

COMMENTARY

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REFINED METHODS FOR ASSESSMENT OF NUTRIENT RESERVE USE AND REGULATION OF CLUTCH SIZE¹

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Abstract. Methods for evaluating regulation of clutch size by endogenous nutrients have evolved rapidly over the last decade. Use of regression and correlation approaches to estimate rate of depletion of nutrient reserves and to test hypotheses about regulation of clutch size represented significant advances. Assessment of relationships between nutrient reserves and clutch size have been substantially improved by controlling for both body size and laying date. We point out potential biases and spurious correlations in current hypothesis tests, which examine the relationship between remaining nutrient reserves and remaining rapidly developing follicles. We recommend new methods to control for clutch size and for estimation of depletion of nutrient reserves, and for testing hypotheses regarding clutch size regulation.

Key words: *clutch size, nutrient reserve dynamics, statistical analysis.*

During the last decade and a half, a substantial literature has developed examining the role of nutrient reserves (herein somatic lipid, protein, or mineral) in the regulation of clutch size, especially for waterfowl (see Alisauskas and Ankney 1992). Alisauskas and Ankney (1985) proposed, for females collected in the field, regressing nutrient reserves at time of collection against nutrients already invested in reproduction, which included nutrients present in the ovary and oviduct plus nutrients in eggs already laid. This method of analysis was superior to earlier methods because it eliminated biases associated with comparison of mean nutrient levels among females in different reproductive states, such as pre-laying and laying (see Alisauskas and Ankney 1992 for further discussion of such problems).

Alisauskas and Ankney's (1985) method also allowed for direct estimation of rate of depletion of nutrient reserves as these reserves were invested in reproduction.

A further refinement in analysis of nutrient-reserve dynamics of breeding birds was adoption of statistical techniques that allowed researchers to statistically control for variation in body size when estimating size of reserves. Alisauskas and Ankney (1987) used Principle Components Analysis of measurements of several body parts to derive, for each bird, a PC₁ score as a measure of its body size. Subsequently, Ankney and Afton (1988) regressed lipid, protein, and mineral reserves of female Northern Shovelers (*Anas clypeata*) on PC₁ scores and used residuals from these regressions to correct for among-female variation in reserves that were due to variation in body size. Currently, researchers routinely use this approach in studies of nutrient-reserve dynamics.

For populations in which females deplete nutrient reserves, two contrasting hypotheses can be envisioned with respect to regulation of clutch size by nutrient reserves. We do not advocate either hypothesis, here, but merely examine how the two alternatives influence methods of testing hypotheses about clutch size regulation. If a nutrient reserve regulates clutch size, then females that begin laying with larger nutrient reserves will produce larger clutches because these females can invest more nutrient into eggs before reaching a minimum threshold that terminates egg laying (Fig. 1a). In nature, we anticipate that both rate of decline in nutrient reserves and minimum reserve threshold will vary among females. Alternatively, if females cease laying at a target clutch size, variation in nutrient reserves at the beginning of egg laying would not be correlated with clutch size (Fig. 1b). Rather, females would vary in the size of remaining nutrient reserves at end of egg laying. Figure 1 essentially represents contrasting hypotheses (or modifications thereof) for regulation of clutch size in birds with precocial young (Fig. 1a) and those with altricial young (Fig. 1b) originally proposed by Lack (1947, 1967), although Lack did not know about use of stored nutrients by laying females. For precocial species, Lack (1967) proposed that clutch size was regulated by nutrients available to females at the time of egg laying. His original hypothesis was modified to include use of stored nutrient reserves (Ryder 1970). The dichotomy represented in Figure 1 is one in which most females are either held below maximum potential fitness by availability of nutrients before or during breeding (Fig. 1a) versus one in which most females are at some fitness optimum for clutch size, defined by selection occurring on clutch size during, or following, egg laying (Fig. 1b).

Although depletion of a nutrient reserve is a necessary condition for regulation of clutch size by that nutrient reserve, nutrient reserve depletion does not, by itself, demonstrate that a given nutrient reserve reg-

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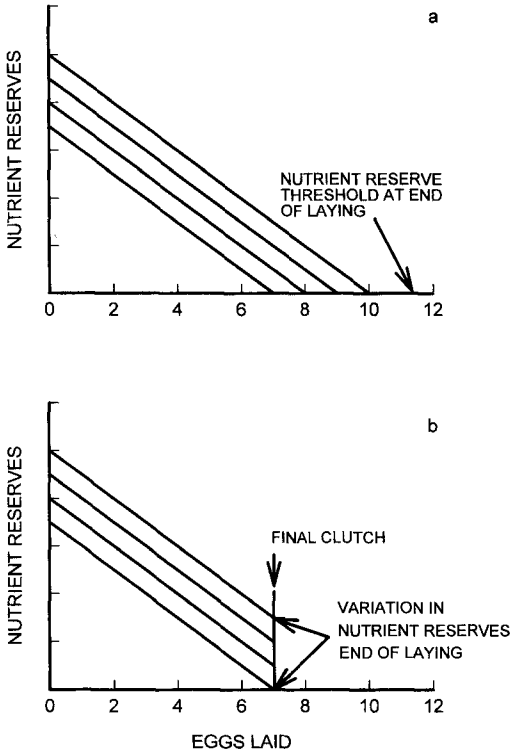


