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A microfluorometric method for quantifying RNA and DNA in terrestrial insects

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Abstract

Evidence is accumulating for a mechanistic linkage between body phosphorus content and growth and reproduction of individual organisms, due in part to variation in allocation of resources to ribosomal RNA. Testing this connection requires reliable methods of quantifying the nucleic acid content of individual organisms. Although methods for quantifying nucleic acids are available for a wide array of organisms, adaptation of such methods for study of insects has been neglected. Sensitive stains and high throughput fluorometric measurements are now available that substantially improve past methodologies. Here we present methods for the extraction and quantification of insect RNA and DNA based on the use of N-lauroylsarcosine and sonication for extraction, the nucleases RNase and DNase, and the use of microplate fluorescent assays to quantify nucleic acids as percent of body weight in insects. We illustrate the method using *Drosophila* and curculionid weevils.

Keywords: Ribogreen, *Drosophila* sp., Curculionidae, Growth Rate Hypothesis, microplate fluorometry, N-lauroylsarcosine, nucleic acids

Introduction

Recent research suggests a mechanistic linkage between body phosphorus content and the growth and reproduction of individual organisms (Elser et al., 2000). Specifically, the growth rate hypothesis posits that variation in organismal body phosphorus content arises in part from variation in allocation to ribosomal RNA (rRNA) among organisms with different growth or development rates (Elser et al., 1996, 2000). Because RNA is rich in phosphorus and is often a major contributor to biomass (Sutcliffe, 1970; Dobberfuhl, 1999), it may contribute significantly to total phosphorus content in many organisms (Hessen and Lyche 1991; Elser et al., 1996). Thus, the growth rate hypothesis predicts that fast-growing organisms should be rich in RNA and phosphorus, therefore exhibiting a higher demand for phosphorus from their environment than slow-growing organisms, leading to a higher likelihood of limitation by insufficient phosphorus supply in their resource base (Hessen, 1992; Sterner and Schulz, 1998; Sterner and Elser, 2002). Clearly, testing the growth rate hypothesis requires a reliable method of quantifying the nucleic acid content of individual organisms. Methods currently exist that allow measurement of nucleic acids in some organisms, however, these methods are difficult to apply to many species, and are particularly inappropriate for use on small insect species. Our objective in this paper is to describe a new method for fast and reliable quantification of nucleic acids in such species.

primarily focused on larval fish (Bergeron, 1997; Westerman and Holt, 1994). In these studies, the availability of large samples made it possible to use spectrophotometric methods to quantify nucleic acid contents. Unfortunately, these methods lack the sensitivity required to detect nucleic acids in single small individuals (e.g. < 200 µg) and also require use of large quantities of reagents. Applying these methods to study smaller organisms necessitated the pooling of multiple individuals, that eliminated obtaining data from single individual animals (Chin-Leo and Kirchman, 1990; Samis et al., 1971; Church and Robertson, 1966). Over the last several years, methods have been developed to overcome this limitation in the study of crustacean zooplankton (Wagner et al., 1998 and 2001; Gorokova and Kyle, 2002; Vrede et al., 2002). Because of small individual body sizes of their study animals, zooplankton researchers have focused not on spectrophotometric methods, but on fluorometric methods (Dortch et al., 1983; Mordy and Carlson, 1991; Fara et al., 1996; Wagner et al., 1998). Fluorometry in combination with sensitive fluorochromes, use of microplates, and double staining of DNA and RNA with the same fluorochrome, permit rapid and sensitive assays of multiple samples using small sample volumes (Wagner et al., 1998; Gorokova and Kyle, 2002; Vrede et al., 2002). These advances have significantly improved the reliability of RNA and DNA quantification for individual zooplankters, but have yet to be applied to insects.

Past studies of nucleic acid levels in insects have used complicated extraction methods involving toxic chemicals (Church

Early method development of RNA and DNA quantification

and Robertson, 1966; McKee and Knowles, 1988; Schmidt and Winkler, 1991) as well as the of pooling many individuals to increase nucleic acid yield to detection level (Church and Robertson, 1966; Price, 1965; Banks et al., 1994; Premkumar et al., 1991; Rai et al., 1986). Here we present a method of measuring RNA and DNA in insects that uses new molecular stains and simple extraction protocols. We also describe the application of nucleases that allow the measurement of both RNA and DNA from a single sample, and new fluorochromes that allow quantification of nucleic acid content in individuals as small as 100 micrograms (dry weight).

