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Analysis of nucleic acids in *Daphnia*: development of methods and ontogenetic variations in RNA-DNA content

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New sensitive fluorochromes, PicoGreen and RiboGreen (Molecular Probes, Inc.), were used to detect ng ml^{-1} concentrations of nucleic acids (RNA and DNA) in single Daphnia individuals of all developmental stages. Nucleic acids were assayed by fluorescence with a microplate reader using (i) RiboGreen (non-specific dye) and PicoGreen (DNA-specific dye) together and (ii) RiboGreen with and without RNase. The two methods yield similar values for DNA and RNA. The second method has the advantage of determining both RNA and DNA within a single sample aliquot, allowing maximum use of available sample. In parthenogenically reproducing Daphnia pulex, RNA and DNA concentrations were related to changes in metabolic activity associated with moulting cycle and ontogenetic development. In juveniles, elevated DNA content was observed in the early postmoult, followed by an increase in RNA during intermoult and premoult. RNA concentration peaked at the latest stages of embryonic development, followed by a gradual decline during juvenile development, with the lowest values in adult females (eggs removed). Similarly, DNA concentrations were highest during the early phase of postnatal development, decreasing when body size increased. Our results suggest that ontogenetic variations have implications for the use of nucleic acids as a measure for growth in zooplankton and may provide useful insights into mechanisms of growth on the cellular level.

INTRODUCTION

Estimation of RNA concentration or total RNA content is an increasingly widespread method for assessing growth and production rate of an organism in both field and laboratory settings. The basis for an overall association between growth and RNA concentration is: (i) RNA makes up 50–60% of the ribosome, the machine of protein synthesis and subsequent cell growth (Becker, 1986), and (ii) this ribosomal RNA (rRNA) comprises 80–90% of the total cellular RNA, with increasing total allocation at high growth rate (Alberts *et al.*, 1983). Since the amount of DNA per nucleus is quasi-constant and each nucleus corresponds to one cell, RNA levels may be normalized through division with the DNA concentration. Thus, the RNA : DNA ratio reflects the level of metabolic activity and can be used as an index of both nutritional status and growth rates of an organism. Also, other ratios, such as RNA : protein, protein : RNA : DNA, protein : DNA, carbon : DNA, DNA : carbon, etc., are used to provide more complete information about cell multiplication and cell enlargement (Bergeron 1997).

To date, a number of studies in ecology, toxicology, aquaculture and fisheries have used this approach to assess the growth status of different organisms, including fish [see reviews (Bergeron, 1997; Buckley *et al.*, 1999)], decapods (Juinio and Cobb, 1994), mysids (Båmsted, 1983), copepods (Båmsted and Skjoldal, 1976; Ota and Landry, 1984; Saiz *et al.*, 1998; Wagner *et al.*, 1998), *Daphnia* (McKee and Knowles, 1987; Barber *et al.*, 1994), molluscs (Wright and Hetzel, 1985; Dahlhoff and Menge, 1996), cephalopods (Clarke *et al.*, 1989), chaetognaths (Båmsted, 1983), cnidarians (Buckley *et al.*, 1999), phytoplankton (Dortch *et al.*, 1983), and bacteria (Kato, 1994; Massana *et al.*, 1998). These studies have shown that RNA-based indices and growth rates can co-vary in response to environmental variability, particularly temperature (Saiz *et al.*, 1998), food availability (Wright and Hetzel, 1985; Malloy and Targett, 1994; Wagner *et al.*, 1998), and toxicants (McKee and Knowles, 1986).

Although the method has been tested on a variety of species, estimation of growth rates based on RNA determination has had variable success, particularly when used on wild populations of crustaceans (Båmsted, 1983; McKee and Knowles, 1989). In these animals, a major problem with interpreting variations in RNA and DNA concentrations is the individual variability related to instantaneous growth status and ontogenetic development. Indeed, ontogenetic variations of DNA and RNA concentrations are expected and have been observed in many arthropods, including insects (Church and Robertson, 1966; McKee and Knowles, 1989), cladocerans (McKee and Knowles, 1987), amphipods (Chandran and Michael, 1968), copepods (Båmsted and Skjoldal, 1976), euphausiids (Skjoldal and Båmsted, 1976), and decapods (Sulkin *et al.*, 1975). Moreover, nucleic acid concentrations are affected by moulting hormones that may stimulate the synthesis of both RNA (Gorell and Gilbert, 1971) and DNA (Freeman *et al.*, 1983), resulting in different concentrations in individuals of the same size and same developmental stage. In particular, previous laboratory studies have revealed that a positive correlation between RNA concentration and growth was limited to specific life stage (Dagg and Littlepage, 1972; Anger and Hirche, 1990), moult stage (Sulkin *et al.*, 1975; Anger and Hirche, 1990; Juinio *et al.*, 1992), or culture conditions (Ota and Landry, 1984). Thus, there are several possible reasons why applying this method to the study of zooplankton in nature has been difficult. These include: (i) working with mixed populations of different species or demographically heterogeneous samples, (ii) ignoring the moult-cycle-related dynamics of RNA synthesis, (iii) insufficient calibration with independent estimates of growth rates under controlled laboratory conditions, and (iv) use of low-sensitivity biochemical methods. Therefore, an investigation of the basic physiological dynamics of nucleic acids during specific life stages, moult cycle, and in relation to ambient environmental conditions is a prerequisite for a use of nucleic acids as biochemical markers of growth in field and laboratory studies in crustaceans. Also, use of a well-defined material, preferably a single individual, would decrease the contributions of individual variation and therefore increase the predictive capacity of the method.

Current fluorometric procedures for RNA and DNA determination are based on the binding of fluorescing compounds (e.g. ethidium bromide, Hoechsht H3358) to nucleic acids. Total sample fluorescence is usually partitioned into RNA and DNA components either by use of DNA-specific and non-specific fluorophores or by use of a non-specific fluorophore in combination with digestion of RNA by ribonuclease. The recent development of highly sensitive microplate fluorometric assays has allowed the analysis of individual copepods (Wagner *et al.*, 1998). Currently, a suite of new sensitive dyes is available, including a non-specific dye (RiboGreen, Molecular Probes, Inc.) and a DNA-specific dye (PicoGreen, Molecular Probes, Inc.) that allow detection of as little as 1 ng ml^{-1} of a nucleic acid, surpassing the sensitivity achieved with ethidium bromide by 200-fold (Singer *et al.*, 1997; Jones *et al.*, 1998). This greatly facilitates fluorometric measurements of individual zooplankters when quantities of sample material are limited.

