Testing Small Clutch Size Models with *Daphnia*

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ABSTRACT: Life-history theory predicts that for small clutches, variance in egg size (between individuals) should decrease in a predictable invariant manner as clutch size increases. To test this, we studied *Daphnia magna* at 350 different food treatments and recorded the number of eggs and the volume of each egg for their first clutch. As predicted, we found that the relationship between clutch size and resources devoted to reproduction was linear, variance in egg volume decreased with increasing clutch size, and resources were shared relatively equally between the eggs in a clutch. However, we found that the rate at which the range of egg volumes decreased with clutch size was slower than predicted. We discuss possible explanations for this discrepancy, including a lower limit on the volume of eggs that are produced and selection for smaller eggs when food is abundant. Consistent with this, we found that mean egg volume decreased with increasing clutch size.

Keywords: clutch size, *Daphnia magna*, egg size, litter size, resource allocation, trade-off.

Examining how mothers allocate resources to their offspring can shed light on how natural selection shapes life histories (Roff 1992; Stearns 1992; Godfray 1994). Evolutionary models exploring optimal clutch (litter) size have typically used the approach described by Smith and Fretwell (1974), which models the trade-off between offspring size and offspring number assuming that mothers have a finite amount of resources available for each clutch and that better-provisioned offspring are fitter. However, because the predicted optimal clutch size is often not an integer value, these models must round to the nearest clutch size. Although this approach generally makes useful

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approximations at larger clutch sizes, it breaks down for smaller clutch sizes (Smith and Fretwell 1974). For example, if a mother has resources intermediate to those required for producing a clutch size of one or two, she then has to decide between producing one offspring that is larger than optimum size or two offspring that are smaller than optimum size. Recently, a number of theoretical models have addressed this problem and explicitly examined the consequences of small integer numbers for clutch size evolution (Ebert 1994; Charnov and Downhower 1995; Charnov et al. 1995; Downhower and Charnov 1998; West et al. 2001).

A novel and useful feature of these small integer models is that they make quantitative predictions that do not depend on difficult-to-measure life-history details and underlying trade-offs. Specifically, Charnov, Downhower, and colleagues have developed two life-history invariant rules. First, variance in offspring size (between clutches) is predicted to decrease with increasing clutch size, whereby the ratio of the range of offspring sizes for clutches of size i and j offspring is the reciprocal of the ratio of clutch sizes (Charnov and Downhower 1995; Charnov et al. 1995; West et al. 2001). This is given formally by

$$\frac{I_{\max i} - I_{\min i}}{I_{\max j} - I_{\min j}} = \frac{C_j}{C_i}, \qquad (1)$$

where I is offspring size, C is clutch size, $I_{\max i}$ and $I_{\min i}$ are the maximum and minimum size of offspring in clutches of C_i young, i and j are clutches of different sizes, and $i = j + 1$. Figure 1 graphically depicts this relationship, which we refer to as Charnov et al.'s invariant rule. A decrease in between-clutch egg size variance was also predicted by Ebert (1994), though Charnov et al. (1995; Charnov and Downhower 1995) were the first to make the invariant predictions. The second invariant rule is that the increase in resources devoted to reproduction required to go from producing a clutch size of C to a clutch size of $C + 1$ is invariant, regardless of clutch size (Downhower and Charnov 1998). We refer to this as the Downhower and Charnov invariant rule.

The usefulness of these two invariant rules is that they are independent of underlying trade-offs, such as the re-
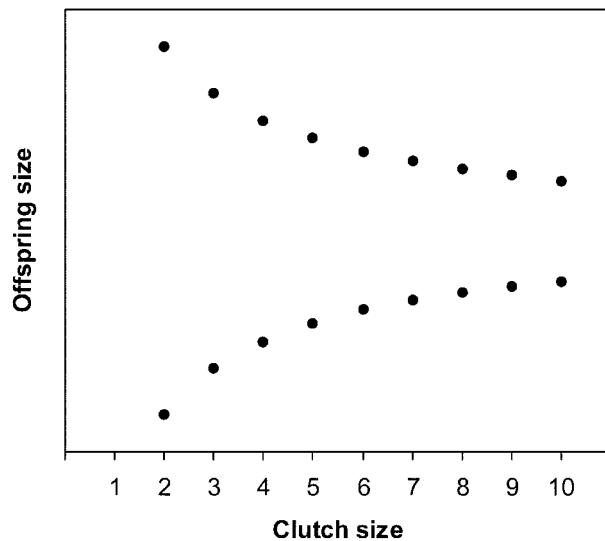
Figure 1: Predicted relationship between range in egg volume and clutch size (as described by eq. [1]). The upper point refers to the largest egg volume and the lower point to the smallest egg volume predicted for that clutch size. The distance between the points represents the total range of egg sizes expected for that clutch size.