FIGURE 1. Two extreme hypotheses regarding the relationship between nutrient reserves and clutch size. In (a), clutch size is regulated by nutrient reserves. Females beginning laying with greater nutrient reserves reach the minimum threshold of reserve level after having laid more eggs than females beginning laying with smaller nutrient reserves. All females end laying with approximately the same nutrient reserves but after having laid different numbers of eggs. In (b), an ultimate factor regulates clutch size to be 7 eggs. Therefore, initial nutrient reserve size is uncorrelated with clutch size, which is constant. Females will vary in the amount of remaining nutrient reserves at the end of laying. An intermediate hypothesis (not shown) is that minimum nutrient reserve thresholds vary among females but that nutrient reserves still regulate clutch size. Under this hypothesis, the relationship between nutrient reserves and clutch size would be more variable than in (a).

ulates clutch size. Additional analyses are required to establish the relationship between clutch size and nutrient reserves. This is especially true for lipid, which is the principal endogenous nutrient used by females to help meet energy requirements during incubation (Afton and Paulus 1992). Therefore, for lipid, the potential exists to tradeoff investment in the clutch against investment in incubation (Ryder 1970, Ankney and MacInnes 1978, Ankney and Alisauskas 1991), and lipid may not directly limit clutch size even though it declines during egg laying. We are not necessarily suggesting that these tradeoffs occur within females, that

is, individual females may conserve relatively more reserves at the end of laying in some years than in others. We believe it is more likely that such tradeoffs occur among phenotypes and genotypes at the population level.

To directly test hypotheses regarding regulation of clutch size by lipids, Ankney and Afton (1988) examined the correlation between number of rapidly developing follicles (RDFs), an estimate of the remaining reproductive potential of females, and remaining lipid reserves in Northern Shovelers (*Anas clypeata*). The analysis was restricted to females late in the laying sequence (≥ 5 post-ovulatory follicles), so the maximum number of RDFs was less than the maximum number (6) detectable in an ovary. Ankney and Afton (1988) interpreted a positive correlation between number of RDFs and reserves as evidence for regulation of clutch size by the nutrient reserve in question. Recently, this method has been modified by replacing number of rapidly developing follicles with nutrient reserves yet to be committed to reproduction (product of nutrients depleted per egg \times number of RDFs) (Esler and Grand 1994).

CORRECTION FOR DATE AND BODY SIZE

DATE

Some index of calendar date, such as Julian date, can be used to quantify lateness in the nesting season. It likely is not date *per se* that is of interest, but rather its relation to exogenous food abundance which increases as spring progresses (Murkin and Kadlec 1986, Murkin et al. 1992). For example, paired ducks arrive before food is abundant, and some species, particularly diving ducks, may store additional nutrient reserves after arrival (Alisauskas et al. 1990, Afton and Ankney 1991, Alisauskas and Ankney 1994). Therefore, measurement of food abundance will make an important contribution to future studies of clutch size regulation.

It is common to detect a seasonal decline in fat reserves during nesting (e.g., Hohman 1986) which may occur in both breeding and nonbreeding females (Alisauskas and Ankney 1994). Because time and nutrients are finite, breeding waterfowl face ecological constraints that amount to processing adequate nutrients at a physiologically feasible rate. Reynolds (1972) reasoned that interplay among (a) nutritional condition of waterfowl on the breeding grounds before nesting, (b) their ability to store additional nutrients on breeding grounds before nesting, and (c) relative dates at which (a) and (b) occur, would modify levels of nutrient reserves that would be critical to possess before attempting to lay eggs. Alisauskas and Ankney (1994) concluded that Reynolds' hypothesis for Ruddy Ducks (*Oxyura jamaicensis*) was correct: those females that nest relatively late in the season begin to do so with significantly fewer fat reserves than do females that nest earlier (Alisauskas and Ankney 1994).

