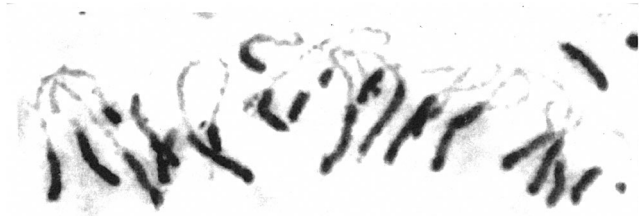


Fig. 5. C-banded chromosomes of an embryo of *Mesocyclops edax* prior to DNA elimination;  $2n = 14$ . Giemsa staining reveals the distribution of constitutive heterochromatin, recognized as densely staining regions, or C-bands, to occur mostly at the terminal ends of chromosomes. Just prior to DNA elimination, these C-bands appear unusually swollen and densely stained relative to the euchromatic regions of the chromosomes during the embryonic cell stage. C-bands mark at a gross scale the chromosomal regions that are scheduled for excision.

Table 2. Timing of DNA elimination and duration of embryogenesis *Mesocyclops edax* at  $22 \pm 0.5^\circ\text{C}$ . Duration of embryogenesis was measured from egg laying to hatching into nauplius I larvae.

Event	Median time (hours)	Number of egg sacs	Number
DNA elimination in 15 presomatic cells	10.30 (10.02-10.33)	6	18 elimination figures
Embryonic duration	58.80 (57.77-61.25)	6	39 embryos

mosome figures with large masses of eliminated DNA at the metaphase plate (e.g., circle in Fig. 2G). Between 10.02-10.33 h, a total of 18 such DNA elimination figures obtained from egg sacs of six females were clearly visible (Table 2). Additional nuclei from these embryos also contained chromosomal figures with eliminated DNA, but we excluded figures that were partially obscured by overlapping DNA material. The median time to DNA elimination among these 18 chromosomal figures was 10.30 h (see Fig. S1 in the Appendix in the online version of this journal, which can be accessed via <http://booksandjournals.brillonline.com/content/journals/1937240x>), reflecting the fact that most DNA elimination figures were observed at 10.30 h. The variable times required to fill an egg sac with meiotic products and the length of the observation intervals influenced the precision of the estimate of the timing of DNA elimination. For example, a single egg sac could contain late stage 17-cell embryos at the proximal end of the sac and early stage 34-cell embryos at the distal end of the sac. Supplemental Fig. S1 (see the Appendix in the online version of this journal, which can be accessed via <http://booksandjournals.brillonline.com/content/journals/1937240x>) illustrates some of the time points at which 1- through 34-cell stages could be observed and could serve as a guide for planning future studies dependent upon harvesting particular cell stages.

Nuclei of embryos from three egg sacs were not included in the above estimate of the timing of DNA elimination. They were observed between 10.17 and 10.25 h after egg laying and were either in metaphase but excised DNA was not yet visible at the metaphase plate, or had progressed to the telophase or interphase stage but still contained large masses of eliminated DNA at the metaphase plate. These data are consistent with the estimated timing of DNA elimination to be within the half-hour window of time between 10.02 and 10.33 h after egg laying.

Just prior to the main elimination event, each of the 15 presomatic nuclei replicated its DNA in preparation for producing 30 somatic cells. The amount of eliminated DNA in the large masses at the metaphase plate of each of the 15 cells that completed DNA elimination was therefore twice the amount excised from each daughter cell. Then each of the two replicated sets of chromosomes excised 12 pg of DNA which resulted in 24 pg of excised DNA at the metaphase plate (Figs. 1, 2G). At this time the excised DNA appeared as large masses. The postdiminished chromosomes retained 3 pg DNA per nucleus each and migrate in anaphase to the opposite pole to form new daughter cells. Metaphase and anaphase figures of somatic cells in postdiminished embryos contained, as expected, 3 pg and 6 pg, respectively (Fig. 3C); amounts between these two values correspond

to different time points in the cell cycle. DNA contents in postdiminished cells should not be expressed in terms of *C* value.