lationship between offspring size and fitness. This means that they can be tested quantitatively with relative ease in any organism that produces small (but variable) clutch sizes. Consequently, cases where the invariant rules do not hold quantitatively can suggest that the fundamental assumptions of clutch size models do not hold. However, despite this possible wide applicability, there has yet to be a comprehensive, experimental test of these invariant predictions (although some tests have been done; see Charnov et al. 1995; Downhower and Charnov 1998; Mayhew 1998; West et al. 2001).

Our aim here is to carry out an explicit experimental test of the predictions and assumptions of the invariant rules. We use a small, freshwater crustacean, *Daphnia magna*. This is an excellent model organism for testing the invariant rules because clutch sizes can be in the relevant range (1–10); we can experimentally manipulate, with extreme precision, the amount of resources a female has for reproduction by varying the amount of available food; and they are clonal, which allows us to compare genetically identical individuals in the absence of paternal effects on offspring.

We use egg volume as our measure of resource allocation on the basis that egg size correlates with eventual neonate size (Goulden et al. 1987; Ebert 1993; Lampert 1993) and that egg size/offspring size correlates with fitness (Tessier and Consolatti 1989; Gliwicz and Guisande 1992), suggesting that larger eggs have more resources. We set out

to maximize variation in egg size within each clutch size by maximizing the variation in resources (food) within each clutch size. We accomplish this by having many food treatments and assigning only one *Daphnia* per treatment. Our statistical tests compare egg volumes between clutches of different sizes, not between food treatments. Because *Daphnia* clutch size is correlated with food availability, we chose food treatments that would result in first clutches of between one and 10 offspring on the basis of the results of a pilot study.

Our specific aims were to test whether variation in egg size decreases with increasing clutch size (Ebert 1994; Charnov and Downhower 1995; Charnov et al. 1995), whether the range of egg sizes follows Charnov et al.'s invariant rule (1995; Charnov and Downhower 1995; eq. [1]), whether clutch size scales linearly with total resources devoted to reproduction as predicted by the Downhower and Charnov invariant rule (1998), and whether resources are allocated equally within a clutch, which is an assumption implicit in all small clutch size models.

Material and Methods

Study Organism

We used a clone of *Daphnia magna* that had originated in the Gaarzerfeld pond, northern Germany. *Daphnia magna* from this pond have been the focus of numerous studies of parasitism and life-history evolution (e.g., Little and Ebert 2001; Little et al. 2002). Throughout, *Daphnia* were fed on chemostat-grown cultures of the green algae *Scenedesmus* sp. and kept in incubators (20°C, 14L : 10D). We raised three generations of *Daphnia* in jars containing 200 mL *Daphnia* media, five *Daphnia* per jar, with 1.5×10^7 algal cells per day per jar (for 84 jars × 5 *Daphnia*/jar = 420 *Daphnia*) to equilibrate the conditions of the *Daphnia* and control for maternal and grandmaternal effects. For each new generation, we combined all neonates produced over a 24-h period (first-clutch neonates were not used) and randomly allocated neonates to jars. We changed *Daphnia* media every other day. *Daphnia* media was a modified version of the Aachener Daphnien Medium described by Klüttgen et al. (1994).