The aims of this study were: (i) to develop further sensitive fluorometric methods for nucleic acid measurements in small zooplankton species using these new fluorochromes, and (ii) to follow RNA and DNA dynamics during the ontogenetic development and moulting cycle of planktonic crustaceans using a laboratory clone of *Daphnia pulex*.

METHOD

Daphnia cultures and test samples

Stock specimens of an obligately apomictic clone of *Daphnia pulex* were obtained from wild populations from Lake 110 in the Experimental Lakes Area (Ontario, Canada) and were maintained in the laboratory for about 1 year. In life-table experiments, females were held individually in polyethylene jars containing 100 ml of zooplankton COMBO medium (Kilham *et al.*, 1998) and fed chemostat-grown *Scenedesmus acutus* (2.2–2.7 mg C l^{-1}, C : N : P ~ 120 : 18 : 1, by atoms) *ad libitum*. Culture medium and food were replaced every other day and all jars were kept at 21 ± 2°C with an ambient light : dark cycle. All neonates were removed within 24 h of their release and grown under identical conditions. To follow ontogenetic changes in RNA and DNA levels, test samples included: (i) eggs and embryos of different stages (E1 to E5), (ii) four juvenile instars (J1 to J4), and (iii) the first five adult instars [from A1 (primaparae) to A5]. Only individuals originated from third and later broods were used for analyses. In these broods, animals were checked daily for exuvia and released neonates to determine instar number and, for each respective instar, individuals from the same brood were taken in five to seven replicates. Only females from which freshly deposited eggs were removed from the brood chamber were used for both weight estimates and nucleic acid analysis. Eggs and embryos were staged according to the criteria of Threlkeld (Threlkeld, 1979) for embryo-age evaluation; they were gently flushed out from females following the technique of Glazier (Glazier, 1992), and handled separately. To analyse moult-related changes in nucleic acid concentrations, daphniids were moult-staged using morphological criteria for determining hypodermal stages in *Daphnia* (Willey and Threlkeld,

1995). Here, four moult stages are denoted as MS1 to MS4, corresponding to stages 1 to 4 defined by Willey and Threlkeld (Willey and Threlkeld, 1995). As ovarian development synchronized with the moulting cycle may affect both RNA and DNA levels (Gorell and Gilbert, 1971; Freeman *et al.*, 1983), only J3 were used. Moult stages were determined for three randomly chosen individuals originating from the same brood and all the remaining siblings were assumed to be in the same stage. From these siblings, five to seven animals were chosen for nucleic acid assay. Therefore, to represent four moult stages during the intermoult period, four independent broods were needed.

Weight measurements

Individual dry weight (DW, mg) was determined according to the length–weight regressions developed for this particular clone: $DW = 0.007*BL^{2.23}$ ($r^2 = 0.92$) for juveniles and $DW = 0.0084*BL^{2.58}$ ($r^2 = 0.90$) for adults. To establish this length–dry weight regression, a body length from the top of the head to the base of the spine (BL, mm) was measured under stereomicroscope (± 0.025 mm), then the animal was transferred with forceps into a pre-weighed foil boat, dried at 60°C overnight, and weighed on a microbalance (± 1 μg). Sample size varied from 10–15 individuals for neonates to a single individual for adult females. Clutches at different developmental stages were processed separately, dried, and the mean egg/embryo mass was calculated as dry weight of the clutch divided by the number of eggs/embryos.

Sample preparation for nucleic acid assay

We first examined the effects of different preservation methods on RNA-DNA determinations using neonates (J1) and third juvenile instars (J3). Two preservation methods were used: (i) deep freezing, when *Daphnia* was placed alive in a nuclease-free Eppendorf tube and immediately into a –80°C freezer; and (ii) preservation with RNA*later* (RNA preservative, Ambion), when *Daphnia* was placed in an Eppendorf tube containing 25 μl of RNA*later*, incubated for 24 h at 4°C, and stored at –20°C until analysis. The results obtained when using these preservation methods were compared with control samples that contained fresh animals of the same life stage. As we found no significant differences between fresh and preserved samples (see 'Results' section), a single individual was processed fresh or preserved depending on the experimental design. Prior to extraction (when fresh animals were used) or preservation, a body length was measured under stereomicroscope and individual dry weight (DW, mg) was determined according to the length–weight regression. When analysing eggs and embryos, they were transferred to a drop of nuclease-free water and consequently pipetted into an Eppendorf tube containing extraction buffer. Care was taken to minimize the volume of water introduced in a sample and it never exceeded 4 μl.

Nucleic acid analysis

Methods for quantification of nucleic acids largely followed those of Bentle *et al.* and Wagner *et al.* (Bentle *et al.*, 1981; Wagner *et al.*, 1998), although a number of modifications were necessary, as described in the following paragraphs.

Working reagents

RNA*later*™ (Ambion, cat. # 7020); RNA (Type III: from Bakers Yeast, Sigma, cat. # R-7125); DNA (calf thymus, Sigma, cat. # D-1501); RNase, DNase-free (Boehringer Mannheim, cat. # 1579681), 50 Kunitz U ml^{-1} in TE buffer; *N*-lauroysarcosine (sarcosyl, Sigma, cat. # L-5125); RiboGreen™ RNA Quantitation Kit (Molecular Probes, cat. # R-11490); PicoGreen™ dsDNA Quantitation Reagent (Molecular Probes, cat. # P-7581); distilled water, DNAase, RNAse free (Gibco, Life Technologies, cat. # 10977–015) was used in all solutions. Working solutions of the dyes were prepared daily according to the manufacturers' instructions and stored in darkness at 4°C until analysis.

Buffers

TE buffer was RNase-free from RiboGreen™ RNA Quantitation Kit; the extraction buffer was 1% w/v sarcosyl in TE buffer; and standard buffer was 0.2% sarcosyl in TE buffer.

Standards

RNA and DNA standard sets were prepared from frozen (–20°C), aliquoted stock. Working solutions were diluted in standard buffer in concentrations ranging from 0.25 to 2.0 μg ml^{-1} for RNA and from 0.02 to 0.65 μg ml^{-1} for DNA. The standard sets were divided into aliquots and stored at –20°C until analysis.

Controls

Negative controls (two or three replicates) containing all chemicals but no *Daphnia* were included in every set of samples and processed in the same way.