The PEC and PGC remain un-diminished at this time. After the diminution of the 15 presomatic nuclei, the PEC nucleus replicated and contained the expected 4*C* amount of DNA. Densitometric measurement of one such PEC at this stage showed a DNA content of 31.4 pg (large circle in Fig. 2H). Similar to the chromosomes of the 15 presomatic nuclei just prior to diminution (Figs. 2F, 5), densely stained heterochromatic regions appeared at the terminal ends of the chromosomes in the PEC (large circle in Fig. 2H). The nucleus of the PGC contained 16.7 pg DNA, appeared diffuse, and was likely in the process of replicating its DNA (small circle in Fig. 2H).

During the third event the PEC underwent a delayed DNA elimination and excised 12 pg from each of its chromosomal complements as described for each of the 15 presomatic nuclei in the first diminution (Figs. 1; 2I, J, circles). This resulted in two additional somatic nuclei that contained 3 pg of DNA per nucleus. Simultaneously, the fourth event occurred when the PGC, which has gonameric chromosomes, divided to produce two germ cells without excising the DNA (Fig. 1; arrow in Fig. 2I). These germ cells contained the expected diploid amount of 15 pg DNA per nucleus in the germline genome. We noted that the timing of one such division figure of the PGC occurred 10.25 h after egg laying. If this single observation of timing is representative of PGC divisions, we can conclude that the DNA elimination in the 15 presomatic cells and PEC and the division of the PGC all occur within minutes of one another.

These four division events resulted in a 34-cell stage embryo (Table 1, Figs. 1; 2I, J). The embryo was comprised of 32 somatic cells (3 pg DNA per nucleus), 30 of which arose from the first diminution division and two of which arose from the delayed diminution of the PEC, along with 2 undiminished PGCs (15 pg DNA per nucleus). A single morphologically distinct polar body was evident throughout the fifth cleavage division (arrow in Fig. 2E, G, H). After these two diminution events, the large masses of eliminated DNA began to break up into small DNA "droplets" (Fig. 2J).

#### Postdiminution Cleavage Divisions

Asynchrony in somatic cell divisions began with the delayed division of the PEC relative to the division of the 15 presomatic cells during the fifth cleavage division and precluded assigning a precise number of cells to any cell stage after DNA elimination. After the fifth cleavage division, the number of nuclei at any single stage was approximated by counting the nuclei and then assigning a value based on an as-



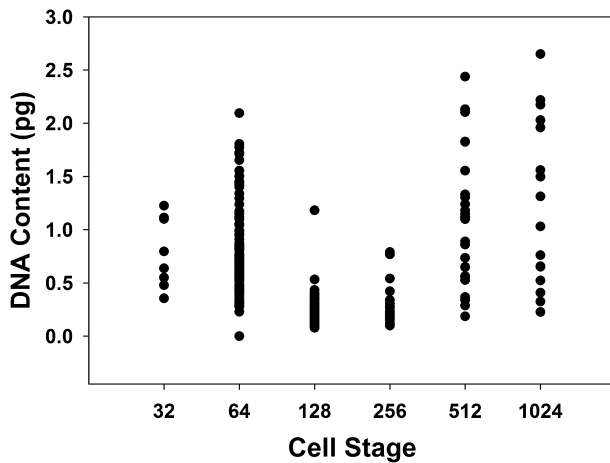


Fig. 6. Nuclear DNA contents of individual excised DNA “droplets” after DNA elimination in *Mesocyclops edax*. The number of cells at each stage are approximate due to asynchronous cell divisions after the DNA elimination at the 16-32 cell stage. Sampling effort among cell stages was not equal and so the number of measurements at each cell stage is not intended to depict a trend in abundance.

sumed doubling of cell number in each cell division. For this reason, the axis labels indicating the number of nuclei after each cleavage division in the graphs that describe the number of polar bodies, DNA contents of excised DNA “droplets,” and the total number of nuclei in the newly hatched nauplius I stage are approximations (Figs. 4, 6, 7B).