Experiment

We collected all offspring produced over a 15-h period from the third generation (first-clutch neonates were not used), placed them in separate jars containing 200 mL *Daphnia* media, and randomly assigned them to a food level. Food treatments ranged between 1.00×10^4 and 3.50×10^6 algal cells per day. Each treatment differed from the nearest food treatments by 1.00×10^4 algal cells, re-
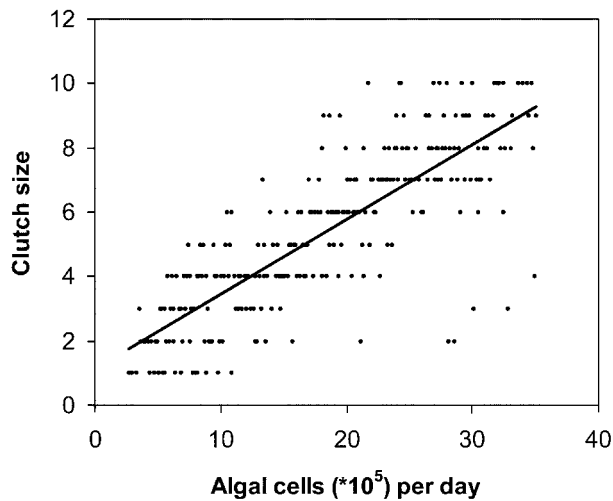
Figure 2: Relationship between food treatment and clutch size. Linear trend line included ($y = 0.2337x + 1.1147$, $r^2 = 0.663$).

sulting in 350 food treatments with one *Daphnia* per food treatment. The *Daphnia* jars were in trays, 12 jars to a tray. We randomized the location of the *Daphnia* within trays and the location of the trays within the incubator. We systematically rotated the jars within the trays and the trays within the incubator each day. We changed the *Daphnia* media in all the jars every other day. We checked the *Daphnia* twice per day, morning and evening, and recorded deaths. When the first clutch of eggs was produced, the mothers (still containing eggs) were photographed using a camera attached to a dissecting microscope. The eggs were then counted, removed, and photographed. All eggs were in stage 1 of development as described by Threlkeld (1979). Later, the photographs were used to measure mother length (top of head to base of spine) and egg diameter. Because many of the eggs were oval in shape, both length (longest axis) and width (perpendicular across middle of length) of the eggs were measured. Egg volume was estimated by modeling the eggs as an ellipsoid shape as described by equation (2):

$$\text{volume} = \frac{4}{3} \times \text{length} \times \text{width}^2. \quad (2)$$

Data were collected for only the first clutch of eggs.

Statistics

We used the SAS system, release 8.0 (SAS Institute 1999), for all analyses not requiring resampling. Resampling was done in Microsoft Excel 2000 using the Poptools add-in (Hood 2002). When appropriate, we averaged egg volume

over clutch to avoid pseudoreplication. We used generalized linear regression to test the relationship between clutch size and food level and between egg size and food level. Because variance was heterogeneous, we used a Spearman's rank correlation to test whether egg volume (averaged over clutch) changed with clutch size.

We calculated the variance in egg volume for each clutch size (using egg volumes averaged over clutch) and used an ordered heterogeneity (OH) test to evaluate whether variance decreased with increasing clutch size. The OH test combines the P value from the Bartlett homogeneity of variance test (P_{Bartlett}) with the Spearman's rank correlation coefficient (r_s) to calculate the OH statistic ($r_s Pc$) as follows: $r_s Pc = r_s \times (1 - P_{\text{Bartlett}})$ (Rice and Gaines 1994).

We used data on the mean egg volume (averaged over clutch) to test whether range in egg volume decreased as predicted by Charnov et al.'s invariant rule (1995; Charnov and Downhower 1995). We tested equation (1) for $\{i, j\} = \{2, 1\}$ through $\{10, 9\}$. We estimated the value of the invariant and the 95% confidence intervals for the left-hand side of equation (1) by bootstrapping 10,000 resamples of egg volume for each clutch size, as described by West et al. (2001). Because sample size affects range, we used the smaller sample size of the two clutch sizes, i and j, for both the numerator and denominator for each test.

