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Instantaneous Reproductive Effort in Female American Oysters, *Crassostrea virginica,* Measured by a New Immunoprecipitation Assay

KWANG-SIK CHOI[1,*], ERIC N. POWELL[1,†], DONALD H. LEWIS[2], AND SAMMY M. RAY[3]

[1] *Department of Oceanography and* [2] *Department of Veterinary Pathobiology, Texas A&M University, College Station, Texas 77843, and* [3] *Department of Marine Biology, Texas A&M University at Galveston, Galveston, Texas 77550*

Abstract. An immunoprecipitation assay was developed for measuring instantaneous reproductive effort in female American oysters, *Crassostrea virginica.* Oysters were injected with ^{14}C-leucine and incubated *in situ* for 1 to 30 h periodically throughout the annual gametogenic cycle. Gonadal protein labeled with ^{14}C-leucine was precipitated from an oyster homogenate with rabbit anti-oyster egg IgG as the primary antibody. Antibody-oyster egg protein complex was further purified by immunoadsorption with staphylococcal protein A cell suspension. The quantity of oyster eggs was determined by single-ring immunodiffusion. A mathematical model was developed to calculate the instantaneous reproductive rate of oysters and to estimate the number of days required from the initiation of gonadal development to spawning. The oyster population was lightly to moderately infected with a protozoan parasite, *Perkinsus marinus.* A negative correlation between the intensity of infection and the rate of gonadal production suggests that *P. marinus* retards the rate of gamete development. The seasonal cycle of gamete production determined by direct measurements of egg protein production was not equivalent to that determined by standard gonadal-somatic index (GSI), except at the most basic level. GSI was highest during the spring spawning peak, but the rate of gamete production was highest in the fall. Accordingly, the two measurements, rate *versus* standing crop (volume of gonad), reveal a substantially different picture about the details of the spawning season. Estimates of the time required to reach spawning condition ranged from several weeks to 1 or 2 months; these values agree with published estimates derived from less direct methods. Direct rate measurements thus seem to accurately reflect the true rate at which gametic tissue is produced in the field. A positive correlation between oyster size and the estimated days to spawn suggests that larger oysters require longer to prepare to spawn. Furthermore, the range in observed somatic and gametic growth emphasizes the conservatism of somatic growth and the volatility of gonadal growth that is borne out by the results of population dynamics models of oysters.

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* Current address: Environmental Radiological Assessment Dept., Korea Institute of Nuclear Safety, P.O. Box 16, Daeduk-Danji, Taejeon, 305-606, Korea.

† Person to whom correspondence should be addressed.

Introduction

Like other bivalves, the American oyster (*Crassostrea virginica*) goes through three stages during the gametogenic cycle: (1) proliferation of germ cells, (2) vitellogenesis, or the growth and enrichment of gametes, and (3) spawning (Sastry, 1979). Egg constituents such as lipids and proteins accumulate during vitellogenesis (Kennedy and Battle, 1964). These constituents are either mobilized and synthesized from nutrient reserves in other organs or synthesized directly from compounds assimilated during feeding (Soniat and Ray, 1985; Shafee, 1989; Thompson and MacDonald, 1990; Suzuki *et al.,* 1992). Spawning occurs when the cumulative reproductive biomass reaches a certain proportion of the standing stock (Hofmann *et al.,* 1992; Choi *et al.,* 1993). Reproduction is an energetically costly process because much of the net production allocated to reproduction is lost as gametes during

spawning. The fraction of net production diverted to gamete production is called the reproductive effort. Reproductive effort in bivalves is often expressed as the total number or weight of eggs or sperm released per individual or biomass (Sprung, 1983; Deslous-Paoli and Héral, 1988; Hofmann *et al.*, 1992; Choi *et al.*, 1993).

Environmental conditions such as food availability, water temperature, salinity, and parasitism can influence growth and reproduction of oysters in various ways. For example, an increase in water temperature increases the filtration rate of oysters and also results in the diversion of more of the net energy to reproduction, resulting in increased fecundity or spawning frequency (Hofmann *et al.*, 1992). *Perkinsus marinus*, a protozoan parasite, exerts a significant impact on oyster growth and reproduction. Higher infection intensities may retard growth, delay reproductive development, or reduce spawning intensity and frequency (White *et al.*, 1988a, b; Wilson *et al.*, 1988). Infection intensity and prevalence is in part governed by salinity and water temperature. Powell *et al.* (1992) reported that regional and temporal shifts in the infection intensity of *P. marinus* in oysters in the Gulf may be controlled by long-term climatic cycles.

The reproductive effort of oysters can be examined using two approaches. Firstly, the number and developmental stage of the gametes can be assessed. This is essentially a standing crop measurement. Histology is one of the most common methods used for this assessment (Ford and Figueras, 1988; Barber *et al.*, 1988; Gauthier and Soniat, 1989; Wilson *et al.*, 1990). However, the analysis, at best, is only semiquantitative. Recently Choi *et al.* (1993) introduced an immunological technique that allows a quantitative evaluation of reproductive effort of oysters. They reported that the weight of gametes produced per spawn by oysters in Galveston Bay, Texas, amounted to as much as 40% (eggs) or 44% (sperm) of the total weight. Secondly, the rate of gamete production can be measured—for example, by measuring the rate of synthesis of gonadal protein. We will term this the instantaneous rate of gamete production. The two approaches are complementary because environmental factors may affect fecundity by varying either the number of gametes per spawn (measured by standing crop) or the spawning frequency (controlled by the rate of gamete development).