Polar bodies were easily identified by their distinctive morphology and persisted until at least the approx. 512-cell stage. The histological preparations at approx. 256-cell stage did not include nuclei that were spread sufficiently apart to accurately identify a polar body. We did not observe polar bodies in embryos after the approx. 512-cell stage, but cannot rule out the possibility that they were obscured by Feulgen-stained nuclei. The sampling effort varied among cell stages and thus no trends in DNA content or number should be inferred from Fig. 4.

Immediately after DNA elimination at the fifth cleavage division, the eliminated DNA appeared as large masses at the metaphase plate (Fig. 2G). The resulting two daughter cells were observed as two sets of half anaphase figures migrating to the opposite poles and each containing 3.0 pg DNA per nucleus, forming the 30 somatic nuclei.

After the fifth cleavage division, the large masses of excised DNA at the metaphase plate were broken down into smaller, dispersed masses, which we refer to as DNA “droplets” of Feulgen-stained material. These droplets were first observed at the late approx. 34-cell and approx. 64-cell stages (Fig. 2J, K) and persisted until at least the approx. 1024-cell stage (Figs. 4, 2L). The DNA “droplets” appeared as condensed, dark ovals scattered throughout the squashed embryos. At the approx. 64-cell stage, the length of these “droplets” varied from 1.07 to 5.61  $\mu\text{m}$  with a mean  $\pm$  SD length of  $3.50 \pm 0.89 \mu\text{m}$  ( $n = 60$ ) in a single embryo. The distinctive morphology and size of the excised DNA “droplets” allowed us to identify them in stages after the fifth cleavage division (Fig. 2J-L). These “droplets” of excised DNA varied in their amounts of DNA both within and among cell stages. DNA contents ranged from 0.1-2.7 pg DNA (Fig. 6). There was no trend in the amount of DNA in each “droplet” among the different cell stages.

The number of DNA “droplets” and their DNA contents at each embryonic cell stage depicted in Fig. 6 should not be interpreted as representative of the population of “droplets” at each stage because as cell number increased, it became increasingly difficult to observe isolated “droplets” suitable for measurement of DNA content (Fig. 2K, L). We believe, however, that the number of DNA “droplets” per nucleus declined over time. What is most notable is that some DNA “droplets” persisted throughout embryogenesis during ontogeny (Fig. 6). We did not observe DNA “droplets” in the newly hatched N1 nauplius larvae either because they were obscured by other Feulgen-stained material or they were absent.

#### Embryonic Development Time

Thirty-nine embryos from the clutches of six females were observed to estimate total embryonic duration. The median time from extrusion of meiotic products into the egg sac until hatching of the egg into a free-swimming nauplius 1 larva was 58.8 h at 22°C (Table 2). Small variations in embryonic duration within a clutch occurred, most likely because some eggs sacs were filled with eggs sequentially within a couple of minutes and others required 20 min. Other sources of variation might be inherent variation among egg sacs laid by different females. The N1 nauplius larva stage had approx. 2048 cells when it hatched from the embryo sac (Fig. 7A, B).

