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# Size and Organic Content of Eggs of Marine Annelids, and the Underestimation of Egg Energy Content by Dichromate Oxidation

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# Size and Organic Content of Eggs of Marine Annelids, and the Underestimation of Egg Energy Content by Dichromate Oxidation

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*Dichromate oxidation is a simple technique that is often used to estimate the energy content of eggs in studies of marine invertebrate life histories (1). We used this method to measure the energy contents of the eggs of 12 species of marine annelids. In combination with measures of egg ash-free dry weight (AFDW), these data yielded estimates of AFDW-specific energy density that were mostly lower than the average weight-specific energy density of carbohydrates. This seemed unlikely to be correct, as invertebrate eggs typically contain little carbohydrate and instead are composed primarily of energy-dense protein and lipid (1, 2). After validating our methods (by using them to estimate energy content and AFDW of the eggs of a previously studied echinoderm) and reexamining published data on the energy contents of echinoderm eggs, we conclude that dichromate oxidation often underestimates the energy contents of small eggs of marine invertebrates. This systematic error, which is likely related to the tendency of the assay to incompletely oxidize proteins, can only be corrected with substantial independent data on egg biochemical composition. We thus suggest that dichromate oxidation should not be used for routine measurement of the total energy content of marine invertebrate eggs.*

Maternal investment of energy per offspring is a variable of fundamental importance in models of the evolution of life histories (3, 4). It is relatively easy to quantify in free-spawning marine invertebrates, where maternal investment is primarily limited to the organic material provided in the egg. Among echinoderms, egg energy and organic content

(as AFDW) are both positively correlated with egg volume in interspecific comparisons (1, 5). Few data on relationships between egg size and organic or energy content are available for members of other phyla of invertebrates (1, 6, 7). We measured these variables in 12 species of marine annelids, with the aim of testing hypotheses on differences between annelids and echinoderms in relationships between maternal investment and larval nutritional mode. We used the technique of dichromate oxidation to estimate egg energy content (8, 9). Though it is prone to several problems (10, 11, 12), previous analyses have suggested that dichromate oxidation estimates agree well with those made by other techniques (1, 13), and it is frequently used to measure egg energy content (reviewed in ref. 1). Studied annelid eggs ranged in diameter from 44 to 352  $\mu\text{m}$  (Table 1), and in egg volume from  $4 \times 10^{-5}$  to  $2 \times 10^{-2}$   $\mu\text{l}$ , a range of 2.7 orders of magnitude (calculated as in 14). Both AFDW and energy content were positively correlated with egg volume in interspecific comparisons (by ordinary least squares [OLS] regression:  $\ln[\text{AFDW}]$  on  $\ln[\text{volume}]$ ,  $r^2 = 0.99$ ;  $\ln[\text{energy}]$  on  $\ln[\text{volume}]$ ,  $r^2 = 0.99$ ).

However, calculation of AFDW-specific energy densities (Table 1) revealed surprisingly low values: the mean AFDW-specific energy density for eggs of the 12 species was 15.2  $\text{mJ}/\mu\text{g}$  (range 10.3–22.5), lower than the average weight-specific energy density of carbohydrates (17.5  $\text{mJ}/\mu\text{g}$  [15]). This result might be correct if annelid eggs were composed primarily of carbohydrates. However, all invertebrate eggs whose composition has been studied (primarily those of echinoderms and crustacean arthropods) contain little carbohydrate (less than 5% by weight: 1, 2, 6) and a great deal of relatively energy-dense protein and lipid. Thus, we focused on two other possible explanations for our low estimates of AFDW-specific energy density in annelid

Table 1

Egg size, ash-free dry weight (AFDW), energy content, and weight-specific energy density for 12 species of marine annelids

Note that we believe our reported energy contents (and energy densities) are significant underestimates of true values; see text for discussion

