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SHORT COMMUNICATION

A revised methodology for prediction of egg production *Calanus finmarchicus* from preserved samples

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We describe here a refinement of the method for the estimation of egg production rates of the planktonic copepod, Calanus finmarchicus, from formaldehyde-preserved samples. We present criteria for classifying pre-spawned clutches as either high, intermediate or low in number. The method allows reproductive assessment of preserved females without the need for staining and is capable of accurate asssessments in food-limited environments.

During maturation, the gonads of planktonic copepods undergo distinct morphological changes associated with oocyte development [e.g. (Sømme, 1934; Kimoto *et al.*, 1986; Runge, 1984, 1985; Niehoff and Hirche, 1996; Niehoff, 1998)]. These changes are readily visible in preserved females and have been used to identify stages of gonad development in studies of copepod reproduction [e.g. (Marshall and Orr, 1955; Runge, 1985; Batchelder, 1986; Smith, 1990; Hirche and Niehoff, 1996)].

Runge (Runge, 1987) established a quantitative reproductive index (RI) which allows the empirical prediction of egg production from preserved samples. Using histological techniques, Niehoff and Hirche (Niehoff and Hirche, 1996) elucidated the reproductive processes underlying the morphological changes and refined the RI approach as applied to the marine planktonic copepod *Calanus finmarchicus*. This method directly predicts the proportion of spawning females, i.e. the spawning frequency.

Since *C. finmarchicus* releases a batch (clutch) of eggs during a spawning event, egg production is a function not only of spawning frequency but also of clutch size (Runge and Roff, 2000). Clutch size at a given body size (Runge and Plourde, 1996) was assumed to be approximately constant relative to the spawning frequency, which was influenced by environmental conditions (Runge, 1987; Carlotti and Hirche, 1997; Hirche *et al.*, 1997). However, recent studies have confirmed that clutch size can vary significantly, by a factor of two or three, with food supply and female age (Niehoff *et al.*, 1999; Rey *et al.*, 1999; Niehoff, 2000). Experiments have demonstrated the correspondence between the number of mature oocytes in female *C. finmarchicus* and clutch size under variable feeding conditions [(Niehoff and Hirche, 1996); B. Niehoff, unpublished data]. Niehoff and Hirche (Niehoff and Hirche, 1996) determined the expected clutch size from histological sections but this method is time-consuming, limiting its application as a standard procedure.

Here we propose a revision to the existing gonad classification system that provides an estimate of the expected clutch size and thus more accurately predicts egg production from preserved samples. We present a modified gonad classification system, which we apply to samples and data collected during the US GLOBEC Northwest Atlantic/Georges Bank Program.

In species of *Calanus* the ovary is located dorsally. From its anterior end, two diverticula extend anteriorly into the head region and posteriorly along each side of the thorax to the genital pore on the first urosomal segment (Hilton,

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1931; Lowe, 1935; Niehoff and Hirche, 1996; Niehoff, 1998). When stage CV moults to female, the diverticula are typically empty. During the active reproductive period, oocytes pass from the ovary into both diverticula, where they increase in size, the most mature stages occurring ventrally. Early oocyte development involves cell growth and vitellogenesis 1, a slow and endogenous synthesis of yolk [(Hilton, 1931; Arnaud *et al.*, 1982; Niehoff and Hirche, 1996; Niehoff, 1998); for review of yolk synthesis in crustaceans see Harrison (Harrison, 1990)]. Niehoff and Hirche (Niehoff and Hirche, 1996) divided oocytes in this phase of development into two stages (OS1 and OS2) according to their size and morphology. Final maturation involves rapid cell growth, incorporation of yolk and lipid droplets, and nuclear changes (Niehoff and Hirche, 1996). These oocytes, which form the most ventral layer in the gonads of mature females, are also distinguishable in two developmental stages, OS3 and OS4 (Niehoff and Hirche, 1996). The stage OS3 is characterized by a prominent follicle cell layer, the occurrence of numerous lipid and yolk droplets and a rapid increase in size. In stage OS4, the nucleus has disappeared and condensed chromosomes in metaphase 1 are visible. These oocytes constitute the next clutch; the next dorsal layer of oocytes starts final maturation either just before or just after release of a clutch, depending on food conditions. The presence of stages OS3 and OS4 thus represents an index of spawning activity (Niehoff and Hirche, 1996; Niehoff, 1998). Detailed morphological differences between OS3 and OS4 can only be observed in histological sections; however, the presence (OS3) or absence (OS4) of a nucleus can be seen under stereomicroscopic examination of the whole female.