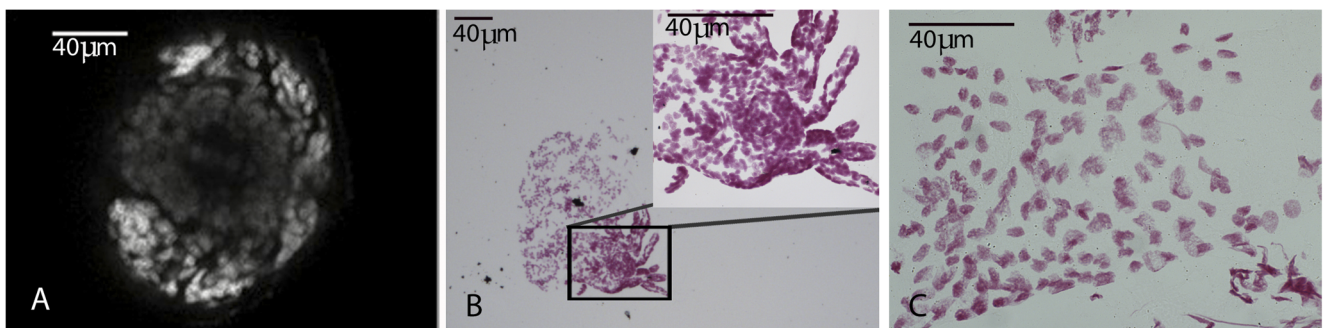


Fig. 7. Nauplius I larva and somatic nuclei of adult female of *Mesocyclops edax*. A, DAPI-stained limb bud stage of intact embryo just prior to hatching, confocal microscopy; B, Feulgen-stained nuclei of entire N1 nauplius larva. The inset in the upper right-hand corner is a magnified view of one of the portions of the larva that was used to enumerate total number nuclei in an individual; C, Feulgen-stained interphase somatic nuclei of adult female typical of those used to measure internal standards.

The nuclei of these cells were easily recognized as the nuclei of adult somatic cells that were used as standards (Fig. 7C).

## DISCUSSION

We used a timetable of early embryonic stages leading up to the DNA elimination event and measurements of the DNA contents throughout embryogenesis to refine the model of DNA elimination (chromatin diminution) proposed by Beermann (1977) and Rasch et al. (2008). DNA elimination in *Mesocyclops edax* spans multiple embryonic stages and confers features at each embryonic stage that are unique to copepods that possess this trait. The timing of the excision of the DNA irrefutably occurs during the fifth cleavage division. We found anaphase figures with large masses of eliminated DNA at the metaphase plate to occur within a half-hour window of time during which the median time was 10.3 h after egg laying at 22°C. Specifying the timing of the key events associated with DNA elimination is necessary to perform mechanistic studies of the DNA elimination process. DNA elimination is also a highly programmed event with remarkably unvarying reductions in DNA amounts in the somatic nuclei within and among embryos. This contrasts markedly with the variable DNA contents of the polar bodies and reveals that polar bodies cannot always be used as a proxy for haploid genome size. The partial breakdown of the large masses of excised DNA into smaller “droplets” that gradually decline in number throughout embryogenesis was observed for the first time in copepods. We hypothesize that the eliminated DNA has been co-opted over evolutionary time to contribute to “fueling” the replication during later embryonic stages, which could explain its adaptive value and persistence in certain species.

### Timetable of Cytological Events

Knowledge of the timing of individual cytological events leading up to the excision of DNA will facilitate molecular studies, such as transcriptomic experiments, that can identify the various RNA transcripts involved in marking the DNA to be excised and assembly of the molecular machinery that excises the DNA. Estimates of the variability in timing of the sequences of events leading up to the DNA elimination within and among embryos will also aid in efficiently designing experiments to study the molecular mechanisms.

The first cytological structures observed in a newly laid egg sac are a polar body and pronuclei. The presence of unfused pronuclei within the first five minutes of the embryo sac being laid is consistent with reports in marine calanoid copepods that fertilization is completed only after the meiotic products are deposited into the embryo sac (Marshall and Orr, 1955; Blades-Eckelbarger and Youngbluth, 1982; Boxshall, 1992). Chromosomes were never visible in newly laid egg sacs of *M. edax* (MKC, GAW, per. obs.) and did not appear in condensed form until the cell was preparing for the first cleavage division, which also agrees with the detailed description of reproduction in *Calanus* (Marshall and Orr, 1955).

Only a single polar body was observed with each embryo from about 5 min after egg laying until at least the approx. 512-cell stage. We cannot eliminate the possibility that a second polar body was present and escaped the cell

membrane within the first few minutes of egg laying. Marshall and Orr (1955) report that two polar bodies are initially present in *Calanus* Leach, 1816 and that one polar body separates from the egg membrane 5 min after egg laying. Polar bodies are present in the marine calanoid *Calanus* at about the 32-cell stage, but the authors make no mention of its presence in later stages (Marshall and Orr, 1955). Overlapping nuclei in squash preparations of the late stage embryos of *M. edax* preclude being certain whether the single remaining polar body persists past the approx. 512-cell stage.