Taxon	<i>n</i>	Diameter ( $\mu\text{m}$ )	Volume ( $\mu\text{l}$ )	AFDW ( $\mu\text{g}$ )	Energy (mJ)	Energy density (mJ/ $\mu\text{g}$ )
Chaetopteridae						
<i>Chaetopterus</i> sp. (FL, plk)	3	94.7 (0.7)	0.00045	0.152 (0.017)	2.19 (0.28)	14.4
Hesionidae						
<i>Ophiodromus pugettensis</i> (WA, plk)	3	85.1 (3.1)	0.00032	0.096 (0.015)	1.01 (0.22)	10.5
Maldanidae						
<i>Axiiothella mucosa</i> (FL, lec)	4	212.4 (3.2)	0.005	1.670 (0.275)	33.95 (6.02)	20.3
Nereididae						
<i>Platynereis bicanaliculata</i> (WA, lec)	1	149.0	0.002	0.652	9.5	14.6
Onuphidae						
<i>Kinbergonuphis simoni</i> (FL, lec)	3	351.9 (7.5)	0.023	10.306 (0.738)	231.60 (20.23)	22.5
Pectinariidae						
<i>Pectinaria gouldi</i> (FL, plk)	2	43.7 (1.1)	0.00004	—	0.16 (0.00)	—
Sabellariidae						
<i>Phragmatopoma lapidosa</i> (FL, plk)	4	84.6 (2.9)	0.00032	0.120 (0.033)	1.90 (0.31)	15.8
<i>Sabellaria cementarium</i> (WA, plk)	3	68.8 (3.4)	0.00017	0.067 (0.010)	0.74 (0.03)	11.0
Sabellidae						
<i>Schizobranhia insignis</i> (WA, lec)	3	155.5 (2.7)	0.002	0.895 (0.025)	15.64 (1.78)	17.5
Serpulidae						
<i>Hydroides sanctaerucis</i> (FL, plk)	3	52.2 (0.5)	0.00007	0.027 (0.003)	0.40 (0.04)	14.8
<i>Protula</i> sp. (FL, lec)	2	86.3 (1.1)	0.00034	0.138 (0.004)	2.07 (0.14)	15.0
<i>Serpula columbiana</i> (WA, plk)	4	69.1 (3.6)	0.00017	0.063 (0.009)	0.65 (0.03)	10.3

Values are means (one standard deviation) of measurements from each of *n* females. "FL" and "WA" refer to the collection location (Florida or Washington); "plk" and "lec" refer to larval nutritional mode (planktotrophic or lecithotrophic).

**Collection of adults and eggs.** Adults were obtained from around the Smithsonian Marine Station, Fort Pierce, Florida, and the Friday Harbor Marine Laboratories, Friday Harbor, Washington. Details of collection sites are available from the first author. Eggs were obtained from pectinariids, sabellariids, and serpulids by removing them from their tubes, after which they spawned. Nereidids and hesionids were captured as epitokes which spawned in the laboratory after capture. Sabellids spawned after warming of the seawater to room temperature. Maldanids deposited embryos in gelatinous masses attached to parental tubes; recently deposited masses (<12 h old) were obtained by frequent visitation to a marked patch of adults in the field, and embryos were removed from masses by dissection. The onuphids were intratubular brooders, and embryos were obtained by searching through parental tubes. Recently fertilized, single-celled zygotes were used for measurements in all cases except the maldanids and onuphids, where early cleavage stage embryos were used. For simplicity, we hereafter call all these stages "eggs." Eggs were cleaned by passing them through a Nitex sieve with a mesh size slightly larger than the egg diameter to remove any large debris, and collecting them on a sieve with a mesh size smaller than the egg diameter to remove smaller debris. They were rinsed twice and resuspended in filtered seawater (0.45- $\mu\text{m}$  mesh size, FSW). Concentrations of eggs in the final suspension were estimated by counts of eggs or embryos in replicated (4–6 $\times$ ) subsamples of known volumes taken with calibrated micropipettors. The large eggs of maldanids and onuphids were counted directly using a dissecting microscope.

**Egg volume.** Linear dimensions were estimated by measuring eggs with a compound microscope and ocular micrometer (final magnification usually 400 $\times$ ). In many cases, unfertilized eggs were not spherical at spawning, but became spherical soon after fertilization. A single axis (diameter) was measured for spherical eggs. Eggs of the maldanid were prolate spheroids (oblong-shaped), and two axes were measured. For each female, means of the linear dimensions of 20 eggs were calculated; reported diameters are among-female means of these values for spherical eggs, or the diameters of spheres of equivalent volumes for the maldanid.