Downhower and Charnov's invariant rule (1998) predicts that going from a clutch size of C to a clutch size of $C + 1$ requires an invariant increase in resources allocated to reproduction irrespective of C. This would lead to a linear relationship between clutch size and resources devoted to reproduction. We tested this prediction by using
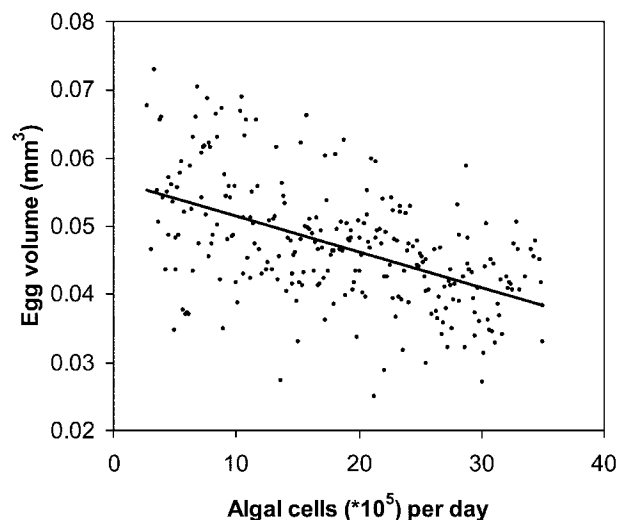

Figure 3: Relationship between food treatment and egg volume. Linear trend line included ($y = -0.0005x + 0.0567$, $r^2 = 0.260$).
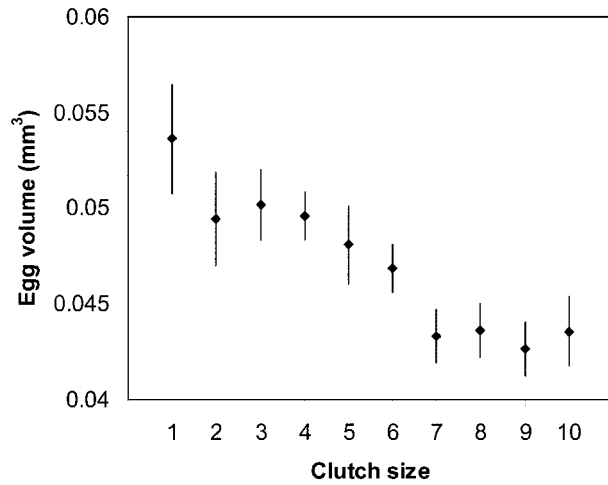
Figure 4: Mean egg volume for clutches of size one to 10. Bars are ±SEM.

generalized linear regression to relate clutch size to resources devoted to reproduction (number of eggs in a clutch multiplied by the mean egg volume for that clutch). We tested for nonlinearity by determining the significance of adding a quadratic (squared) term to the model.

To test whether resources are allocated equally within clutches, we followed the randomization procedure of West et al. (2001). Specifically, we calculated the mean within-clutch variance for each clutch size. Then, within each clutch size, we randomly allocated eggs to clutches and calculated the mean within-clutch variance of our randomized clutches. We repeated this procedure 1,000 times and tested whether the observed variance differed from the variance of our randomized clutches. This procedure was done separately for each clutch size.

Results

No reproduction was seen in animals receiving fewer than 2.70×10^5 algal cells per day, nor was it seen in approximately 8% of *Daphnia* receiving more than this amount of food. We included only *Daphnia* that produced clutches of ≤10 eggs in analyses because few *Daphnia* (<5%) produced >10 eggs. Our analyses contain data from 274 *Daphnia* and 1,336 eggs. We observed the *Daphnia* for 21 d after being placed in their treatments, during which time all *Daphnia* had either produced a clutch of eggs or died (96% of those *Daphnia* that produced eggs did so within the first 14 d of the experiment). Clutch size increased with increasing food ($F = 534.18$, df $= 1, 272$, $P < .0001$; fig. 2). Egg volume decreased with increasing food ($F = 89.2$, df $= 1, 259$, $P < .0001$; fig. 3) and decreased with

increasing clutch size ($r_s = -0.34$, $P < .0001$, $n = 261$; fig. 4).