Extraction procedure

Daphnia were extracted in one of two ways. If analysed fresh, a single individual was placed with forceps in 1.5 ml Eppendorf with extraction buffer (buffer volume was determined according to Table I). When analysing preserved samples, extraction buffer was added directly to the tubes containing either frozen *Daphnia* or frozen *Daphnia* in

Table I: Daphnia life stages, body size as length (BL, mm) and weight (DW, μg), and corresponding volumes (μl) of extraction buffer (ExB) and TE buffer for nucleic acid extraction

Instars	BL (mm)	DW (μg)	ExB (μl)	TE (μl)	Total (μl)
E1–E5; J1–J3	0.5–1.0	2–7	50	250	300
J4–A1	1.2–1.5	10–24	100	500	600
A2–A4	1.7–2.2	33–60	200	1000	1200
A4–A5	2.2–2.5	60–90	300	1500	1800

RNA*later*. Extraction of either fresh or preserved samples followed the same technique after this step. Samples were shaken at room temperature on a multiple vial head for 1.5 h then diluted 1 : 4 with Tris buffer (see Table I), and shaken for an additional 15 min. At this point, the samples could be either analysed immediately or stored at 4°C overnight.

Fluorometric determinations

Fluorescence measurements were performed using fluorometer FL-800T (BioTek, microplate reader, filters: 485/30 for excitation and 528/20 for emission) and black solid flat-bottom microplates (Corning). Staining with Ribogreen followed by RNase digestion was used to estimate RNA by calculating the difference between initial and remaining sample fluorescence. From this difference, RNA concentrations in the samples were calculated based on the RiboGreen standard curve against known RNA concentrations. Two methods were used to measure DNA in extracted samples after RNase pre-treatment: (i) from the remaining fluorescence in PicoGreen-treated samples using a PicoGreen standard curve against known DNA concentrations (two-dye protocol) and (ii) from the remaining fluorescence in RiboGreen-treated samples using a RiboGreen standard curve against known DNA concentrations (one-dye protocol). When DNA was measured with PicoGreen, each plate included (i) extracted samples and controls, four to six replicates, 75 μl well^{-1}; (ii) RNA and DNA standards, two to four replicates, 75 μl well^{-1}. Half of the replicates of each sample/control were designated for RNA determination (hereafter RNA samples/controls) and half for DNA determination (hereafter DNA samples/controls). RNA samples, RNA controls, and RNA standards received 75 μl well^{-1} of RiboGreen followed by 5 min incubation in darkness. Then the plate was read on the fluorometer with sensitivity at 60 (RNA scan #1). After the first scan, 7 μl well^{-1} of RNase working solution was added and the plate was incubated for 20 min in darkness. At the end of the incubation, 75 μl well^{-1} of PicoGreen were added to the DNA samples, DNA controls, and DNA standards and the plate was incubated for another 5 min. The plate was scanned again at sensitivity 60 (RNA scan #2) and 70 (DNA scan).

When DNA was estimated from the RiboGreen-treated samples, the same sample replicates were used for both RNA and DNA estimates as sample fluorescence remaining after RNA digestion was assigned to DNA–RiboGreen complexes. Instead of PicoGreen, RiboGreen was added into DNA standards and a DNA–RiboGreen standard curve was used to calculate DNA values. The entire procedure was as follows: (i) samples, controls and standards (both RNA and DNA) were treated with RiboGreen, (ii) the plate was scanned with sensitivity at 60 (for RNA, scan #1), (iii) RNase was added and the plate was incubated for 25 min, (iv) the plate was scanned again with sensitivities at 60 (for RNA, scan #2) and 70 (DNA scan).

In both methods, RNA concentrations in the samples were calculated according to RNA standard curves (RNA scan #1) using the difference in fluorescence between RNA scan #1 and RNA scan #2. DNA concentrations were calculated according to the respective DNA standard curves (DNA–PicoGreen for the first method and DNA–RiboGreen for the second) and sample fluorescence from the DNA scan. RNase solution was found to increase the background fluorescence somewhat and this increase was factored into the calculations.

Recoveries were determined by spiking 10 subsamples (five for RNA and five for DNA) of *Daphnia* homogenate that was prepared as follows: 10–15 animals were crushed in 50 μl of water. The mixture was centrifuged, the supernatant was aliquoted, and a 5 μl aliquot was used as a subsample in each case. In addition, RNA and DNA standard stocks were added to this homogenate at concentrations of half the expected values for the homogenate. The final yields of internal standard RNA and DNA were high, i.e. 96.2 ± 2.3% and 101.2 ± 3.5%, respectively. We therefore

did not correct concentrations in the samples for per cent recovery of internal standards.

Statistics

Statistical tests were performed with GRAPHPAD PRISM 3.0 (GraphPad Software). Significant outliers were detected using Grubb's test and removed from calculations. The length–weight relationships were determined on non-transformed data by regression analyses. Significant differences between regression lines within a given category were analysed by testing slopes for equality by analysis of variance (ANOVA) and then by testing elevations of lines with homogeneous slopes for equality by analysis of covariance (ANCOVA). When comparing two groups, an unpaired *t*-test was applied followed by an *F* test to compare variances. Comparisons of three and more groups were performed with a one-way ANOVA with Bartlett's test for equal variances followed by Bonferroni's Multiple Comparison Test. Unless specified otherwise, data are presented as means along with standard deviations.

RESULTS

Methodological tests

Performance of RNA and DNA standards was similar to those described by manufacturer (Singer *et al.*, 1997; Jones *et al.*, 1998). Three standards were regularly measured and used: RNA with RiboGreen, DNA with RiboGreen, and DNA with PicoGreen. Typical RiboGreen and PicoGreen measurements with standard calf thymus DNA and yeast RNA are shown in Figure 1. It is clear that the DNA–RiboGreen reaction is at least three times more sensitive than the DNA–PicoGreen reaction. Within the RiboGreen reactions, the reaction with DNA proved to be more sensitive than that with RNA. All standard curves demonstrated a high level of accuracy ($r^2 \geq 0.98$). Internal sample fluorescence was always below the detection limit while negative controls were not significantly different from the blanks (Unpaired t test, $F = 1.181$, d.f. = 9, two-tailed $P < 0.87$). Using the assay procedure described above in combination with a fluorescence microplate reader, we were able to detect as little as 8 ng ml^{-1} (0.6 ng well^{-1}) of DNA and 280 ng ml^{-1} (20 ng well^{-1}) of RNA extracted from a single *Daphnia* egg.