BODY SIZE

Not all of a bird's somatic protein and mineral are a "reserve." Clearly, even under periods of starvation, a bird cannot fully utilize all its somatic protein and mineral. Thus, its true reserve of, say, protein is that difference between (A) what it currently has, and (B)

the amount it would have left if starved to the point of debilitation or death. Thus, of two birds with equal somatic protein, but one bird structurally larger, the larger bird actually has a smaller *reserve* because at point B (above) it would have more somatic protein left than the smaller bird. Therefore, residuals from a regression of somatic protein on some measure of body size provide a better estimate of size of *reserves* than do absolute values of protein mass. Although the foregoing applies equally to somatic mineral, we assert that it applies less well, and perhaps not at all, to somatic lipid.

Unlike somatic protein and mineral, the absolute mass of a bird's neutral lipids (Dobush et al. 1985) represents the size of its lipid *reserve*. Therefore, we conclude that controlling for among-bird variation in body size when analyzing somatic lipids may be, in fact, an "over-correction" that could result in overestimating size of reserves for smaller birds and, vice versa, for larger birds. This could obscure important patterns of interest, e.g., use of lipid reserves during egg production. Body size, however, may define an upper limit to fat storage and such constraints may be of ecological relevance.

TESTS OF REGULATION OF CLUTCH SIZE BY NUTRIENT RESERVES

CORRELATION BETWEEN RDFS AND REMAINING NUTRIENT RESERVE

Examining the correlation between RDFS and remaining nutrient reserves in females late in the laying sequence as a test of the hypothesis that nutrient reserves regulate clutch size contains a potentially spurious correlation, increasing the potential for type I errors. If nutrient reserves decline during egg laying, irrespective of whether the nutrient reserve regulates clutch size, females earlier in their own laying sequence will contain both more nutrient reserves and more rapidly developing follicles. Therefore, both nutrient reserves and RDFS are negatively correlated with stage of laying, which can create a positive correlation between nutrient reserves and RDFS, even if there is no relationship between clutch size and nutrient reserves.

To demonstrate the potential for stage of laying to influence the correlation between nutrient reserves and RDFS, we simulated data, based on a study of Northern Pintails by Mann and Sedinger (1993). We created 100 samples of 100 females drawn from a normal distribution of lipid reserves with the same mean and variance as that for pre-laying females. We then randomly assigned each female a clutch size from the clutch size distribution range for Northern Pintails in interior Alaska (Petrula 1994). Mean (\pm SE) correlation between clutch size and nutrient reserves in our 100 samples of females was $r = 0.034 \pm 0.011$, $P > 0.05$. We randomly assigned each female a stage of laying, as a proportion of her clutch, when she was "collected." Number of eggs already laid, based on stage of laying at the assigned time of collection, was subtracted from each female's assigned clutch size to calculate number of RDFS at time of collection. Each female also was randomly assigned a rate of depletion of nutrient reserves based on the mean slope of the relationship between remaining reserves and nutrients in-

vested in the clutch. We used the mean slope (11.7 g lipid egg⁻¹) (Esler and Grand 1994) with a standard deviation equal to 20% of the mean. We estimated each female's remaining lipid reserves at collection by subtracting the product of grams of lipid depleted for each egg produced \times number of eggs laid, from initial lipid reserves of each female. We excluded any female for which number of RDFS would underestimate number of eggs yet to lay, based on her assigned clutch size, stage of laying, and the maximum number of RDFS (i.e., 6) detectable in an ovary. We then examined the correlation between RDFS and lipid reserves. In the simulated sample, for which there was no relationship between lipid reserves and clutch size, RDFS and remaining lipid reserve were highly correlated (mean $r \pm$ SE = 0.65 ± 0.01 , $P < 0.01$). This simulation demonstrates the potential for correlations between RDFS and remaining nutrient reserves to give misleading results, relative to the role of nutrient reserves in regulation of clutch size. Several studies have relied on this method to test for regulation of clutch size (e.g., Ankney and Afton 1988, Ankney and Alisauskas 1991, Alisauskas and Ankney 1994, Esler and Grand 1994). Alisauskas and Ankney (1994) and Thompson (1996) compared a procedure that eliminated the correlation among RDFS, nutrient reserves, and stage of laying with one that did not and found no difference in results. Other studies, however, should be reevaluated.

USE OF RESIDUALS FROM THE REGRESSION OF NUTRIENT RESERVES ON REPRODUCTIVE NUTRIENT TO TEST FOR REGULATION OF CLUTCH SIZE

The first attempt to eliminate the spurious correlation associated with stage of laying was by Mann and Sedinger (1992), who controlled for stage of laying by using residuals from the regression of nutrient reserves on reproductive nutrients. Females with large residuals, i.e., greater nutrient reserves at a given stage of laying, should have a greater potential to lay additional eggs (more RDFS) than females with smaller residuals under an hypothesis that nutrient reserves regulate clutch size. Therefore, under such an hypothesis, RDFS should be positively correlated with nutrient reserve residuals.