Four gonad development stages have been described that are associated with these oocyte development stages (Niehoff and Hirche, 1996). GS1 to GS3 are characterized by the presence of oocytes in stages OS1 and OS2. GS4 represents mature females carrying stage OS3 and/or OS4, depending on the timing of preservation during the final maturation cycle. The proportion of GS4 females in a preserved sample is an index of the proportion of spawning females in a population.

The presence of stage GS4, however, does not by itself provide information on clutch size (Niehoff and Hirche, 1996). Under food-limited conditions, the number of oocytes, especially in OS3 and OS4, is lower than in well-fed females (B. Niehoff, unpublished data). In a previous approach to the RI, the relationship between egg production rate (EPR) and the proportion of females carrying mature oocytes was obtained empirically by linear regression (Runge, 1987). The empirical relationship is directly applicable to the particular region under study and represents a quasi-average of conditions affecting clutch size during the period in which the calibration measurements were made. Including the number of maturing oocytes in a prediction of spawning frequency would yield a more biologically correct and accurate estimate of EPRs at a given station. In particular, use of the old RI may overestimate EPRs at stations that are severely food-limited, where, although females are predicted to spawn, the clutch size might be significantly lower than the regression mean.

We have modified the classification of gonad development stages presented by Niehoff and Hirche (Niehoff and Hirche, 1996) as follows. Instead of staining females with borax carmine, we used the natural colour from formaldehyde preservation (Runge, 1987) to distinguish between developing oocytes (OS1 and OS2) and oocytes undergoing final maturation (OS3 and OS4). With borax carmine, stages OS1 and OS2 stain dark red; in formaldehyde-preserved females they are clear and

Table I: Modified gonad maturation stages of female C. finmarchicus after Niehoff and Hirche (Niehoff and Hirche, 1996) and Runge (Runge, 1987)

GS1	Anterior and posterior diverticula are empty, diverticula walls visible as thin lines
GS2	Developing oocytes (OS1 and OS2, either transparent or lightly opaque) are present in one row in both anterior and posterior diverticula. In late OS2, multiple layers of OS1 and OS2 are found in the anterior diverticula
GS3	Multiple layers of OS1 and OS2 in both anterior and posterior diverticula. Gonads carrying oocytes in transition to maturity (late GS3 or GS3–4) contain early OS3 visible as a lightly coloured ventral layer; these oocytes are <90 μm in diameter
GS4	Medium to dark brown oocytes >90 μm undergoing final maturation (OS3 and OS4) forming the most ventral layer in the gonads. Layers of OS1 and OS2 are located dorsally
A:	OS3 or OS3 and OS4 are densely packed in multiple layers, pouches are prominent
B:	OS3 or OS4 form one solid row, oocytes touching but less densely packed than GS4A; pouches in posterior diverticula are absent or rudimentary
C:	OS3 or OS4 are loosely packed, posterior pouches are always absent

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opaque. Stages OS3 and OS4, which stain red only lightly, are medium to dark brown in preserved females (Niehoff *et al.*, 1999). The differences in staining properties are the result of the incorporation of lipid and yolk droplets during final maturation (Niehoff and Hirche, 1996), and the same processes cause the different natural colours of preserved oocytes. The general characteristics of GS1, GS2 and GS3 (Table I; Figure 1A–C) are similar to the descriptions by Niehoff and Hirche (Niehoff and Hirche, 1996). Late GS3 can be in a transition stage (GS3–4) to GS4 which represents mature females. The ventral layer in transition GS3–4 consists of early OS3 oocytes, which are just sufficiently coloured to be distinguished from OS1 and OS2. Mature gonads (GS4), borne by females that are predicted to spawn within 24 h, are generally characterized by the occurrence of OS3 and OS4 oocytes appearing as a medium to dark brown band in the ventral part of both anterior and posterior diverticula (Figures 1D and 2).