The presence of large masses of eliminated DNA at the metaphase plate during the fifth division is an obvious indication that germline-soma differentiation has occurred. The segregation of the presumptive primordial germ cell from cell lineages destined for the soma likely occurs prior to this cell stage. Whether the PGC has inherited maternal germ plasm containing determinants during the first cleavage division, which seems more likely (Extavour and Akam, 2003; Seydoux and Braun, 2006; Rebscher, 2014), or is specified from an undifferentiated cell by inductive signals in an epigenetic fashion, will need to be tested with markers for maternally supplied mRNAs or their protein products as suggested by Sagawa et al. (2005) in a general discussion of invertebrate germline development.

Homozygous gonomic divisions were observed in the second through fourth cleavage divisions, as previously reported for early cleavage divisions in *M. edax* (Rasch and Wyngaard, 1997) and other cyclopoid copepods that have DNA elimination (Häcker, 1894; Beermann, 1977; Rasch and Wyngaard, 2008). In the case of *Cyclops strenuus* Fischer, 1851, some populations showed heterozygous gonometry and Beermann (1977) assigned the larger amount of DNA to the paternal set. Here we report homozygous gonometry also in the PGC during the fifth cleavage division of the presomatic cell lineage of *M. edax*. Gonometry seems to be associated with the presence of DNA elimination in copepods (Rasch and Wyngaard, 2008). An exception is the marine harpacticoid *Tigriopus californicus* (Baker, 1912). This species lacks DNA elimination (Rasch and Wyngaard, 2006), but also has gonometry, which, however, is restricted to the zygotes (Ar-rushdi, 1963).

The prolonged duration of the cell stage immediately prior to the cleavage division during which DNA elimination occurs has been observed in every species that has DNA elimination and for which such data are available (Beermann, 1977; Leech and Wyngaard, 1996). These prolonged stages can last as long as seven hours in the case of *C. strenuus* (Beermann, 1977) to be useful as an assay for the presence of DNA elimination (Leech and Wyngaard, 1996). During the prolonged cell stage in *M. edax*, the terminal ends of the chromosomes that are scheduled for elimination become swollen, likely reflecting assembly of the molecular machinery for DNA excision.

Knowledge of the timing of DNA elimination and the events leading up to it will significantly increase the efficiency and efficacy of studies aimed at targeting specific molecular mechanisms involved in what is a complex process spanning several cell stages. Chinnappa (1980) reported DNA elimination to occur during the fourth cleavage di-

vision (8 to 16-cell stage) in an Ontario, Canada population of *M. edax*, but provided no information regarding how the timing of DNA elimination was ascertained. Leech and Wyngaard (1996) used Chinnappa's statement in their study, which reported a prolonged duration during the 8-cell stage for *M. edax*, as found in the present study. Efforts to replicate Chinnappa's finding failed to find diminution figures at the fourth cleavage division, leading Rasch et al. (2008) to suggest that the diminution might occur at the fourth or fifth cleavage division. The present study observed dozens of egg sacs and we report that the DNA elimination unequivocally occurs during the fifth cleavage division.

We have identified a half-hour window of time during which DNA elimination occurs in the 15 presomatic cells. DNA elimination in these 15 cells was always observed to be synchronous within an embryo. Embryos within an egg clutch sometimes were present at slightly different cell stages in the elimination process, likely due to their slightly different ages. An embryo sac that was filled over a 20 min period, for example, likely contained embryos of different ages. Future studies that use egg sacs that fill with embryos within only a minute or two and very short observational intervals could further narrow the time window during which DNA is eliminated.

The durations of cell stages and other events described here pertain to this Virginia population of *M. edax* at 22°C. We chose to describe the cytological events at 22°C as it is close to room temperature. Embryonic duration in copepods is largely temperature dependent (Herzig, 1983; McLaren et al., 1989) and so the timetable of development at other temperatures would have to be adjusted accordingly. Genetic differentiation among widely separated geographical populations inhabiting lakes with different trophic states and thermal regimes influences development time from hatching to adult in *M. edax* (see Wyngaard, 1986). Adaptation to different thermal regimes in embryonic duration has yet to be explored.