**Ash-free dry weight.** Known numbers of eggs were transferred to 1.5-ml microfuge tubes, with three replicate tubes for each female. Tubes were centrifuged briefly and the supernatant FSW removed. To remove residual seawater, eggs were resuspended in milli-Q filtered H<sub>2</sub>O (mqH<sub>2</sub>O), immediately centrifuged again, and the supernatant decanted. This rinsing process was repeated once more. Samples were stored at –80 °C for up to a month before further processing. Samples were eventually transferred to pre-ashed foil pans and dried to constant weight at 65–75 °C (6–14 days). Dried samples were weighed with a Cahn electrobalance, then ashed for 5 h at 500 °C. Ashed samples were weighed and AFDW was estimated by subtraction. Per-egg AFDW was obtained by division. Egg AFDW for each female was taken as the mean of three separate measurements; reported values are among-female means. For *Axiiothella* and *Phragmatopoma*, AFDW measurements were made on eggs from only three females. Insufficient tissue was available to measure AFDW of eggs of *Pectinaria*.

**Energy content.** Known numbers of eggs were transferred to 1.5-ml microfuge tubes, with three replicate tubes for each female, and rinsed in mqH<sub>2</sub>O as described above. Rinsed eggs were transferred to pre-ashed glass test tubes, where they were stored at –80 °C for up to a month before energy assays were conducted. Energy assays were carried out by dichromate oxidation following McEdward and Coulter (9), except that samples and standards were lyophilized prior to assays (preliminary experiments showed that lyophilization had no effect on energy content). For each sample, organic matter was estimated against glucose standards (0–857  $\mu\text{g}$ ) as the weight of glucose ( $\mu\text{g}$ ) yielding equivalent reduction in dichromate. Division of this value by 2.5 gave sample content in equivalents of organic carbon ( $\mu\text{g}$ ) in glucose, and this was converted to energy content using the relationship 1  $\mu\text{g C} = 39 \text{ mJ}$ . Per-egg energy content was obtained by division. Egg energy for each female was taken as the mean of three measurements; reported values are among-female means.

Note that eggs for AFDW and energy content measurements for each female were drawn from the same container of eggs of known concentration, and eggs destined for both assays were rinsed in mqH<sub>2</sub>O using the same methods. Errors associated with estimating egg concentration or with rinsing eggs should thus be of the same magnitude and direction for both assays for each female, and should cancel in estimates of energy density.

=2348.5,  $n = 11$ ) than in those whose egg energy contents were estimated by biochemical component analysis (mean = 7100.5 mJ/ $\mu$ l, SD = 2214.9,  $n = 14$ ; Student's  $t$  test,  $P = 0.011$ ). This difference was not evident in the lecithotrophs, where mean volume-specific energy density was similar in both groups (dichromate oxidation mean = 11655.2 mJ/ $\mu$ l, SD = 1303.2,  $n = 9$ ; biochemical component mean = 12893.5 mJ/ $\mu$ l, SD = 3434.8,  $n = 13$ ;  $t$  test,  $P = 0.318$ ).

We can think of two explanations for this result. First, many of the data on energy contents of small eggs of echinoderms are derived from studies by only two groups of investigators: Strathmann and Vedder (16), who used dichromate oxidation, and Turner and Lawrence (17), who used biochemical component analysis. It is possible that errors associated with either group, not with the techniques used, produced the apparent differences in egg energy content. However, Strathmann and Vedder's (16) results agree well with ours for eggs of a sea urchin and two annelids (*Sabellaria cementarium* and *Serpula columbiana* [as *S. vermicularis*]); this consistency suggests that the dichromate oxidation results are correct, at least within the limits of the method. And because Turner and Lawrence's (17) data yield realistic estimates of AFDW-specific energy density that are consistent with results from direct calorimetry (18), we do not doubt that they are accurate. Instead, we suggest that dichromate oxidation systematically underestimates the energy content of small eggs. Our reasoning is as follows. Though dichromate oxidation performs well in oxidizing carbohydrates and lipids, it is known to incompletely oxidize proteins, and thus to underestimate energy contributed by proteins (10, 11, 12). In echinoderms, at least, protein concentration varies with egg size, with the smaller eggs of planktotrophs containing proportionally more protein than the larger eggs of lecithotrophs (1). Underestimates of energy content attributed to incomplete oxidation of protein by dichromate oxidation should thus be more severe for smaller eggs, consistent with the pattern that we have observed in previously published data for echinoderms (Fig. 1) and in our data on annelids (Table 1). Note that eggs of 11 of the 12 species of annelids we studied fall in the range of sizes of planktotrophic echinoderm eggs shown in Figure 1. Further, even in the limited size range of annelid eggs we studied, there is a significant positive relationship between AFDW-specific energy density and egg size (by OLS regression,  $\ln[\text{AFDW-specific energy density}]$  on  $\ln[\text{volume}]$ ,  $r^2 = 0.60$ ), consistent with the hypothesis that underestimates of energy content associated with the technique of dichromate oxidation are more severe for smaller eggs. We believe that the data that we have reported here on egg volume and AFDW in annelids (Table 1) are accurate, but we have no confidence in the accuracy of our energy content or energy density estimates and report

them only to illustrate this systematic problem with dichromate oxidation.