DNA measurements made with PicoGreen and RiboGreen were compared using *Daphnia* individuals with different body sizes (Figure 2). On average, DNA concentrations were 7.2% higher when measured with PicoGreen

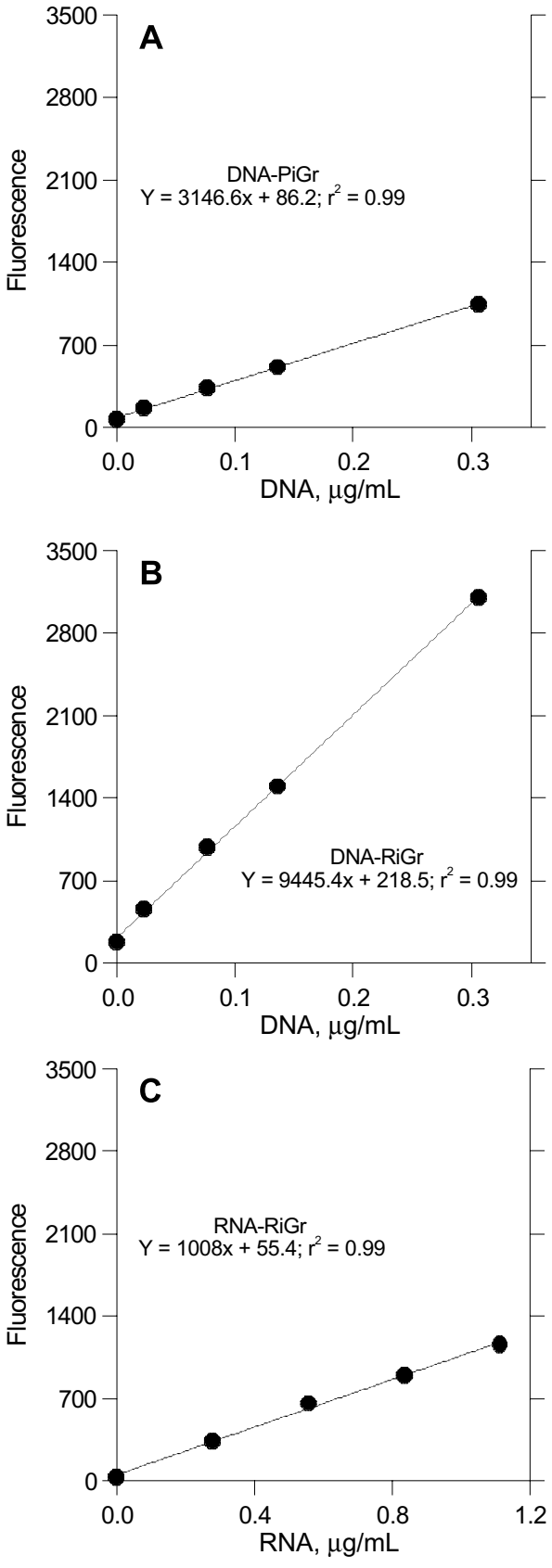

Fig. 1. Calibration lines for PicoGreen–DNA (A), RiboGreen–DNA (B), and RiboGreen–RNA (C).

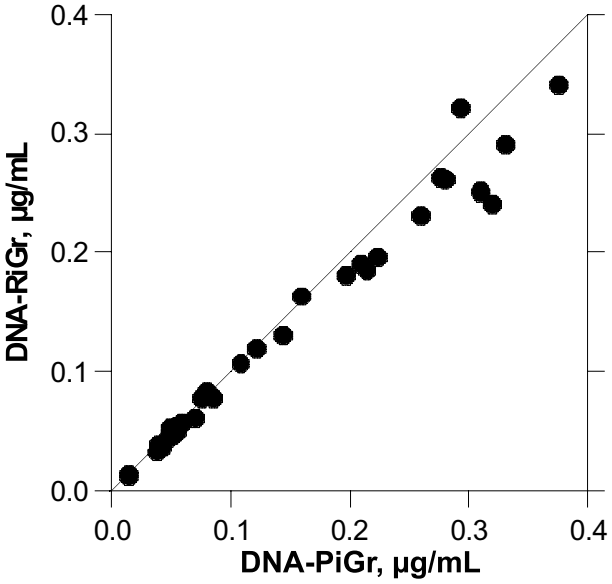

Fig. 2. Comparison of DNA concentration (µg ml^{-1}) measurements using PicoGreen and Ribogreen. The line represents 1:1 ratio.

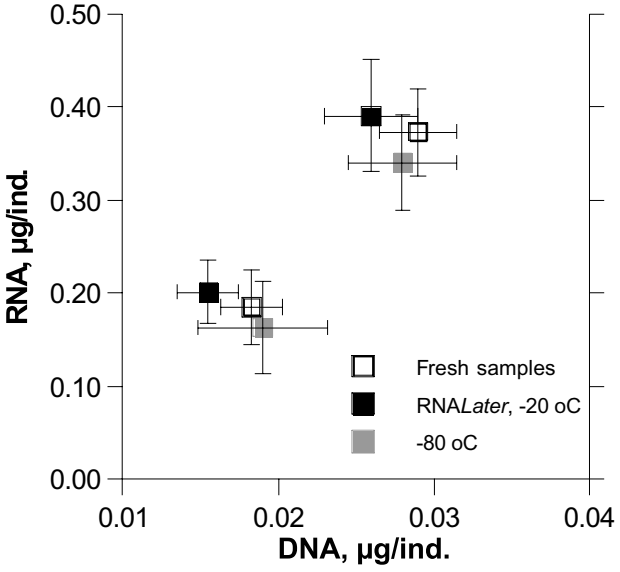

Fig. 3. Effect of preservation methods on RNA and DNA measurements (µg ind.$^{-1}$) in juvenile instars J1 and J3. See Method section for explanations.

relative to measurements with RiboGreen. However, at low DNA concentrations (< 0.1 µg ml^{-1}, i.e. in the samples containing juveniles), this difference was only 6.1%. Higher DNA levels in the samples with large females produced greater differences, with average value of 12.4%.

Relative to fresh samples, preservation of material in RNA*Later* with subsequent storing in freezer at –20°C until analysis as well as immediate freezing of the samples at –80°C did not cause any detectable differences in the measurements of both RNA (two-way ANOVA, $P < 0.39$, $F = 0.9695$) and DNA ($P < 0.5662$, $F = 0.5837$). As there were no significant differences found between preservation methods tested (Figure 3), all data obtained using either fresh animals or preserved with any of these methods were pooled for further analyses.

Nucleic acid dynamics during the moulting cycle

Under these experimental conditions, the duration of the third instar was approximately 31 h. There was a general increase in both RNA (46.3%) and DNA (30.8%) content during the moulting cycle (Figure 4). During the first third of the intermoult period (IMP), DNA levels increased significantly by about 20% from MS1 to MS2 (unpaired t test, $F = 1.757$, d.f. = 12, one-tailed $P < 0.05$). No significant further changes were observed until MS4 (one-way ANOVA, $P > 0.3$). With a slight general increase during MS3 and MS4, the average total DNA levelled off at approximately 0.034 µg ind.$^{-1}$. In contrast, RNA content during the postmoult period was relatively constant and demonstrated a significant increase only from MS2 to MS4 (Unpaired t test, $F = 3.870$, d.f. = 10, two-tailed $P < 0.04$) reaching highest value of 0.44 ± 0.12 µg ind.$^{-1}$ in MS4 (Figure 4).