Methods

“Wagner Method”

The method presented here builds on the approach described by Wagner et al., 1998 (“Wagner method”, hereafter) for copepod RNA and DNA quantification. A brief description of the Wagner method follows. Nucleic acids were extracted from individual copepods in a solution of N-lauroylsarcosine (sarcosyl) in Tris buffer (10mM Tris, 1mM Na₂EDTA, pH 7.5) on a vortex mixer for 1 hour. Samples were diluted 1:4 with Tris buffer (final concentration 0.2% sarcosyl) and returned to vortex for an additional 5 minutes followed by centrifugation (12,000 g for 10 minutes) to separate undissolved particles. Standard buffer (0.2% sarcosyl) was used to dilute RNA and DNA standards to bracket expected concentrations of samples. Samples and nucleic acid standards (75µl in each well) were run in duplicate in a 96-well microtiter plate. Ethidium bromide stain was applied for 15 minutes and the plate was then scanned for fluorescence (530 nm excitation, 590 nm emission filters). RNase was added and after 20 minutes the plate was rescanned. Calculations were made by subtraction of the two readings to estimate fluorescence due to binding of probe to RNA. Residual fluorescence was assumed to reflect fluorescence due to binding of probe to DNA with background fluorescence considered negligible.

To test methods developed here, we compared them to a modified Wagner method, which included substituting Ribogreen™ (Molecular Probes, Inc) for ethidium bromide, and gentle shaking on ice for extraction. Alternative methods were also tested. These included sonication, extractions with commercial reagents, and use of DNase. In order to compare all methods Ribogreen was used throughout. Exact details are described below.

Standards

As noted by Buckley et al. (1999), DNA and RNA standards can produce different intensities of fluorescence depending on the source and preparation of the standard nucleic acids. One factor affecting fluorochrome binding to nucleic acids is fragment length. To create DNA of approximately uniform length, DNA stock (calf thymus, Sigma, www.sigmaaldrich.com) was prepared by diluting 100 mg DNA overnight in 20 ml of nuclease-free water (Gibco Life Technologies, www.lifetech.com). This solution was placed into an iced sonication bath (30 min) to shear the DNA into smaller pieces. The product was centrifuged at 12,000 g for 15 min to separate out the lighter smaller fragments. The base pair length of the DNA supernatant was determined by agarose gel electrophoresis with the goal of producing DNA fragments with a relatively uniform length of 200-500 bp. This size range allows for repeatable results with

low variation when stained with Ribogreen. Aliquots of the standards were made and stored at –80 °C. RNA (Type III from Baker’s yeast, Sigma) was diluted, aliquoted, and stored at –80 °C. To compare results across laboratories, stock standards for both RNA and DNA were prepared at the Arizona State University lab and distributed to the lab at the University of Arizona for analysis.

Nucleases

While the Wagner method made use of RNase only, we used both DNase and RNase to improve quantification accuracy by correcting for background fluorescence (Bentle et al., 1981). A highly concentrated DNase I (GibcoBRL) was used to decrease the potential for increased background fluorescence from the addition of glycerol used in most DNase products. The DNase I was tested with and without magnesium and calcium. A concentrated Mg/Ca buffer was prepared and stored at room temperature and diluted using nuclease free water as needed. This buffer was either added independently to sample wells or mixed with DNase I and then added to sample wells in combination at concentrations of 0.8 mM calcium and 0.6 mM magnesium (ultra pure grade, Sigma). In both addition methods, DNase I spike concentration was between 17 and 28 units per well (125 units per µl). RNase A (4 mg/ml, Promega, <http://www.promega.com/>) was diluted 1:400 using nuclease free water and 0.14 µl added per µl sample.