As predicted (Ebert 1994; Charnov and Downhower 1995; Charnov et al. 1995; fig. 1), variation in mean egg size (between clutches) did indeed decrease with increasing clutch size (OH test: Spearman r_s value $= -0.87$, Bartlett P value $= 0.11$, $r_sPc = -0.77$, two-tailed P value $< .001$; fig. 5). We used a random resampling technique to test the extent to which this led to a decrease in range of mean egg size with clutch size, as predicted by Charnov et al.'s invariant rule (1995; Charnov and Downhower 1995). The 95% confidence interval of the decrease in range included the invariant value predicted by equation (1) for all clutch sizes tested (table 1). However, confidence intervals were large, and in all cases, the 95% confidence intervals included 1. Consequently, when considering each pair of clutch sizes (i.e., comparing C with $C + 1$), individually there was no statistically significant change in range. Overall, six of the nine observed values were larger than the expected values. This lack of a close fit to Charnov et al.'s prediction is also shown by the fact that the range did not show a symmetrical convergence around the mean; while the ninetieth percentile of mean egg size significantly decreased with increasing clutch size (slope $= -0.0019$ mm³/clutch size, $r_s = -0.92$, $n = 10$, $P = .0002$), the tenth percentile did not significantly increase with increasing clutch size (slope $= 0.0003$ mm³/clutch size, $n = 10$, $r_s = -0.61$, $P = .060$; fig. 5).

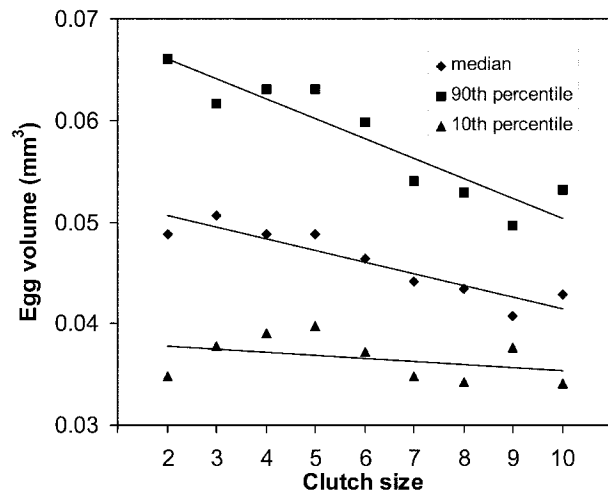We carried out two further tests to ensure the robustness of the negative relationship between variance in egg vol-

Figure 5: Median (*diamonds*), ninetieth percentile (*squares*), and tenth percentile (*triangles*) for egg volume for clutches of size one to 10. Linear trend lines included (median: $y = -0.0011x + 0.053$, $r^2 = 0.851$; ninetieth percentile: $y = -0.0019x + 0.0699$, $r^2 = 0.853$; tenth percentile: $y = -0.0003x + 0.0384$, $r^2 = 0.148$).

Table 1: Test of equation (1)

Clutch sizes compared	Sample size	Expected $\dfrac{C_i}{C_j}$	Observed $\dfrac{I_{\max i} - I_{\min i}}{I_{\max j} - I_{\min j}}$	Lower confidence limit (2.5%)	Upper confidence limit (97.5%)
2 vs. 1	15	.5	1.09	.71	1.65
3 vs. 2	22	.67	.83	.57	1.10
4 vs. 3	23	.75	.99	.70	1.41
5 vs. 4	24	.80	1.28	.72	1.78
6 vs. 5	24	.83	.72	.46	1.21
7 vs. 6	35	.86	1.41	.72	1.85
8 vs. 7	28	.88	.70	.43	1.39
9 vs. 8	17	.89	.81	.37	1.40
10 vs. 9	16	.90	1.21	.64	2.68

Note: The observed values (median of the 10,000 resamples) and 95% confidence intervals for $(I_{\max i} - I_{\min i})/(I_{\max j} - I_{\min j})$ were obtained by resampling the egg volume data (averaged over clutch).

ume and clutch size. First, we tested that this pattern was not simply a statistical artifact due to averaging over more eggs as clutch size increased. To do this, we randomly chose one egg from each clutch and calculated the regression line relating variance in egg volume to clutch size. We repeated this 100 times. All 100 regression lines had a negative slope, leading us to conclude that variance does indeed decrease with increasing clutch size ($P < .01$). Second, we tested that the relationship did not arise merely as a result of differences in egg shape. We did this by testing for relationships between clutch size and egg length and between the variance in egg length and clutch size. The

Figure 6: Relationship between clutch size and resources devoted to reproduction (number of eggs in a clutch multiplied by the mean egg volume for that clutch). Linear trend line included ($y = 0.0406x + 0.0275$, $r^2 = 0.826$).

results of both these tests were similar to those reported for egg volume and supported the robustness of our results.