#### Quantitative Behavior of DNA

The invariable amount of excised DNA in *M. edax*, 12 pg DNA per chromosomal complement, is the same amount reported in previous studies of widely separated geographical populations (Rasch et al., 2006b). This is perhaps the most persuasive line of evidence to date that DNA elimination in *M. edax* is highly programmed.

Several kinds of cells have been used by investigators to estimate the amount of excised DNA. The direct method of measuring the large masses of DNA remaining at the metaphase plate immediately after the DNA is eliminated leaves little question about the amount eliminated. Such measurements were made in *M. edax* and verified by indirect methods that compare nuclear DNA contents of: 1) embryos prior to DNA elimination containing the full germline DNA content with somatic cells of embryos after DNA elimination containing the 2C–4C amounts according to the point in their cell cycle or 2C amounts in adult somatic cells which do not molt, and 2) mature adult germ cells of known 2C or 4C values in oviducts of adult female somatic cells. Both of these indirect methods yield estimates consistent with the direct method (Rasch and Wyngaard, 2008; present study).

An alternative method of estimating the amount of eliminated DNA relies on using sperm DNA contents to define the 1C amount of DNA and comparing this with somatic nuclei after DNA elimination. This is used less often in copepods because spermatophores are rarely found attached to cyclopoid copepods and sperm are sometimes difficult to identify with confidence once deposited into the genital pore. Additionally, sperm nuclei often have DNA compaction, leading to underestimates of DNA content. In *C. strenuus*, for example, Beermann (1977) compared DNA contents in sperm and late telophases in embryos to estimate the amount of eliminated DNA. She reported the 1C value for sperm to be 2.2 pg DNA. Rasch and Wyngaard (2006a) examined a different German population, and reported large germline oocytes in the adult female oviduct to have a DNA content 9.7 pg DNA per nucleus, to which they erroneously assigned a 2C value. We now believe that these oocytes contained the 4C amounts of DNA, in which case the sperm content would be calculated to be 2.4 pg DNA, a value very close to Beermann's measurement of sperm DNA content of 2.2 pg DNA.

Sufficient data have accumulated to put to rest the unconventional model of endoreduplication in the germline described in *M. edax* proposed by Rasch and Wyngaard (2001) and Rasch et al. (2008). It was not known at the time that copepods that have DNA elimination begin DNA replication during meiosis as early as the juvenile CIV stage. They mistook the increasing DNA contents and absence of mitoses in germ cells during the juvenile copepodid stages as endoreduplication, rather than the replication that occurs from 2C to 4C during meiosis. This unusually early and prolonged timing of meiosis contrasts with the typical timing of gametogenesis in copepods that lack DNA elimination, exemplified by *Macrocyclops albidus* (Jurine, 1820), which contains the entire sequence of DNA replication during meiosis within the adult stage (Rasch and Wyngaard, 2006a).

We have no definitive measurements of sperm DNA content in *M. edax*, but note that Rasch and Wyngaard (2001) assigned a value of 3.0 pg DNA to what they called "putative" sperm found in the genital segment of *M. edax*. This unexpectedly low estimate of sperm DNA content would require a diminution during spermiogenesis and unequal contributions of DNA by males and female to the embryo, two rather extraordinary and unlikely events. Nevertheless, in the absence of material from sperm or early embryonic cleavage divisions, the DNA contents of 4C oocytes in adult female copepods, can be compared with adult somatic DNA contents to infer the presence and amount of DNA elimination. Such an approach would be useful in exploratory surveys of the phenomenon and would avoid the necessity of laboratory culture. Perhaps new applications of confocal microscopy to reproductive systems in copepods will enable sperm and other germ cells to be more confidently recognized (Fitzer et al., 2012).