Ontogenetic variations of nucleic acid concentrations

Variations in weight-specific RNA and DNA during prenatal and postnatal developments and their stage-specific

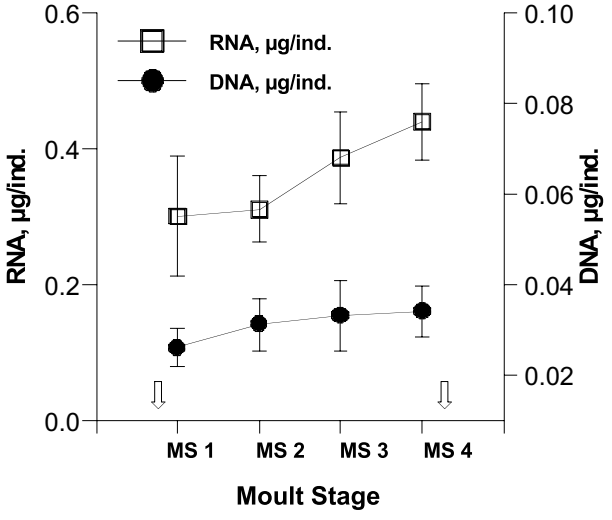

Fig. 4. *Daphnia pulex*: variations in nucleic acid content (µg ind.$^{-1}$) during moulting cycle (stages MS1 to MS4) of juvenile instar J3. Arrows indicate ecdyses.

content are shown in Figures 5 and 6, respectively. Generally, both RNA and DNA concentrations increased during embryonic development and decreased from earlier instars to older instars in growing *Daphnia*. At the same time, RNA and DNA content per animal largely follow the DW dynamics.

In actively reproducing females (adult stage AIII and further), the duration of embryonic development was approximately 27 h. During this period, a highly significant (one-way ANOVA, $F = 22.30$, $P < 0.0001$) decrease in embryo DW of was observed, from a DW of 2.66 ± 0.08 μg ind.$^{-1}$ in newly deposited eggs (E1) to 2.1 ± 0.09 μg ind.$^{-1}$ in embryos of the most advanced stage (E5) with an overlap between neighbouring stages (Fig. 5A). As free-living individuals, neonates were released at a mean size of 2.06 ± 0.12 μg ind.$^{-1}$. Thus the embryo undergoes a substantial dry weight reduction (23% in average, Figure 5A) during prenatal development, likely reflecting utilization of lipid energy reserves that support embryonic metabolism.

The changes that accompany embryogenesis are reflected in nucleic acid dynamics. During this period, RNA levels increased significantly (one-way ANOVA, $F = 22.88$, $P < 0.0001$) from 0.07 ± 0.009 μg ind.$^{-1}$ in E1 to 0.18 ± 0.035 μg ind.$^{-1}$ in E5 (Figure 5A). Similarly, a highly significant eight-fold increase was observed in DNA content of developing embryo (one-way ANOVA, $F = 31.60$, $P < 0.0001$) from 0.002 ± 0.0004 μg ind.$^{-1}$ in the earliest stage to 0.016 ± 0.003 μg ind.$^{-1}$ in the most advanced stages. No significant differences were found between this last embryonic stage and released neonates in either DNA (unpaired *t*-test, $F = 2.195$, d.f. = 18, $P > 0.3$) or RNA (unpaired *t*-test, $F = 3.063$, d.f. = 18, $P > 0.8$) contents.

Due to the decrease in embryo DW accompanied by the increase of RNA and DNA content, the concentrations of both nucleic acids significantly increased (one-way ANOVA, $P < 0.0001$) during embryonic development. Notably, the increase in DNA concentration was nearly 12-fold while the RNA increase was only three-fold (Figure 5B). Thus, a progressive and significant decrease in RNA : DNA (one-way ANOVA, $F = 14.72$, $P < 0.0001$) and DW : DNA (one-way ANOVA, $F = 32.06$, $P < 0.0001$) ratios occurred during embryogenesis, reflecting a higher rate of DNA accumulation compared to that of RNA (Figure 5C).

Juvenile development of *D. pulex* under laboratory conditions lasted on average 6.5 days. Neonates were released at a body size of 0.59 ± 0.02 mm (2.06 ± 0.12 μg) while females achieved maturity at 1.5 ± 0.02 mm (24.9 ± 1.97 μg), resulting in an average juvenile growth rate of 3.8 μg ind^{-1}a day^{-1}. Following the onset of reproduction, somatic growth rates slowed down so that no