These results lead us to suggest that dichromate oxidation should not be used to estimate total egg energy content in marine invertebrates. One problem, illustrated for echinoderms in Figure 1, is that the magnitude of the error associated with incomplete oxidation of protein may vary with egg size. One can try to correct this potential error by independently measuring egg protein content and then applying a correction factor to account for incomplete oxidation of that material, as suggested by several authors (11, 12). However, a second problem may complicate this correction—that is, different proteins appear to be incompletely oxidized to different degrees by dichromate oxidation (12). Thus there is potential error associated not only with variation in protein concentration in eggs, but also with variation in the types of proteins present. Eggs of different species may vary in both types and quantity of protein present (22, 23). These issues, as well as others such as the sensitivity of dichromate oxidation to residual chloride in samples (12), suggest that when estimates of the energy contents of eggs or other protein-rich tissues are desired, other techniques will generally be preferable to dichromate oxidation. If only relative estimates of egg energy content are required for a particular study (e.g., do eggs of species A contain more or less energy than those of species B?), then, because energy content generally appears to be correlated with egg size (1, 6, 7), simple estimates of egg volume may well be sufficient. If absolute measures are required (e.g., for studies of the scaling of energy content with egg size), one can use direct calorimetry (10) or elemental analysis (24), or one can independently measure carbohydrate, protein, and lipid fractions and sum their energetic contributions. The latter technique, in particular, is suitable for analyzing the relatively small amounts of tissue often available in studies of marine invertebrate eggs. Further, though it is more laborious than dichromate oxidation and is subject to its own potential problems (in particular, how to deal with the "remainder" fraction of egg AFDW that is often unaccounted for by the separate component assays: 1, 9), biochemical component analysis has the pleasing property of yielding plausible data on energy content for eggs of all sizes.

### Acknowledgments

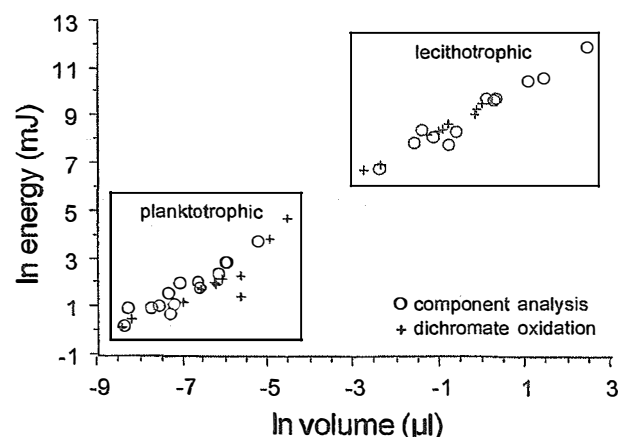
We thank D. Duggins, K. Fauchald, K. Hayes, W. Lee, S. Reed, H. Reichardt, J. Simon, and C. Staude for help collecting annelids. H. ten Hove kindly identified *Hydroides sanctaecrucis*. Discussions with M. Jacobs, B. Miner, R. Strathmann, and K. Zigler helped to clarify our argument and writing, as did the comments of two reviewers. We thank the Smithsonian Marine Station at Fort Pierce and the Friday Harbor Laboratories for laboratory space and sup-