Reagents

As in the Wagner method, an extraction buffer solution containing sarcosyl was used. Tris buffer was prepared by diluting 200 mM Tris-HCl, 20 mM EDTA, pH 7.5 (Molecular Probes, <http://www.probes.com/>) with nuclease-free water at a dilution of 1:20. The extraction buffer was prepared by adding N-lauroylsarcosine (ultrapure, Sigma) at 1%w/v to Tris buffer. Extraction buffer was frozen in small volumes in 50-ml centrifuge tubes. As needed, extraction buffer was thawed and added to samples for the extraction process, which were then further diluted following extraction, to a final concentration of sarcosyl of 0.1%w/v for plating. Extraction buffer volumes were scaled to the mass of the organism using weight to volume ratios similar to Wagner (see Table 1 from Wagner et al., 1998). A separate standard buffer was prepared by diluting extraction buffer to a concentration of 0.1%w/ final concentration and used to create a suite of standards. Concentration of sarcosyl in each well was 0.1%w/v.

While the Wagner method uses ethidium bromide for quantification of nucleic acids in the extracts, we chose to use a

Table 1. “Artificial” flies were prepared by mixing RNA and DNA standards to target insect nucleic acid concentrations (n=3 unless noted in table) and processing as a sample. RNA and DNA concentrations (µg ml⁻¹) ± standard errors are reported. RNA/DNA ratios are calculated from initial estimated RNA/DNA. Percent recovery is percent of nucleic acid measured compared to actual. Mean values for the 5 experiments are given with standard errors.

Experiment	RNA:DNA	RNA (µg ml ⁻¹)	% Recovery	DNA (µg ml ⁻¹)	% Recovery
1	4.17	0.97±0.002	96.5	0.25±0.003	106.3
2	1.25	0.186±0.006	93.0	0.193±0.004	121.0
3	6.25	0.901±0.012	90.1	0.20 (n=1)	126.2
4	1.25	0.201±0.015	100.5	0.166±0.001	103.7
5	6.25	1.07±0.013	107	0.174±0.002	108.7
Mean			97.4±2.9		113.8±4.4

less toxic, more sensitive stain, Ribogreen™ (Molecular Probes, Inc.). Ribogreen was frozen in aliquots, thawed as needed, and diluted according to manufacturer directions. This approach eliminated possible variation in Ribogreen fluorescence generated by repeated thawing and freezing of stock solutions. Ribogreen was used to stain both RNA and DNA following the double staining techniques of Berdalet and Dortch (1991) and Gorokova and Kyle (2002).

Quantification

Standards were diluted to bracket concentration of samples (typically 0–2 µg/ml for RNA and 0–0.5 µg /ml for DNA). Samples and standards were plated in duplicate onto black 96-well plates using 50 or 75 µl volume. Three identical plates were created: Plate 1 for RiboGreen alone; Plate 2 for RNase and Ribogreen; and Plate 3 for RNase, DNase I, Mg/Ca, and Ribogreen (Fig. 1). Volumes in all plates were equalized using nuclease-free water. Nucleases were added to plates 2 and 3 and incubated for 25 to 60 minutes for RNase and 1 hour for DNase, both at room temperature. Ribogreen was added in a 1:1 proportion of sample volume to dye and allowed to stain for 5 minutes before reading using a fluorometer (Bio-Tek, FLx800) at the highest sensitivity possible (typically 80). Fluorescence due to RNA was calculated by subtracting fluorescence of Plate 2 from that of Plate 1, while fluorescence due to DNA was calculated by subtracting fluorescence of Plate 3 from that of Plate 2 (Fig. 1). Concentrations were then calculated based on standard curve regressions of fluorescence vs. known standard concentrations. RNase was always added to plates involved with DNA determination as the DNase I/buffer complex inhibits fluorescence of extant RNA, thus giving a falsely high estimate of DNA in samples that contain intact RNA.

“Artificial Insects”

To test for potential interference when measuring both RNA

and DNA from a single sample, RNA and DNA stocks were mixed in known concentrations and proportions. These samples (“artificial insects”, hereafter) were assayed as if they were insect extracts and % recovery of RNA and DNA was calculated.

Spike Recovery

A subset of weevils (see below) and cohorts of age and size matched male *Drosophila mojavensis* were spiked with RNA and DNA standards and sonicated for 60 seconds then assayed for recovery of spike. Target concentration of spike was calculated to ensure that spike plus insect nucleic acid concentration was within the normal range of standards. Unspiked insects served as controls.