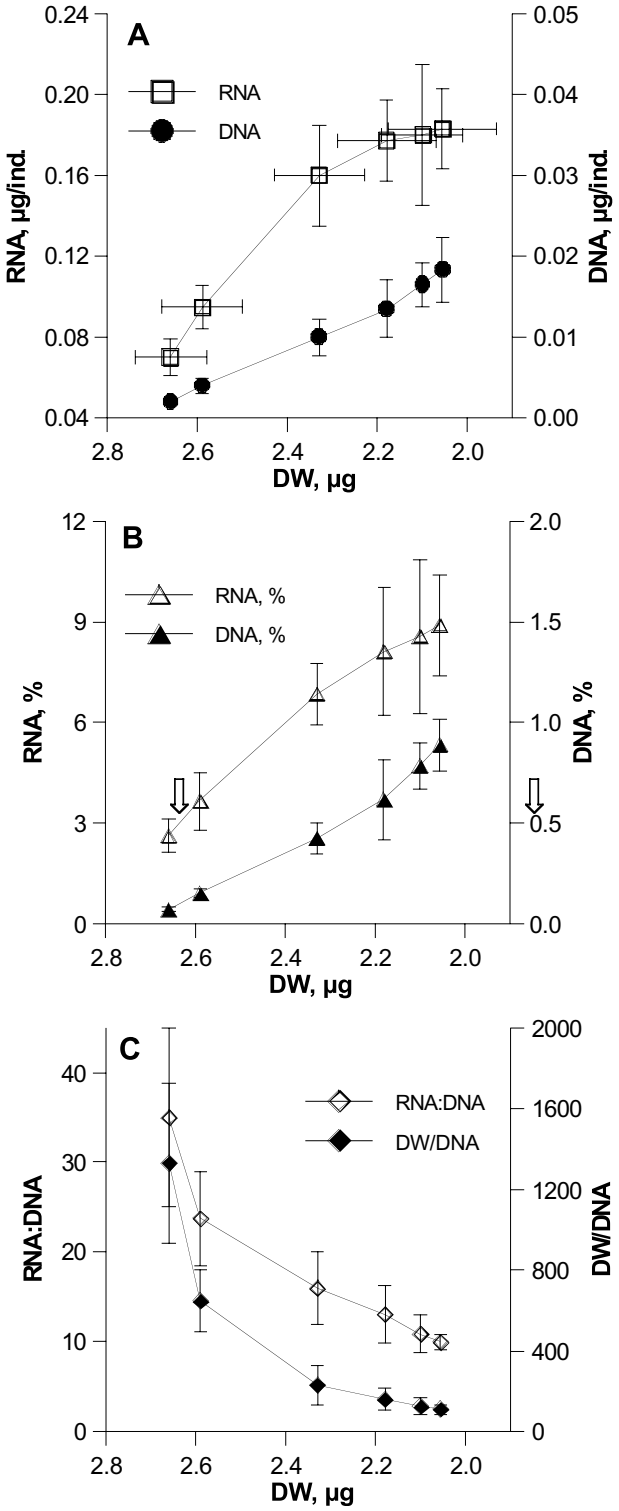

Fig. 5. *Daphnia pulex*: RNA and DNA content (A), concentrations (B), RNA : DNA and DW : DNA ratios (C) in developing embryos (E1 to E5).

significant difference in body size was observed between adult instars A4 and A5 (unpaired *t*-test, $F = 1.005$, d.f. = 7, $P > 0.4$). Overall growth of *Daphnia* was best described with the logistic model: $DW = 88.5/(1 + 63.6e^{-0.8}$ Instar Number); $r^2 = 0.93$).

In juvenile instars, RNA levels progressively increased (Figure 6A) from 0.18 ± 0.02 μg ind.$^{-1}$ in neonates to 0.98 ± 0.26 μg ind.$^{-1}$ in primaparae (one-way ANOVA, $F = 37.63$, $P < 0.0001$). Although, the overall increase during adult stages was also significant (one-way ANOVA, $F = 5.042$, $P < 0.007$), the differences between successive adult stages A2 to A5 were not (Bonferroni test, $P > 0.05$ in all cases). In adult instars, the rate of increase in RNA levels became significantly lower than that in juveniles, as indicated by the significantly greater slope of linear regression between RNA content and body DW for juveniles (primaparae included) than that for adults (ANCOVA, $F = 45.98$, DFn = 1, DFd = 6, $P < 0.0005$).

Changes in individual DNA content during postnatal development occurred somewhat differently (Figure 6A). The DNA content increased significantly (one-way ANOVA, $F = 22.56$, $P < 0.0001$) from 0.018 ± 0.004 μg ind.$^{-1}$ in neonates to 0.062 ± 0.017 μg ind.$^{-1}$ in primaparae. In adults, DNA levels continued to increase, reaching 0.22 ± 0.038 μg ind.$^{-1}$ in the oldest individuals. Following the DW trend, DNA values in the last two adult stages were not significantly different (unpaired *t*-test, $F = 1.717$, d.f. = 7, $P > 0.4$). Unlike RNA synthesis rates that appeared to decrease after the onset of reproduction, the rates of DNA increase during juvenile and adult periods were similar. An analysis of covariance showed that DNA to DW regression slopes for J1–A1 and for A1–A5 lines were not significantly different ($F = 4.68$, DFn = 1, DFd = 6, $P > 0.07$), with an average slope of 0.0027.

Concentrations of both RNA and DNA were highest in neonates (8.9 ± 1.2 and 0.89 ± 0.19 %, respectively) followed by a sharp decrease (Figure 6B). The declines were exponential ($r^2 = 0.98$ and $r^2 = 0.99$ for RNA and DNA, respectively (Figure 6B) and occurred mostly during the juvenile period, resulting in relatively constant concentrations in older juveniles and in adults (Bonferroni test, $P > 0.05$ for both RNA and DNA concentrations in successive J4 to A5).

Variations in the RNA : DNA ratio reflected simultaneous changes in RNA and DNA content (Figure 6C). Initially, RNA : DNA ratios showed a significant linear increase (one-way ANOVA, $F = 35.48$, $r^2 = 0.78$, $P < 0.0001$). The highest values (15.8 ± 2.7) were observed in primaparae stage and were followed by a significant linear decline (one-way ANOVA, $F = 11.84$, $r^2 = 0.74$, $P < 0.0001$) with the lowest values (9.5 ± 1.8) in the oldest stages (Figure 6C). In addition, DW : DNA ratios rose linearly throughout the juvenile period to primaparae (one-way