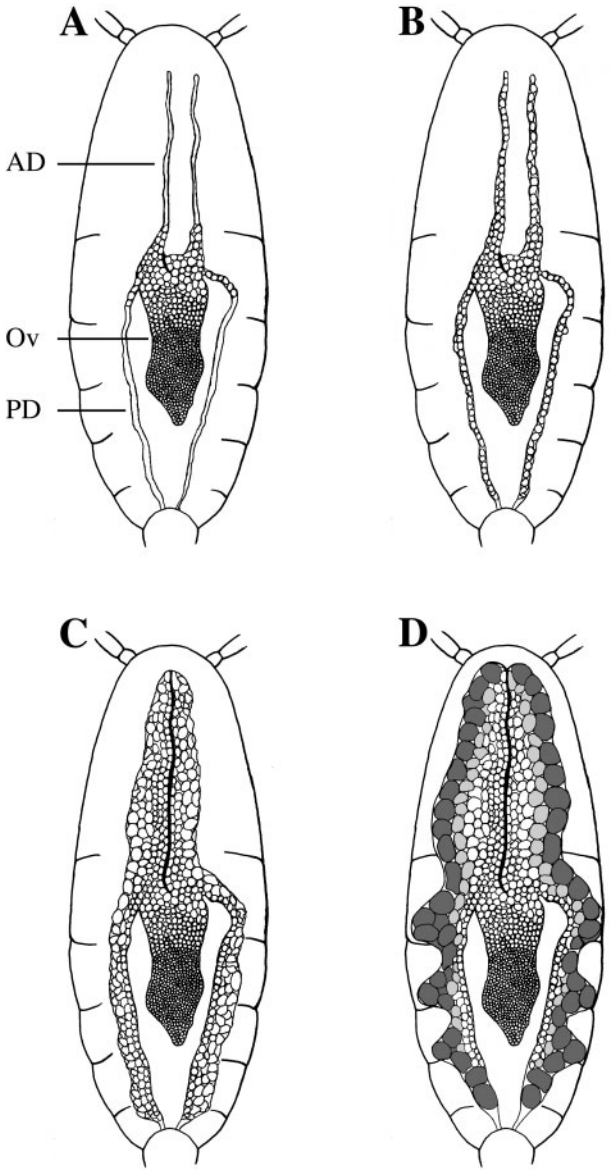

Fig. 1. Gonad development stages (GS) of *C. finmarchicus* (dorsal view): non-spawning females, (**A**) GS1, (**B**) GS2, (**C**) GS3; mature females ready to spawn, (**D**) GS4. The appearance of GS4 as presented here is that of GS4A as characterized by two layers of oocytes in final maturation processes [light grey, early or advanced oocyte development stage (OS) 3; dark grey, late OS3 or OS4]. AD, anterior diverticulum; PD, posterior diverticulum; Ov, ovary.

The GS4 stage is subdivided into three new categories, GS4A, GS4B and GS4C (Table I; Figure 2), according to the quantity of oocytes predicted to be released during the spawning event. GS4A represent well-fed females carrying high numbers of maturing oocytes. Maturing oocytes are densely packed and form several layers, especially visible in the anterior diverticula. In the posterior diverticula, prominent pouches of OS3 or OS4 oocytes are present (Figure 2A,B). Often, in these females, a layer of maturing OS3 can be found above the ventral layer of either OS3 or, closer to the actual spawning event, OS4 oocytes. Females in GS4A carried >50 maturing oocytes (>25 per side); however, dense packing makes an accurate count difficult. In the intermediate state, GS4B, only one row of either OS3 or OS4 oocytes, forming a continuous brown band in the anterior and posterior diverticula, was found (Figure 2C,D). The number of mature oocytes per side ranged from 15 to 24. Pouches in the posterior diverticula, if present, were only rudimentary. In females in GS4C, representing clutch sizes observed under moderate to severe food limitation, OS3 or OS4 were very loosely packed and not necessarily touching (Figure 2D,E). Usually there were 15 <OS3 or OS4 oocytes per side.