Sample materials

The method was tested on several species of insects, including two *Drosophila* (*D. mojavensis* and *D. busckii*), and two Curculionid weevils (*Sabinia setosa* and *Apion ventricosum*). *D. mojavensis* and *D. busckii* were raised in the laboratory from long-term stock populations while individual *Sabinia* and *Apion* (“weevils”, hereafter) were caught in the field (Sonoran desert northeast of Phoenix, AZ) in May and September 2000.

D. mojavensis were used in basic methods development studies, which were then applied to a series of life stages of *D. busckii* and to adult weevils. Individual adult *Drosophila* were weighed (wet weight) and flash frozen in liquid nitrogen and stored at -80 °C until later nucleic acid extraction and analysis. A separate set was weighed, dried, and re-weighed to establish a fresh weight to dry weight conversion factor used to estimate the dry weight of the samples used for determination of nucleic acids.

D. busckii were used to apply the method to a study of individual insect RNA and DNA during development. Adult females were allowed to oviposit on standard yeast/banana food for three hours. Flies were then removed, and larvae were collected for assay every 24 hours starting at 48 h post-oviposition. Larvae were picked from medium with forceps, rinsed in distilled water for ten minutes, and flash frozen in liquid nitrogen followed by storage at -80 °C until assay. Cohorts were dried and used to create estimates of individual dry weight.

Field collected weevils were frozen at -80 °C, removed and held on ice for photodocumentation and identification and returned to -80 °C until processing for nucleic acids. A separate set of weevils was frozen, digitally photographed for determination of body size as projected body area (using NIH imaging and Image-Pro software), dried, and then weighed. From these data, a mass to body area regression was established and used to estimate the body mass of photodocumented weevils that were used for nucleic acid analysis. To demonstrate the application of the method to field studies, weevils were collected in April and September 2000 to evaluate seasonal differences in their nucleic acid content.

Alternative Methods of Extraction

Two methods of extraction with sarcosyl in Tris buffer were tested. In one, insects were crushed in extraction buffer and shaken on ice for 90 minutes prior to 1:10 dilution with Tris buffer (hereafter modified Wagner method). A second method used sonication (Fig. 1). Frozen samples were crushed in extraction buffer, brought to at least 3 ml with ice cold Tris buffer, and sonicated (Branson 450 or

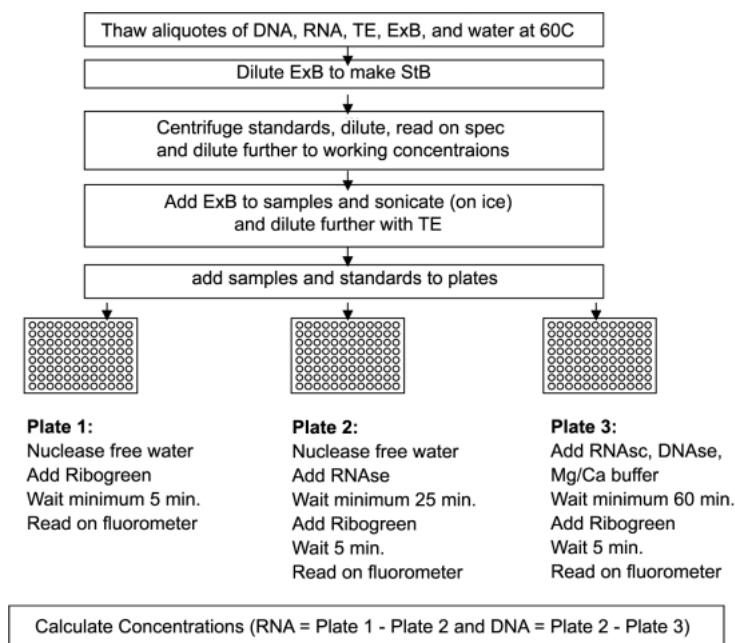


Figure 1. A schematic flow diagram of the sonication-sarcosyl approach to quantifying RNA and DNA. See methods for more detail.