Downhower and Charnov's invariant rule (1998) predicts that the amount of extra resources needed to produce an additional egg remains constant over clutch size. This therefore predicts a linear relationship between resources devoted to reproduction and clutch size. Resources devoted to reproduction were significantly related to clutch size (fig. 6; $F = 35.1$, df $= 1, 259$, $P < .0001$), with a linear regression explaining 82.6% of the variation in the data. We then tested for nonlinearity by adding a squared term (clutch size squared) to the model; the squared term did not significantly improved the model ($F = 3.12$, df $= 1, 258$, $P = .078$).

Clutch size models assume that each egg in a clutch receives equal resources. Our randomization procedure allowed us to test this statistically by comparing variation between eggs from the same clutch to that of eggs from different clutches (within the same clutch size). Eggs from the same clutch were more similar in volume than were eggs from different clutches ($P < .01$) for every clutch size tested (two to 10), supporting the hypothesis that mothers allocate resources relatively equally between eggs in a clutch (table 2).

Discussion

We have carried out an experiment designed explicitly to test the predictions of small clutch size models and in particular their invariant rules (Ebert 1994; Charnov and Downhower 1995; Charnov et al. 1995; Downhower and Charnov 1998; fig. 1). In support of these invariants, we found that variance in egg volume decreased with clutch size (fig. 5) and clutch size showed a linear relationship to resources devoted to reproduction (fig. 6). As assumed
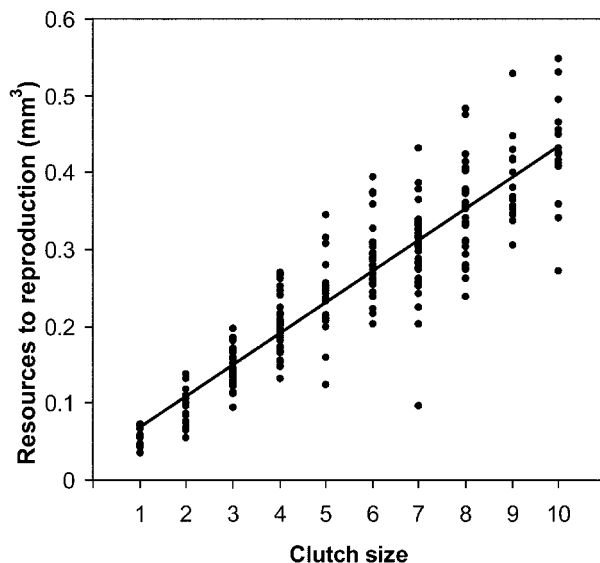
Table 2: Observed mean within-clutch variance in egg volume compared to sampled mean within-clutch variance in egg volume by clutch size

Clutch size	Sample size	Observed mean variance ($\times 10^{-5}$)	Sampled mean variance ($\times 10^{-5}$)	Lower confidence limit (2.5%) ($\times 10^{-5}$)	Upper confidence limit (97.5%) ($\times 10^{-5}$)	P value
2	22	2.81	11.25	6.46	16.18	<.01
3	23	2.68	9.51	6.76	12.62	<.01
4	45	2.65	7.87	6.58	9.34	<.01
5	24	4.93	13.17	10.71	15.95	<.01
6	35	4.55	8.27	6.15	10.28	<.01
7	36	4.48	8.77	7.58	10.44	<.01
8	28	4.61	8.82	7.66	10.40	<.01
9	17	3.52	6.60	5.65	7.77	<.01
10	16	3.47	8.19	7.41	9.06	<.01

Note: The 95% confidence intervals are for sampled values.

by these models, we found that eggs from the same clutch were more similar in volume than were eggs from different clutches (within a clutch size), suggesting that mothers allocate resources relatively equally between eggs in a clutch (table 2). However, mean egg volume decreased with clutch size (fig. 4), and the rate at which the range of egg volumes decreased with clutch size did not show a strong fit to the predictions of the Charnov et al. invariant rule (1995; Charnov and Downhower 1995; fig. 1) regarding how the range of egg volumes should change with clutch size (table 1). Specifically, although the 95% confidence intervals of our data include the values predicted by equation (1), the confidence intervals are large and in all cases include 1 (table 1). Moreover, the tenth and ninetieth percentiles of mean egg size per clutch did not converge symmetrically around the mean with increasing clutch size (fig. 5). There are several possible hypotheses that could explain why range of egg sizes decreased more slowly than expected.