The mean expected clutch size was determined for each GS4 state, A, B and C, from process cruises conducted on various dates between 1994 and 1999 during the US GLOBEC Northwest Atlantic/Georges Bank Program (Wiebe *et al.*, 2002). Clutch size was measured by direct observation [(Runge and Roff, 2000) procedural details provided below]. The mean clutch size differed significantly between stations and dates (Runge *et al.*, unpublished data). For each station and sampling date, the proportion of GS4A, GS4B and GS4C was determined by assessment of females preserved at the time of collection of animals for direct observation. The clutch size data for each station and date were then grouped according to the percentages of GS4A, B and C in the preserved sample. For example, at a station with 60% females determined to be GS4A, 30% females in GS4B, and 10% females in GS4C, the upper 60% of the clutch sizes were assumed to be spawned by the females in A,

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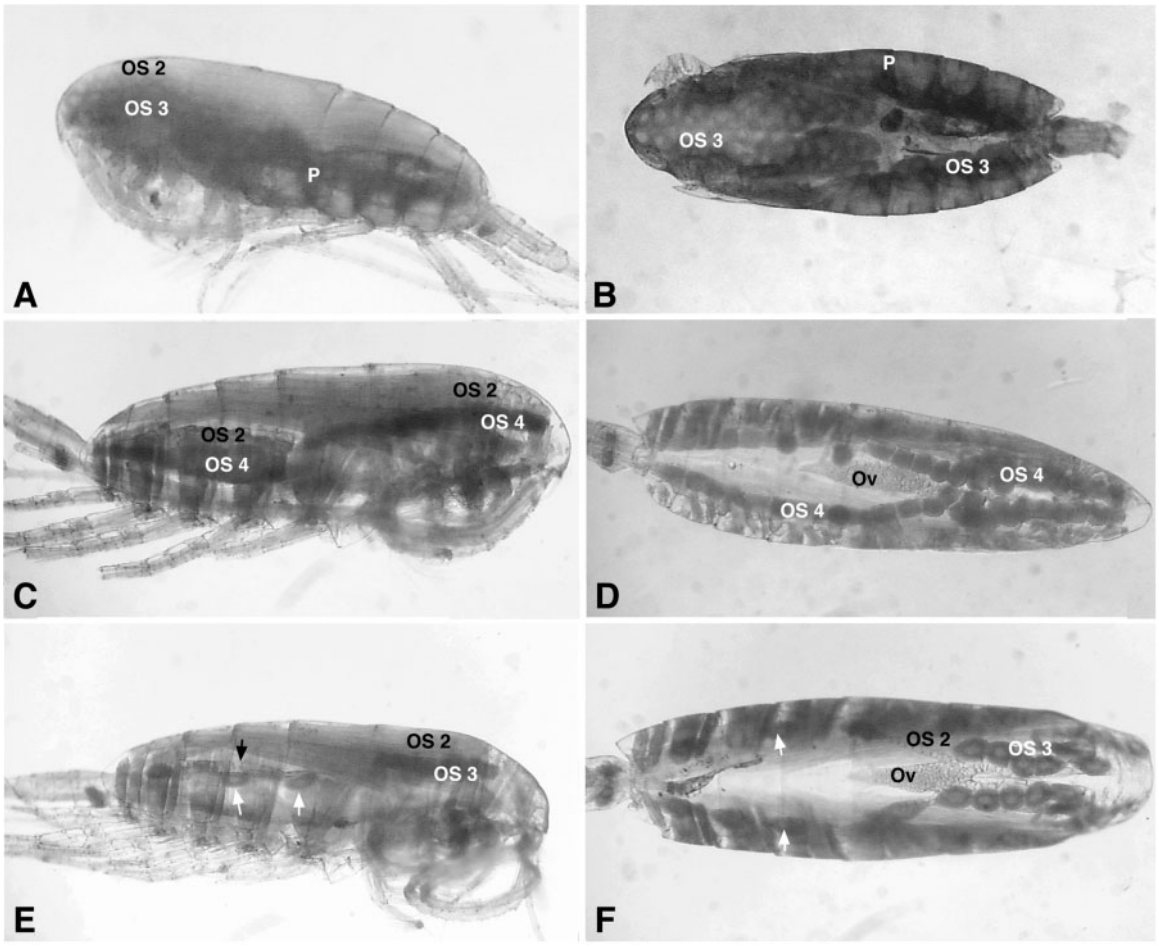