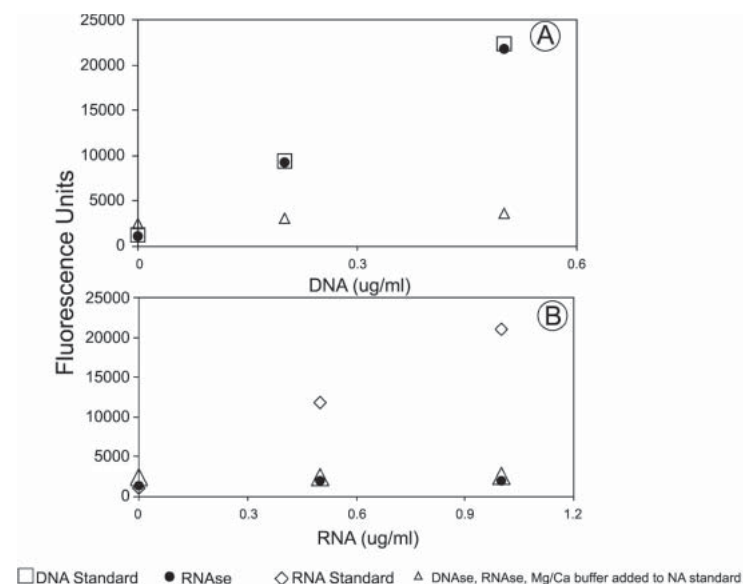


Figure 2. Fluorescence readings for A) DNA standard (squares) and B) RNA standard (diamonds) alone and in combination with RNase (solid circles) and a mixture of DNase, RNase, and Mg/Ca buffer (triangles). Standard error bars are too small to be discerned relative to the size of the symbols (n=3).

Branson 200 sonifier, <http://www.amtechultrasonic.com/>) in ice at 90% duty cycle, output 3, pausing once to maintain cold sample temperature. The duration of sonication was varied (60, 90, and 120 sec). Samples were diluted to a final concentration of 1:10 with Tris buffer and held on ice until processing was complete.

To test the extraction efficiency of sarcosyl, commercially available extraction kits, RNAzol B (Tel-Test, Inc.) and DNAzol (Molecular Research Centers, Inc.), were used. Both are considered high yield methods of extracting high quality nucleic acids. Following extraction as suggested by the manufacturer, the extracted pellet was dissolved in extraction buffer and diluted 1:10 with Tris buffer following the sarcosyl extraction method. A comparison was then made between these extracts and those using sarcosyl extraction by assaying the nucleic acids with Ribogreen as described above.

Results

Standard curves using processed calf thymus DNA (concentration range 0-0.5ug/ml) and bovine RNA (concentration range from 0-2 ug/ml) had consistently repeatable linear correlation coefficients (r) of greater than 0.99 with little variation in slope and intercept when stained with Ribogreen (data not shown). The combination of RNase, DNase I, and Mg/Ca buffer (0.6 mM magnesium and 0.8 mM calcium final well concentration) effectively diminished the fluorescence of the high DNA standard (Fig. 2a) to less than 10% of the original fluorescence. The addition of RNase alone, or RNase, DNase I, and Mg/Ca buffer to the high RNA standard also decreased fluorescence by 90% (Fig. 2b).

The recovery of RNA and DNA from artificial insects was high ($97.4\% \pm 2.9$ SE for RNA, $113.8\% \pm 4.4$ for DNA, Table 1). There appeared to be a consistent slight overestimation of DNA, which may be due to an interaction with RNA, however an increasing RNA:DNA ratio (1.25 to 6.25) didn't appear to effect the estimation

of DNA concentrations.

Spike recovery was high in all cases. For flies, RNA spike recovery was $97.3\% \pm 4.8$ SE (n=4), while DNA spike recovery was $95\% \pm 3.1$ SE (n=4). Weevil RNA recovery was $96\% \pm 5.2$ SE (n=4) and DNA recovery $95\% \pm 5.1$ SE (n=4).

Sonication at 60, 90, and 120 seconds of *D. mojavensis* did not result in significantly different yields for either DNA or RNA than the modified Wagner method (Fig. 3). Sonication did, however, result in significantly higher extraction of DNA than DNAzol (Fig. 3a). No significant differences were found in RNA extraction between any of these methods (Fig. 3b). The effects of sonication on the fluorescence of standards (Fig. 4) were small and varied between RNA and DNA. Fluorescence of DNA increased slightly with increasing sonication time (Fig. 4a), however, the only significant difference was between 0 sec vs. 120 sec. Sonication caused no significant changes in fluorescence of RNA standards (Fig. 4b) but there was a decrease in fluorescence at 120 sec. Sonication for 90 seconds showed optimal recovery of RNA and DNA standards.