We believe that accounting for clutch size in tests of hypotheses of clutch size regulation (see below) is superior to the use of nutrient reserve residuals in most cases. Nevertheless, there may be circumstances, such as relatively small samples, when use of nutrient reserve residuals provides superior statistical power in hypothesis tests of clutch size regulation.

USE OF ANALYSIS OF COVARIANCE TO TEST FOR REGULATION OF CLUTCH SIZE BY NUTRIENT RESERVES

If nutrient reserves regulate clutch size and there is an average minimum threshold that determines cessation of egg laying, then females with different clutch size phenotypes must lie on different trajectories of nutrient reserves versus investment in reproduction having different y-intercepts (Fig. 1a). Females that initiate laying with smaller reserves will, on average, reach the minimum nutrient reserve threshold after laying fewer eggs than will females initiating laying with larger nu-

trient reserves. Under this model, the appropriate test for regulation of clutch size by nutrient reserves is Analysis of Covariance (ANCOVA) with final clutch size as a fixed factor and investment in reproduction as the covariate. Significant variation in adjusted mean levels (relative height of nutrient reserve trajectories) is evidence for regulation of clutch size by nutrient reserves if females with larger nutrient reserves, at a given stage of egg laying, have more RDFs and will ultimately have larger clutches.

Slopes of nutrient reserve trajectories may vary among clutch size phenotypes, which will result in a significant interaction between clutch size and investment in reproduction in the ANCOVA. Also, among-female variation in minimum nutrient reserve thresholds or in egg size and composition (Alisauskas and Ankney 1994) will result in unexplained variation around trajectories. Recent studies (Alisauskas and Ankney 1994, Esler and Grand 1994) have detected seasonal trends in depletion of nutrient reserves; later females rely less on nutrient reserves to produce eggs than do earlier nesters. Initiation date of RFG could be incorporated into the ANCOVA as a second covariate. The result would be a series of planes responding to different clutch size phenotypes. Sections through these planes corresponding to a specific date would produce a series of parallel lines as in Figure 1a.

COMPLICATING FACTORS

At least three different factors may contribute variation in analysis of use of nutrient reserves during clutch formation. First, if the reserve threshold that terminates laying varies among females (Ankney and Alisauskas 1991), variation will exist in the relationship between remaining reserves and RDFs. Phenotypes that terminate laying at lower nutrient reserve levels can, on average, produce more eggs from a given initial nutrient reserve. Lipid and protein are used to meet energy/nutritional requirements both during egg production and incubation, and we, therefore, anticipate the potential for tradeoffs between investment of these nutrients in eggs and investment in incubation.

Second, if females vary in their access to exogenous nutrients, rate of depletion of endogenous reserves could vary among females with the same clutch size (or be equal among females with different clutch sizes). Correlation between initial reserve levels and habitat quality could cause the slopes of trajectories in Figure 1a to vary systematically with initial reserve size. For example, if "poor quality" females begin laying with small nutrient reserves and occupy poor quality habitats, thereby reducing availability of exogenous nutrients, they would deplete nutrient reserves more rapidly than would higher quality females. Such a correlation would produce a divergence of trajectories, compared to parallel trajectories in Figure 1a, whereby females initiating laying with smaller reserves would have a steeper trajectory of nutrient reserves compared to females beginning laying with larger reserves. Such a divergence in nutrient reserve trajectories would produce greater variation in clutch size relative to initial nutrient reserves than indicated in Figure 1a.

Third, the specific nutrient (lipid, protein or mineral) limiting clutch size may vary among females within a

population. Such variation among females would cause apparent minimum nutrient thresholds to vary among females. For example, if clutch size in female A was limited by mineral reserves while that of female B was limited by lipid reserves, female A might have larger lipid reserves at cessation of laying than did female B. Variation among females in the most limiting nutrient might also contribute to variation in rate of decline in nutrient reserves when considered singly.

For many species of waterfowl, the analysis proposed here must be restricted to females late in laying when final clutch size can be estimated, as is true for the correlation approach currently in use. This restriction will increase the difficulty of acquiring an adequate sample in the field. Nonetheless, the ANCOVA approach we advocate, especially when date and body size are controlled for, offers numerous advantages over previous analytical methods. Most importantly, the approach presented here eliminates the potential for spurious correlations, associated with stage of laying, inherent in the correlation between remaining RDFs and nutrient reserves. ANCOVA also allows explicit considerations of models that simultaneously account for stage of laying, date of initiation of RFG, rate of depletion of nutrient reserves, and clutch size. We recommend this approach for future studies of nutrient reserve dynamics and regulation of clutch size.

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