Fig. 2. Refinement of gonad develpment stages in mature *C. finmarchicus* females (GS4): (**A**,**B**) substage GS4A; (**C**,**D**) substage GS4B; (**E**,**F**) substage GS4C. (A,C,E) Lateral view. (B,D,F) Ventral view, limbs and ventral part of the carapace removed. White arrows indicate OS3, black arrows OS2 (E). OS, oocyte development stage; P, pouches; Ov, ovary.

the following 30% by females in B and the lowest 10% by females in C. From these groupings over all stations and dates, a mean clutch size was calculated for each of these three substages (Table II). By this procedure, the mean expected clutch sizes were found to be 61, 36 and 24 eggs, for substages GS4A, GS4B and GS4C, respectively. Although a quantitative comparison was not conducted, these results corresponded well to the direct counts of maturing oocytes in the females in the various substages.

In accordance with Runge (Runge, 1987) and Niehoff and Hirche (Niehoff and Hirche, 1996), the EPR (eggs per female per day) of *C. finmarchicus* on Georges Bank was estimated from a preserved sample by the proportion of females in each of the GS4 states and the clutch size assigned to each state, using:

$$\mathrm{EPR} = CS_{\mathrm{A}}(\mathrm{GS4A}/N) + CS_{\mathrm{B}}(\mathrm{GS4B}/N) + CS_{\mathrm{C}}(\mathrm{GS4C}/N) \quad (1)$$

where CS_{A}, CS_{B} and CS_{C} are, respectively, 61, 36 and 24 eggs per clutch, GS4A, GS4B and GS4C are the numbers of subsampled females in each of the GS4 states and N is the total number of preserved females in the subsample. It was found that, in some instances, it was difficult to decide whether a female was GS4B or

Table II: Mean expected clutch sizes (CS: no. of eggs per clutch) of C. finmarchicus females estimated from GS4 substages

Substage	CS	SE	Minimum	Maximum	*N*
GS4 A	61	0.59	10	147	849
GS4 B	36	0.70	3	59	262
GS4 C	24	1.57	2	46	62

Standard error (SE), minimum and maximum CS attributed to each substage and total number of CS used to determine each category mean.

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GS4C, in which case it was placed in a GS4BC category, with a clutch size of 30 eggs, and added to the terms in the equation above.

The among-investigator reproducibility of the new stage classification was tested during a 5-day workshop. Workshop members were trained in the revised methodology, including the opportunity to discuss and provide feedback for refinement of staging criteria using individual specimens displayed by a camera–microscope hook-up to a video screen. After the training session, four to seven blind subsamples, each containing 25–38 preserved females, were given to members of the workshop for intercomparison. Each observer independently evaluated the proportion of females in each of the GS4 states. The resulting estimates of EPR (Table III) were close, with a coefficient of variation between 2 and 32%. The higher value was associated with a low EPR; the absolute values of estimates on this test sample were very similar (Table III). It can be concluded from this test that the criteria for evaluation of spawning states and clutch size classification are robust and reproducible among trained observers.