Applications of the method

Nucleic acid concentration decreased strongly over the life cycle of *D. busckii* (Fig. 5). Larval DNA concentrations peaked at or before 48 hours post-oviposition and decreased more than seven fold over the larval life span (Fig. 5a). Larval RNA concentrations peaked at approximately 3 days after oviposition, and declined more than three fold over the larval life span (Fig. 5b). Adults had a slightly elevated RNA concentration relative to late stage larvae, and females

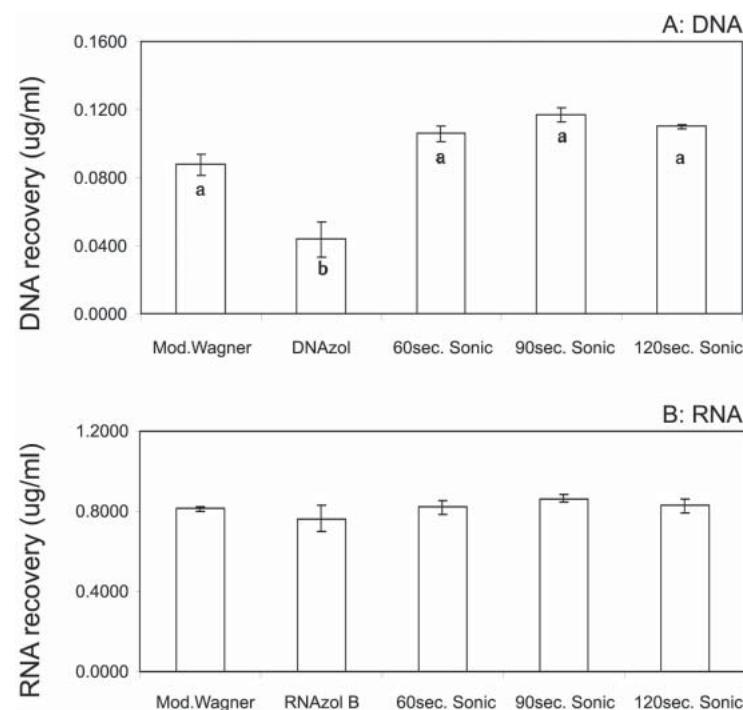


Figure 3. Recovery of (A) DNA and (B) RNA from *Drosophila mojavensis* using the modified Wagner extraction method, extraction by sonication, and two commercial extraction kits (DNAzol and RNAzol B). Error bars represent SE (DNAzol, n=4; all others, n=3). Letters indicate significant differences (ANOVA followed by Tukey's multiple comparison; p<0.05).