Amounts of excised DNA per chromosomal complement in cyclopoid copepod show extraordinary variation. Approximately 1 pg in *Paracyclops affinis* (G. O. Sars, 1863) now accepted as *Ectocyclops affinis* (G. O. Sars, 1863) and three species of *Cyclops* O. F. Müller, 1776 (Beermann, 1977; Grishanin et al., 2004), 5 pg in *Mesocyclops longise-*

*tus* (Thiébaud, 1912) and *Metacyclops mendocinus* (Wierzejski, 1892) (Rasch and Wyngaard, 2006b), and 74 pg in *C. kolensis* (see Wyngaard et al., 2011) of DNA are excised from each chromosomal complement. Yet, the adult 2C somatic DNA contents are all within the range of 1-2 pg of DNA per nucleus. This considerable variation among species suggests an interesting evolutionary dynamic in either increases or decreases in the amounts of DNA responsible for the different germline genome sizes. Transposable elements (TEs) and DNA repeats with sequence complexity account for much of the excised DNA in the predominate genome in *M. edax* (Sun et al., 2014). To the extent that the TEs are active, it is reasonable to hypothesize that their replication and movement explains the variation in distribution patterns of heterochromatin on the chromosomes before DNA elimination and in germline genome size. The broadest survey of the presence of DNA elimination in any genus of copepod is that of *Cyclops* by Einsle (1996b) who observed DNA elimination in all of the approximately one dozen species he examined. It is not known whether germline genome size shows any trends over evolutionary time.

An often overlooked example of DNA elimination in copepods is the report by Robins and McLaren (1982) in which DNA elimination was observed just prior to the fusion of pronuclei in a species which then was of uncertain identity, but now is believed to be *Pseudocalanus acuspes* (Giesbrecht, 1881) (I. A. McLaren, per. comm.). It is noteworthy to note that *P. acuspes* has one of the smaller adult somatic DNA contents among marine calanoids, approx. 7 pg DNA per nucleus, which have diploid somatic genome sizes as high as approx. 24 pg DNA per nucleus (McLaren et al., 1989; www.rgregory@genomesize.com).

One unexpected finding related to DNA amounts concerns the nuclear DNA contents of polar bodies that sometimes contained the haploid amount of DNA, but more often did not. This suggests that caution must be used when using polar-body DNA content to estimate the haploid size of the germline genome, and hence the amount of eliminated DNA. Semishen et al. (2011) was fortunate in obtaining numerous measurements of polar bodies in a Russian population of *C. insignis*, all of which had the expected haploid germline genome size based upon metaphase and anaphase figures of chromosomes prior to DNA elimination. Much remains to be elucidated about this byproduct of oocyte meiotic division (Schmerler and Wessel, 2011).

#### Genome Yolk Hypothesis

The breakdown of the large masses of eliminated DNA at the metaphase plate into smaller DNA “droplets” that appeared dispersed throughout the nucleus and which persisted throughout embryogenesis is perhaps the most intriguing finding of this study. These DNA “droplets,” called granules, have also been observed in *C. strenuus* and contained between 0.5 and 1.5 pg DNA (Grishanin et al., 1994). No mention of the persistence of these granules throughout embryogenesis was noted. These granules and the DNA “droplets” described in the present study might be the same or products of the “chromatin rings” described in *Cyclops furcifer* Claus, 1893 by Beermann and Meyer (1980). Using electron microscopy, these authors observed numerous loop-forming