The estimated EPR based on the above equation compares well with the EPR observed with shipboard incubations made during the US GLOBEC Northwest Atlantic/Georges Bank process cruises. Zooplankton for the direct observations were captured with a 1 m diameter ring net towed at low speed either vertically or obliquely from near bottom to the surface. The catch was diluted into gallon jars containing filtered sea water maintained at a temperature close to the ambient temperature found at 10 m depth. Female copepods were immediately sorted (usually within 1 h of capture) under a binocular microscope. Care was taken to select females at random, without regard for appearance, unless there was evidence of damage to the exoskeleton. Forty *C. finmarchicus* females were placed individually into polystyrene Petri dishes (50 ml capacity) containing filtered sea water. The dishes were kept in a 12 h dark:12 h dim light, controlled temperature incubator at a temperature approximating to ambient at 10 m depth. They were inspected at ~8-h intervals for the presence of eggs, which were counted and then removed at each interval. The number of eggs per spawning event (clutch size, used in the determination of clutch number in states A, B and C described above) was counted. The daily EPR was calculated from the total number of eggs produced in 24 h divided by the total number of incubated females. Typically, subsamples of the live catch at each station were preserved in 4% borax-buffered formaldehyde for analysis of gonadal state, although occasionally, preserved females were sorted from plankton samples collected with oblique water column net tows either just before or during the 24 h incubation. The slope of a least-squares regression of directly observed EPR on the predicted EPR is not significantly different from 1 (Figure 3).

Table III: Among-observer comparison of egg production estimates based on new criteria for reproductive assessment (Table I)

Sample	No. of observers	Mean	CV (%)	Range
1	5	40	8	35.4–42.7
2	5	3	32	1.9–4.2
3	5	14	7	12.5–15.2
4	5	34	2	32.7–34.8
5	4	47	8	42.5–51.0
6	3	46	11	40.9–50.4
7	3	42	25	32.0–53.0

Each observer was given four to seven samples (25–38 female copepods per sample) to analyse for number of GS4A, B and C females in each sample. Mean egg production rate (eggs per female per day, calculated as described in text), coefficient of variation (CV as percentage of mean) and range of estimates.

The designation of clutch sizes for GS4 states A, B and C (Table II) was based on a large number of observations of *C. finmarchicus* on Georges Bank. A significant relationship was recorded between clutch size and female prosome length in these data (Runge *et al.*, unpublished data), but the r^2 was low (0.2). The adjustment of the mean clutch number in each maturity state for variations in body size was recorded, but could not improve upon the relationship shown in Figure 3. Similarly, it was not found that consideration of ambient temperature improved the results presented here.

While the clutch size data used to designate the number of eggs in each clutch and the direct observations of EPR were obtained from the same females, the two estimates are independent under the assumption that the clutch size designations for states A, B and C are generally applicable to *C. finmarchicus* on Georges Bank (at least for the period of study). Neither the number of eggs per clutch nor the measurement of gonadal state determining the frequency of spawning was used in the direct determination of EPR. Strictly speaking, the relationship in Figure 3 represents a calibration, valid for Georges Bank during the period between 1995 and 1999. We have confidence that the RI method applied to preserved samples of female *C. finmarchicus* collected during this period accurately represents *in situ* EPRs. However, the appropriateness of the clutch size designations for regions other than Georges Bank has not yet been determined. We recommend empirical measurement of clutch size and calibration of predicted to observed EPR for each particular region under study, until such time as generalities become clear.