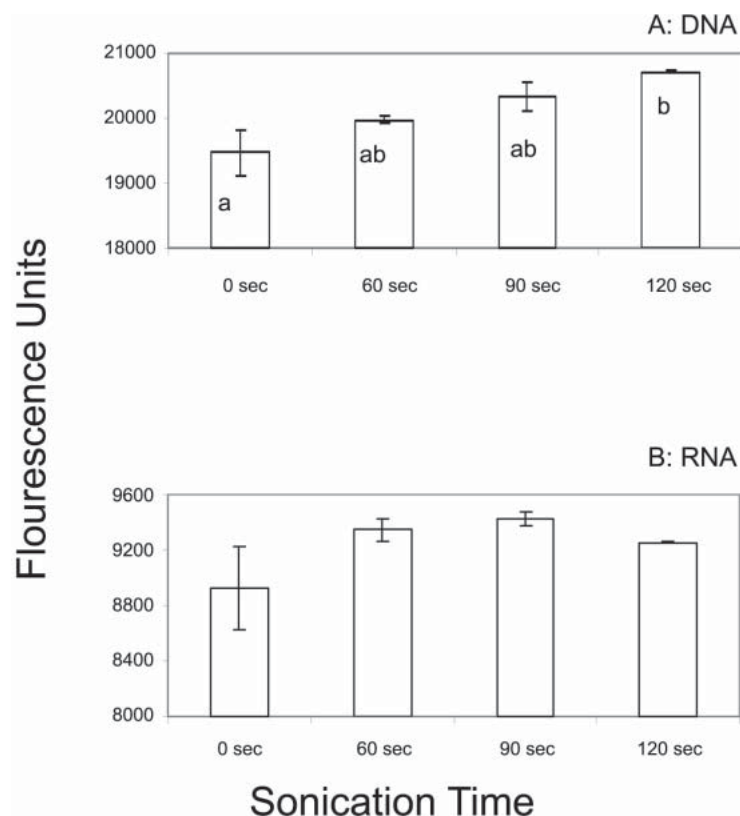


Figure 4. Effects of sonication time on fluorescence of 0.5 µg/ml standards of (A) DNA and (B) RNA. Error bars represent SE (n=3). Letters indicate significant differences (ANOVA followed by Tukey's multiple comparison; $p < 0.05$).

contained strikingly higher RNA than males. This may be a consequence of the biosynthetic demands of oogenesis. As with RNA, DNA concentrations of adults were slightly higher than in late stage larvae; however, the sexual divergences in concentrations seen in RNA were not present.

Nucleic acid concentrations differed between weevil species and showed seasonal variations (Fig. 6). DNA concentration in both *Apion ventricosum* and *Sabinia setosa* changed significantly between spring and fall but neither species showed a significant difference in RNA concentration on the two sampling dates.

Discussion

This study shows that a microplate method, originally developed for *Daphnia* (Wagner et al. 1998), can be modified to quantify RNA and DNA for small-bodied insects. Our data indicate that both the 90-min extraction approach of Wagner and our more rapid sonication procedure were equally effective in extracting nucleic acids. Quality control measures indicate that yields were high and sample degradation during extraction and quantification was minimal. The use of Ribogreen to stain RNA and DNA combined with the use of microplate fluorometry allows the quantification of both RNA and DNA with a less hazardous stain while maintaining the ability to scan large numbers of samples. Use of DNase further refines the method and allows for more accurate assessment of DNA concentrations by correcting for background fluorescence. Without the use of DNase (as with the Wagner method), background

fluorescence in these insect samples (13 to 21% of total) would result in significant overestimates of DNA concentrations.

The use of three simultaneous plates, one for Ribogreen, one for RNase, and a third for DNase, RNase, and DNase buffer means that with this method we do not have to correct for possible sample fading that occurs on plates exposed for multiple readings. Preliminary tests found that if a sample plate was scanned, allowed to sit for 30 minutes (the time required for RNase reaction), and rescanned, fluorescence decreased by 10%, thus requiring the addition of control wells to estimate a "fading factor" correction. This "fading factor" is eliminated by the use of the three-plate method. While the use of three plates increases reagent requirements, this cost is more than offset by increased confidence in the data.

This method provides a means of quantifying RNA and DNA in small individual insects and can detect ontogenetic and ecological patterns in such animals (Figures 5 and 6). The method is simple, relatively fast, and effective. In light of new work showing a relationship between body phosphorus content, growth rate and RNA content, and the influence of that relationship on the interaction between individual organisms, population dynamics and ecosystem-level nutrient cycling (Elser et al., 2000), this method is a critical step forward in our ability to test these hypotheses and increase our