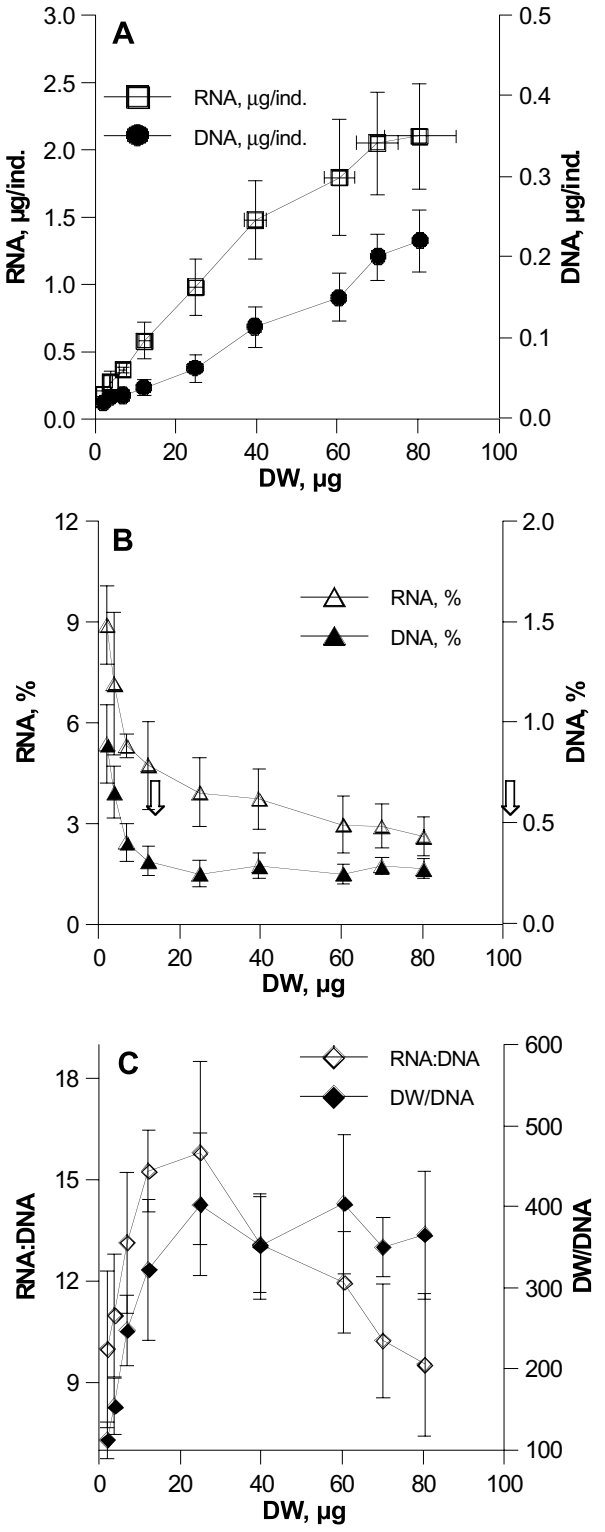

Fig. 6. *Daphnia pulex*: RNA and DNA content (A), concentrations (B), RNA : DNA and DW : DNA ratios (C) in successive juvenile (J1 to J4) and adult (A1 to A5) instars.

ANOVA, $F = 28.34$, $r^2 = 0.80$, $P < 0.0001$) levelling off thereafter, so that no significant trend in DW : DNA was observed in adult *Daphnia* (one-way ANOVA, $F = 0.61$, $P > 0.6$).

DISCUSSION

Fluorescence measurements and sample preparation

A primary objective of this study was to develop further the method described by Wagner *et al.* (Wagner *et al.*, 1998) to provide a low variability and high sensitivity for measuring RNA and DNA in small individual zooplankters. This objective was accomplished by using new sensitive fluorochromes, i.e. RiboGreen RNA quantification reagent and PicoGreen dsDNA quantification reagent. As RiboGreen has been found to be a non-specific nucleic acid dye with strong fluorescence enhancement upon binding to dsDNA (Jones *et al.*, 1998), we attempted to quantify both RNA and DNA sequentially following the method of Bentle *et al.* (Bentle *et al.*, 1981) and using RiboGreen as a fluorescent indicator. To compare DNA values obtained by RiboGreen reaction (one-dye protocol), subsamples were treated with PicoGreen (two-dye protocol) and DNA concentrations were calculated using their respective standard curves. Our results suggested that in combination with RNase treatment both dyes may provide reliable DNA estimations. In samples of *Daphnia* with varying body size and representing a working range of DNA concentrations, the values obtained with the two protocols were generally very similar, being slightly higher in PicoGreen-treated samples (Figure 2). The discrepancies seem to increase with DNA concentrations in the samples and were minimal (~6%) at concentrations below 0.1 μg ml^{-1}. The one-dye method, however, has the advantage of determining both RNA and DNA within a single sample aliquot, allowing maximum use of available sample and proper replication of measurements at the level of individual animals. Since the modes of binding of neither RiboGreen nor PicoGreen are well known (Singer *et al.*, 1997; Jones *et al.*, 1998), it is difficult to speculate whether the differences between DNA concentrations measured using these two dyes could be attributed to the particular affinity to the base composition of DNA. However, substantially different effects of contaminants that might be present (e.g. detergent, urea, some salts) in the samples after extraction procedure on fluorescence signal were reported for RiboGreen and PicoGreen (Singer *et al.*, 1997; Jones *et al.*, 1998). In our assay, 0.1% sarcosyl was present during fluorometric measurements. This might decrease the signal of RiboGreen while increasing or decreasing to a lesser extent the PicoGreen signal, as has been shown for other detergents [e.g. Triton X-100 and sodium dodecyl sulphate; see Table 1 in (Singer *et al.*, 1997) and Table 3 in (Jones *et al.*, 1998)]. These differences may at least partially have accounted for the small observed discrepancy in DNA determination between the two dyes.

Sample preparation, preservation, and storage are critical steps in nucleic acid quantification. It is well known that nucleases, and particularly RNases, are very aggressive and can rapidly digest significant amounts of sample RNA and DNA. In addition to normal laboratory precautions (i.e. use of gloves, sterile and/or nuclease-free labware and solutions), convenient and reliable preservation methods are much needed. We evaluated the effects of preservation and storage, comparing nucleic acid content in (i) fresh animals, (ii) animals deep-frozen and stored at –80°C, a commonly used method in nucleic acid analysis, and (iii) animals kept in RNA*Later*, a newly introduced and commercially available storage reagent that stabilizes and protects cellular RNA in intact, unfrozen tissue samples; preserved samples were then stored at –20°C, as recommended by the manufacturer. Both of the preservation methods tested produced results statistically indistinguishable from those obtained with fresh samples, suggesting that they both can be used routinely in field collections and laboratory studies. Slightly lower RNA values obtained for deep frozen samples indicate a possible loss during the sample preparation procedure despite our efforts to minimize nuclease activity by keeping the samples on ice before transferring them to the freezer. On the contrary, by placing the animals directly into RNA*Later* solution after their length had been measured, 'instant' preservation was achieved.

RNA and DNA variations during the moulting cycle

In crustaceans, the main biological processes are cyclical, particularly those involved in growth through moulting. The stage of the moult cycle therefore can significantly influence metabolic rates and biochemical composition (Passano, 1960; Skinner *et al.*, 1985) including nucleic acid levels (Sulkin *et al.*, 1975; Anger and Hirche, 1990; Juinio *et al.*, 1992). It has been shown for decapods that moulting hormones stimulate the synthesis of both RNA and DNA (Gorell and Gilbert, 1971; Freeman *et al.*, 1983), resulting in increasing rates of DNA synthesis and cell division occurring during late intermoult and/or early premoult (Freeman, 1986) and accompanied by increasing RNA and protein levels (Anger and Hirche, 1990). However, Beaton and Herbert observed a differing pattern of DNA synthesis and mitotic activity during the intermoult of *Daphnia* [*D. pulex* and *D. magna*; (Beaton and Herbert, 1994)]. They found that DNA replication occurred shortly