The method we describe here addresses limitations in the previous methods for the estimation of *Calanus* spp. EPR from preserved samples (Runge, 1987; Niehoff and

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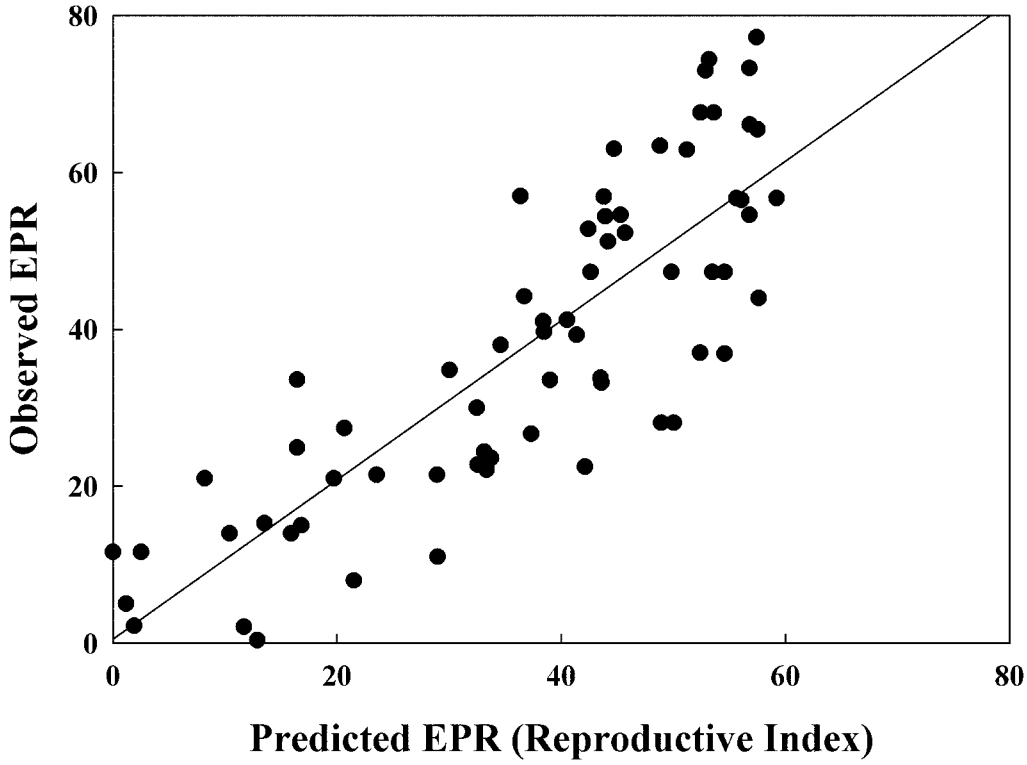

Fig. 3. Predicted egg production rate (EPR; eggs per female per day), estimated from analysis of preserved samples of females and calculated as described in the text, compared with the observed EPR at 45 stations during the US GLOBEC process cruises to Georges Bank between 1995 and 1999. The total number of comparisons in the plot is 68 because some stations were sampled on successive days. Results of a least-squared regression analysis: observed EPR = 1.02(predicted EPR) + 0.5 (r^2 = 0.70).

Hirche, 1996). The criteria for distinguishing which females will spawn derive from the gonad maturity system described by Niehoff and Hirche (Niehoff and Hirche, 1996) and are quick to apply because they are based on a straight microscopic examination without the need for previous staining with borax carmine. The empirical procedure for specifying clutch sizes provides a method for estimating the numbers of eggs released under different food conditions without laboriously counting the mature oocytes in the gonads.

The estimation of copepod EPR from preserved samples has a number of applications in studies of copepod population dynamics and trophic linkage. In the Georges Bank Northwest Atlantic/Georges Bank Program, the method provides data for two-dimensional mapping of EPR (eggs per female per day) and population EPRs (eggs m^{-2} day^{-1}) from broadscale survey cruises made during the same time period as the process cruises (Wiebe *et al.*, 2002). For the broadscale surveys, preserved zooplankton samples were collected at a grid of stations across Georges Bank, but no shipboard incubations conducted. In addition to providing information on the spatial and temporal variation in copepod recruitment, predicted EPRs from the RI can be used to estimate mortality rates of early life stages (Ohman *et al.*, 2002), and potential production of prey for fish in the early stages of life (Runge *et al.*, 2000).

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