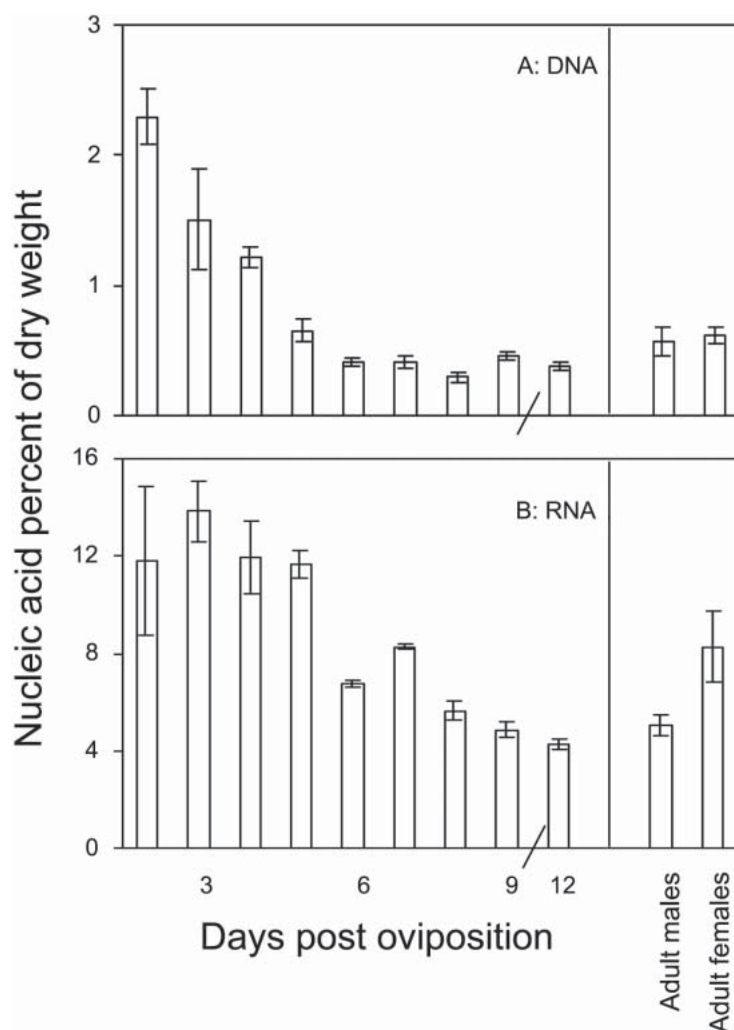


Figure 5. Nucleic acid concentration (percent of dry weight) of *Drosophila busckii* during its life cycle. Error bars represent SE, n=3

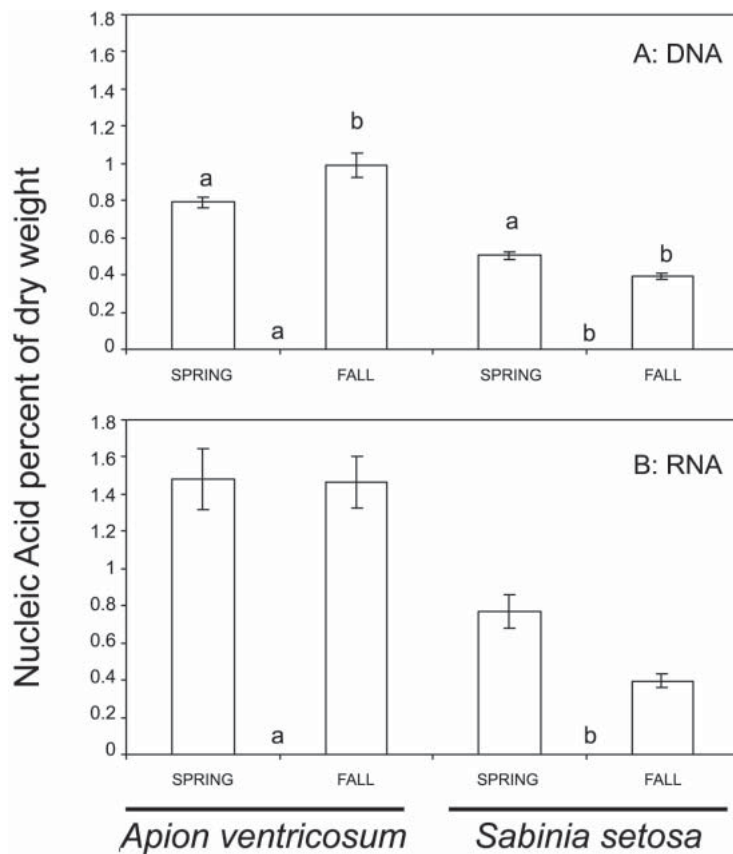


Figure 6. Changes in (A) DNA and (B) RNA between spring and fall for two species of weevils, *Apion ventricosum* and *Sabinia setosa*. Significant differences between seasons are noted above columns while significant differences between species are noted at tick marks between columns.

understanding of the link between biogeochemistry, population ecology, and evolutionary ecology in insect-dominated food webs. It also opens up the possibility of developing a database for nucleic acid contents of terrestrial insect species. Coupled with recent development of similar methods for studying aquatic organisms (Gorokhova and Kyle, 2002), we can now begin to form a general picture of the relationship between life history characteristics and nucleic acid contents of a greater diversity of species. This will improve our ability to develop a general understanding of the interaction between organisms and their environment.

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