rings made up of 250-200 Å fibers with typical lengths between 0.5 and 2.5 μm. They interpreted these fibers to be excised heterochromatic chromosome segments of intact DNA and noted the similarity to chromatin ring formation in the ciliate *Stylonychia mytilus* (O. F. Müller, 1773), which also undergoes DNA elimination (Meyer and Lipps, 1980). While the present study does not present quantitative data on the numbers of DNA “droplets” from the time of DNA elimination to hatching of the nauplius N1 larvae in *M. edax*, we believe our increased difficulty in locating DNA “droplets” in later cell stages reflected a real decrease in abundance and was not solely due to increased incidence of overlapping Feulgen-stained cells. We propose that the eliminated DNA could serve as a resource of nucleotides or their constituents, particularly nitrogen and phosphorus, throughout embryonic development. Fitness in copepods subject to intense invertebrate and vertebrate predation is maximized by reduced age at first reproduction. In much the way that adult female copepods sequester lipids which they supply to their embryos (Brett and Müller-Navarra, 1997; Lee et al., 2006), copepods with expanded germline genomes might sequester DNA to be used by embryos for replication and protein synthesis. DNA elimination might contribute to the unusually wide distribution of *M. edax* compared to other freshwater planktonic cyclopoids. Development times and clutch sizes in *M. edax* are significantly reduced in lakes with low or medium-level trophy (Wyngaard, 1986), yet this species is abundant in many lakes regardless of trophic state. Sequestration of nucleotides could be especially important in species such as *Cyclops kolensis* that sometimes inhabit lakes so depauperate in nutrients that they require multiple years to mature and are the only crustacean in the zooplankton community, as proposed by Wyngaard et al. (2011). The approx. 150 pg of DNA remaining at the metaphase plate in each DNA elimination figure of *C. kolensis* is in fact sufficient to complete the replication of somatic cells until hatching into a nauplius I larva. Such co-option of a novel DNA elimination process might explain its persistence over evolutionary time in some species.

#### *Mesocyclops edax* as a Model Organism for Studying DNA Elimination

*Mesocyclops edax* is a widely distributed, warm-water species that inhabits permanent lakes from southern Canada to Central America (Gutiérrez-Aguirre and Suárez-Morales, 2001). It can be cultured in the laboratory under defined conditions and has egg sacs containing 20-50 embryos, according to laboratory and field conditions (Wyngaard and Chinnappa, 1982; Wyngaard, 1986). Synchrony in the timing of DNA elimination facilitates collection of pure extracts of germline and somatic DNA and RNA. The well-studied population biology of copepods affords the opportunity to discover any adaptive significance of DNA elimination that is related to its ecology. What is an advantage for cytological study, its enormous germline size and amount of eliminated DNA, is currently also a disadvantage to its study at the genomic level, as tools are lacking to sequence and assemble large germline genomes replete with DNA sequence repeats and transposable elements. When these challenges are overcome, there will be the possibility to perform manipulative studies that reveal the molecular mechanisms respon-

sible for this intriguing example of genome reorganization. Organisms that excise high copy numbers of transposable elements and other repeat sequences that are often the classes of DNA responsible for variation in eukaryotic genome size might be key to understanding genome stability, modulation of genome size, and dynamics of repeat elements (Gregory, 2005; Kidwell, 2005; Feschotte and Pritham, 2006; Sun et al., 2014; Elliott and Gregory, 2015). We hope that this study propels forward not only studies of DNA elimination in *M. edax*, but also other copepods, especially those with bioinformatically tractable germline genomes.

#### CONTRIBUTIONS BY AUTHORS

MCK and ASH contributed equally to collection of data and preparation of manuscript.

GAW planned the work and co-wrote the manuscript with MKC, ASH, and RT. RT discovered the persistence of DNA “droplets.” MKC, ASH, and RT collected material and measured DNA contents. ASH measured timing of cytological events; MKC and ASH performed data analyses.

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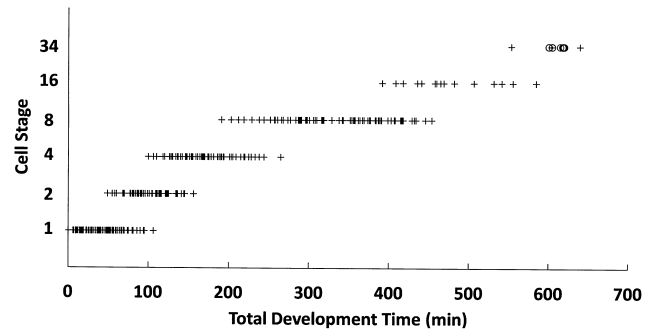


Fig. S1. Time since egg laying at which particular cell stages of *Mesocyclops edax* were observed. This figure includes some of the data in Table 1 and provides a guide to the investigator as to the range of times a particular cell stage can be found and harvested for study. Cell stage duration should not be inferred from this graph.