after moulting and was followed by intense mitotic activity so that proliferation was restricted to a short period during the postmoult. In our study, using juvenile individuals at different moult stages, a similar trend, with a significant increase in DNA levels during postmoult and relatively constant values during the rest of the intermoult period (Figure 4), was observed. Based on the frequencies of mitotic figures and interphase nuclei in epidermal cells, Beaton and Herbert [see Figure 5; (Beaton and Herbert, 1994)] found that cell densities in the fourth juvenile instar of *D. pulex* increased by approximately 60%. However, from our data, the relative increase in total DNA content from MS1 to MS4 during IMP of the third instar, appeared to be almost two-fold lower (Figure 4). Although it is possible that the animals used in our experiment had lower mitotic activity, this seems unlikely as high juvenile growth rates were observed and IMPs were similar [32 h in our study versus 36 h in the study of Beaton and Herbert, (Beaton and Herbert, 1994)]. This discrepancy might rather be related to the lack of accuracy in chronology of *Daphnia* moult staging. The relative amount of elapsed intermoult time for the first hypodermal retraction stage (= MS1) has a midpoint 0.17, meaning that an individual scored as being at MS1 could have already completed 17% (more than 5 h) of the IMP. If DNA replication occurs most intensively immediately after ecdysis (1–9 h) as suggested previously [(Beaton and Herbert, 1994); Figure 5], then our values for MS1 are underestimated and the overall increase in individual DNA content during the early postmoult might be higher. The gradual increase in RNA levels occurring mostly during the second half of the IMP (Figure 4), corresponded well with the pattern observed in juvenile decapods (Anger and Hirche, 1990; Juinio *et al.*, 1992). The rise in RNA content was about 45% and indicated that the period of particularly high mitotic activity during postmoult was followed by cell growth due to protein synthesis.

Most data reported in the literature for RNA and DNA levels in crustacean zooplankton (and for many other arthropod species as well) are based on single measurements made in an unspecified stage of the moult cycle. Such values are often highly variable and therefore inconclusive. To increase the predictive power of nucleic acid analysis as a means of assessing nutritional status and growth rates *in situ*, moult-cycle-related variations should be considered in comparative analyses of such data. In addition, when collecting field samples animals at particular moult stages should be selected for analysis. For example, when collecting cladocerans, either hypodermal retraction or stage of parthenogenetic eggs carried by females could be used to determine elapsed intermoult time (Willey and Threlkeld, 1995). Therefore, by selecting females with freshly deposited eggs (i.e. at MS1), a large portion of moult-cycle-related variability would be eliminated.

RNA and DNA variations during ontogenetic development

Different growth patterns were shown by and within the embryonic and postembryonic ontogenetic stages under constant experimental conditions. The loss of embryo DW during embryogenesis amounted to 23%, which is typical for daphniids: decreases of 16–25% were observed in *D. magna* and *D. curvirostris* (Green, 1956), 34% in *D. parvula*, and 48% in *D. ambigua* (Lei and Armitage, 1980). This phase is characterized by steep increases in both RNA and DNA (Figure 5) indicating particularly high cellular proliferation rates probably accompanied by high rates of protein synthesis. Accumulation of RNA was proportionally lower than of DNA, suggesting cell multiplication and morphogenetic differentiation to be the main processes.

In general, the amounts of nucleic acids during the postnatal period tended to parallel the growth curve, which indicated that there is a steady increase in cell number and mass throughout the life period studied (Figure 6). Steep decreases in both RNA and DNA concentrations and increases in RNA : DNA and DW : DNA ratios were observed during juvenile development, concurrent with declining growth rates. Decrease in DNA and RNA concentrations probably reflects a greater proportion of acellular tissue (e.g. lipids, chitin), while the increase in DW : DNA ratios indicates continuous cell enlargement. Moreover, as RNA accumulation rate was consistently higher than that of DNA, the RNA : DNA rose throughout the juvenile period. However, high variability of nearly all parameters within each juvenile instar (which could be in part explained by moult-cycle-related differences between replicate samples) makes most adjacent instars statistically indistinguishable.

A decisive event in the postembryonic ontogenetic development relative to nucleic acid dynamics seems to be the start of the reproduction, which in fact reveals a main change in growth pattern (Lynch *et al.*, 1986). With the onset of reproduction, DNA concentrations level off and the rate of decrease in RNA concentrations slows down (Figure 6). Cells, as indicated by DW : DNA values, appear to reach a definitive size that remains relatively constant for the remaining adult period. Thus, it appears that growth during juvenile development of *D. pulex* is due primarily to an increase in cell size, while in reproducing females, growth is due to increase in both cell size and number.

Gonad production usually involves marked changes in nucleic acid levels (Båmsted and Skjoldal, 1976). As could be seen from our data, large broods may comprise more

than half of the mature female in terms of DW, and, depending on the embryo developmental stage, 50–150% of RNA and 20–120% of its DNA contents. In addition to eggs/embryos that females carry in the brood chamber, ovarian cells undergoing a cycle in preparation to the next brood may also contribute substantially as well as altering RNA : DNA ratios, similar to findings in copepods (Biegala *et al.*, 1999). Thus, in environmental studies seeking to assess overall growth status of the *Daphnia* population, using females at different stages of their brood development and ovarian cycle could potentially mask the environmental effects on growth and nucleic acid content. To standardize sampling material, for example, only females with freshly deposited eggs (i.e. the initial stage of ovarian development) might be used. Then, eggs could be either removed from the female or subtracted from the total estimate later if DW, RNA and DNA contents of eggs were known.

Another area of concern with respect to variability of DNA is a high incidence of endopolyploidy observed in *Daphnia* cells (Korpelainen *et al.*, 1997; Beaton and Herbert, 1999). For example, the average proportion of polyploid nuclei in adult *D. pulex* was found to be as high as 27% (Korpelainen *et al.*, 1997). Moreover, ploidy levels were affected by growth rate and age, with adults expressing more extensive polyploidy than did juveniles. Beyond the fourth instar, however, the number of polyploid cells remained relatively uniform (Beaton and Herbert, 1999). This might in part explain the observed DNA concentration reaching asymptotic levels with low variability between individuals after the fourth instar in our experiment (Figure 6B) and makes comparison between adult individuals more reliable than between juveniles.

Several studies have tested the applicability of nucleic acid analysis for growth rate assessment of single or multispecies zooplankton samples using RNA concentrations (Dagg and Littlepage, 1972; Båmsted and Skjoldal, 1980) or RNA : DNA values (Wagner *et al.*, 1998). Sources of variability other than growth rates and nutrition include natural physiological changes related to development, and maturation are likely to affect nucleic acid content and potentially preclude field applications of the method. Nevertheless, accumulating research suggests a characteristic biochemical signature of rapid organism growth (Elser *et al.*, 2000), manifested in elevated RNA concentrations, which is driven by the fundamental association of rapid growth with ribosomal RNA. Through the study of the timing and mechanisms of this association and greater consideration of physiological development, we may begin to actually assess *in situ* growth of planktonic organisms, populations, and communities.

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