First, there might exist a lower boundary on egg volume below which eggs are not viable. A lower limit on egg size in *Daphnia* has been postulated previously (Glazier 1992), and at small egg sizes, there is a strong positive correlation between egg size and likelihood of hatching in *Daphnia pulex* (Bell 1983). The consequences of a minimum egg size is that in some circumstances, *Daphnia* females would reduce their clutch size by one rather than produce eggs smaller than this minimum egg volume, hence decreasing the possible egg volume/egg number combinations. Consistent with this, we found that while the ninetieth percentile of egg volumes decreased with increasing clutch size, there was no change in the tenth percentile (fig. 5). A similar pattern has been observed in a fish (Charnov et al. 1995).

Second, the optimal egg size may vary with food and resource availability and therefore clutch size. In our study, mean egg volume decreased with increasing clutch size.

Decreasing egg (or neonate) size with increasing clutch size (or increasing food availability because clutch size and food availability are correlated) has been found previously in *Daphnia* (e.g., Bradley et al. 1991; Gliwicz and Guisande 1992; Ebert 1993; although other patterns have also been observed, e.g., Boersma 1995; Lynch 1989; Tessier and Consolatti 1991; Glazier 1992). Equation (1) assumes that mean egg size remains constant. Our results are consistent with the hypothesis that mothers in bad environments (in this case, low food) produce larger eggs than do mothers in good environments (high food). This would occur if the fitness benefit of being a large offspring is greater at lower food (Hutchinson 1951; Green 1966; Goulden et al. 1987; Gliwicz and Guisande 1992; Ebert 1994).

Third, the relationship between resources allocated to an egg and egg volume might be nonlinear. Egg volume is correlated with offspring fitness (as discussed in the introduction to this article), suggesting that larger eggs do indeed receive more resources than do smaller eggs. However, the carbon : nitrogen ratios in neonates differ with the mothers' food availability in *Daphnia pulicaria* (Tessier and Consolatti 1991), suggesting that resource partitioning may differ between eggs of different sizes. In addition, size can affect predation risk for *Daphnia*, and *Daphnia* can adjust age and size at maturity in line with predation risk (Weider and Pijanowska 1993; Stibor and Lüning 1994). In other organisms, complicated size-fitness relationships have been described, for example, where fitness depends on an individual's size relative to competitors (Westoby et al. 1992; Rees and Westoby 1997).

Fourth, a female might be simultaneously optimizing the amount of resources that she allocates to reproduction with the resources allocated elsewhere, such as to growth. If this is the case, then she might not be constrained to put a fixed amount into reproduction, dependent on food levels.

Conclusions

Quantitative life-history predictions often require a detailed knowledge of life-history details that are difficult to measure. Recently, it has been shown that this problem can in some cases be resolved through the use of a dimensionless approach (Charnov 1993, 1997). This method can predict life-history invariants that permit quantitative tests in the absence of a detailed knowledge of underlying trade-offs and without the inclusion of biological complexities. As well as the small clutch size invariants tested here, life-history invariants have been successfully utilized to explore topics as varied as age at maturity in parasitic nematodes (Gemmill et al. 1999), timing of sex change in fish (Charnov and Skuladottir 2000; Allsop and West 2003*a*, 2003*b*), and life-history characteristics (age at maturity, cellular maintenance rate) in mammals (Charnov 2001).

In this study, Charnov et al.'s invariants (1995; Charnov and Downhower 1995; Downhower and Charnov 1998) have proven useful for expanding our knowledge specifically about *Daphnia* reproduction and generally about resource investment into offspring. We found qualitative support for the prediction that variance in egg volume should decrease with increasing clutch size (Ebert 1994; Charnov and Downhower 1995; Charnov et al. 1995; fig. 5). Where the data do not fit the invariant predictions quantitatively has led to several hypotheses about *Daphnia* reproduction and about resource allocation in general. These observations require further investigation. In particular, Is there a minimum viable egg volume? Does the relationship between egg volume and offspring fitness vary with food availability and thus clutch size? To what extent are our findings generalizable to more species? In addition, it would also be extremely useful to explore the theoretical implications of a minimum viable egg volume and variation in the optimal egg volume with clutch size.

Acknowledgments

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