eggs—that we had systematically underestimated egg energy content or overestimated egg AFDW. To distinguish between these possibilities, we estimated the energy content and AFDW of eggs of the sea urchin *Strongylocentrotus droebachiensis* from San Juan Island, Washington, using the same techniques we had used for annelid eggs. Eggs of *S. droebachiensis* had previously been studied by other investigators using several different methods (16, 17, 18), and their data provided a useful check on our methods. The eggs we obtained were 152.25  $\mu\text{m}$  in mean diameter (SD = 1.77,  $n = 4$  females), slightly smaller than those studied by these other workers (157, 156, and 157  $\mu\text{m}$  in refs. 16–18, respectively). Our estimate of egg energy content in *S. droebachiensis* (mean = 8.16 mJ, SD = 0.66,  $n = 4$  females) was similar to the published value obtained by dichromate oxidation (7.02 mJ, calculated from data in 16), and our estimate of egg AFDW (mean = 0.494  $\mu\text{g}$ , SD = 0.074,  $n = 4$  females) was similar to the sole published value (0.531  $\mu\text{g}$ , calculated from data in 17). Again, however, our data yielded a very low AFDW-specific energy density, 16.5 mJ/ $\mu\text{g}$ . Our estimate of the energy content of eggs of *S. droebachiensis* is undoubtedly incorrect, as eggs of this species contain substantial protein and lipid but little carbohydrate (17, 19). Indeed, an energy content estimate for eggs of *S. droebachiensis* made by summing the energetic values of measured biochemical components and the remainder fraction was 12.18 mJ, 1.5 times greater than the estimate yielded by dichromate oxidation (17). This higher estimate of energy content yielded a more realistic AFDW-specific energy density of about 23 mJ/ $\mu\text{g}$ , which is very similar to the weight-specific energy density of eggs of *S. droebachiensis* measured by another technique, direct calorimetry (22.5 mJ/ $\mu\text{g}$  dry weight [18]; note that these authors report *dry weight*-specific energy density, which we expect to be a slight underestimate of AFDW-specific energy density). The striking difference in energy content and density estimates made by dichromate oxidation, on the one hand, and biochemical component analyses and direct calorimetry, on the other, led us to suspect that we had consistently underestimated energy contents of our samples due to an error associated with the dichromate oxidation technique itself.

It is also possible that we (and other workers: 17, 18) had consistently overestimated egg AFDW, perhaps because water associated with intracellular salts was not removed by drying at 65–75 °C, but was removed when samples were ashed at 500 °C (20). However, eggs of marine invertebrates contain only small amounts of salts (2, 21), and even if all of these are hydrated, this potential error cannot account for the large underestimates of AFDW-specific energy density we observed (Table 1). Further, our AFDW measurements yield realistic estimates of AFDW-specific energy density when combined with energy content estimates made by biochemical component analysis (17), and these are consis-

tent with those made by direct calorimetry (18), suggesting that we did not seriously overestimate egg AFDW.

We hypothesized that this possible systematic underestimation of energy content by dichromate oxidation might be apparent in published data on egg size and energy content in echinoderms, a phylum in which both dichromate oxidation and biochemical component analyses have been used to estimate egg energy contents. To test this hypothesis, we examined data compiled by McEdward and Morgan (5) on the energy contents of eggs of 47 echinoderm species. Because AFDW is rarely measured in dichromate oxidation studies of egg energy content, we were unable to compare relationships between energy content and AFDW for the two methods; instead, we used egg volume as a measure of egg size. Eggs in this sample fall into two groups: 24 species with planktotrophic larval development and relatively small eggs (75–274  $\mu\text{m}$  diameter), and 23 species with lecithotrophic development and large eggs (496–2799  $\mu\text{m}$  diameter). Inspection of these data (Fig. 1) suggests that for the small eggs of planktotrophic species, energy content estimates made by dichromate oxidation are often lower than those made by biochemical component analysis. For the larger eggs of lecithotrophs, the two methods yield similar estimates. Comparisons of egg volume-specific energy densities support these impressions. Within the planktotrophs, mean volume-specific energy density was significantly lower in species whose egg energy contents were estimated by dichromate oxidation (mean = 4566.1 mJ/ $\mu\text{l}$ , SD



**Figure 1.** Relationships of egg energy content (estimated by dichromate oxidation [+] or biochemical component analysis [o]) to egg volume in 47 species of echinoderms. Data were taken from McEdward and Morgan (5), with three changes: we replaced their value for the energy content of eggs of *Clypeaster rosaceus* with the higher value recently reported by Miner *et al.* (25); we excluded *Notasterias armata* from our analysis, as McEdward and Morgan (5) concluded that it was an extreme outlier in their dataset; and we excluded *Perknaster fuscus* from our analysis, since published estimates of egg energy content for this species vary considerably (26, 27). None of these modifications significantly affect our conclusions.

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### Acknowledgments

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