No studies have reported the instantaneous rate of gamete production in oysters because an adequate method was lacking. Accordingly, we developed a technique to measure the rate of gonadal synthesis of gametic protein in oysters based upon a radiolabeled amino acid tracer and an immunological probe. Rabbit anti-oyster egg IgG (Choi *et al.*, 1993) and staphylococcal protein A, an immunoadsorbent, were employed in a radio-immunoprecipitation assay to measure the rate of production of gonadal protein. The present study focuses on the seasonal variation in the rate of gonadal synthesis in female oysters and the effect of environmental conditions such as water temperature and *Perkinsus marinus* parasitism on that process.

Materials and Methods

Field procedures

Adult oysters (7 to 12 cm in length) were collected from Confederate Reef, Galveston Bay (Fig. 1) from August 1990 to July 1991. Collections were made monthly during the reproductive season and less frequently during the remainder of the year. Water temperature and salinity were recorded during each sampling. To administer the ^{14}C-labeled leucine, a rock saw was used to make a v-shaped notch at the posterior end of each oyster shell, and 1.5 to 2 μCi of ^{14}C-labeled L-leucine was injected into the adductor muscle. The oysters were then placed in a

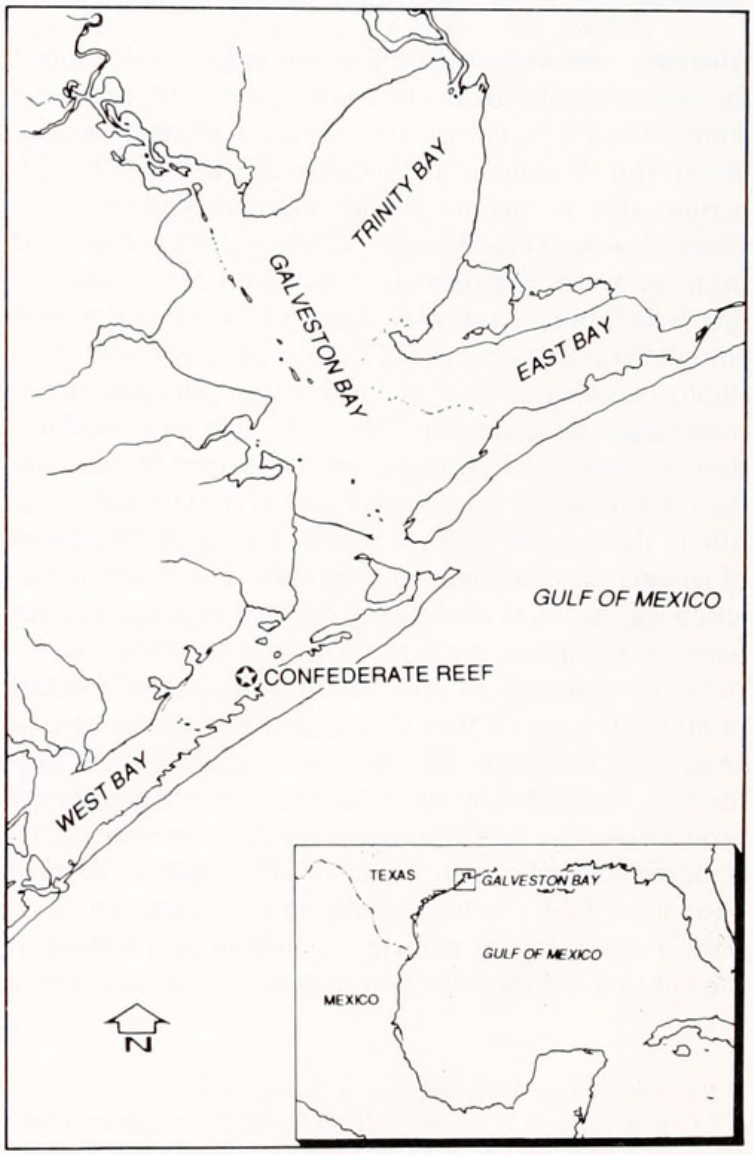

Figure 1. Location of study area, Confederate Reef in West Bay, Texas.

nylon-mesh bag and incubated *in situ* according to the experimental design. Table I summarizes the data collection efforts. After incubation, oysters were opened and the maximum length of the upper shell was recorded. A piece of mantle tissue was taken from each oyster and placed into a 10-ml culture tube containing fluid thioglycollate medium fortified with antibiotics according to Ray (1966) to assess the intensity of infection by *P. marinus*. A narrow cross-sectional slice was taken from the central part of the body and fixed in a 25-ml Bouin's solution for the examination of gametogenic stage. The remaining oyster flesh was frozen on dry ice, transported to the laboratory, and stored at −40°C until used.

Table I

Data collection efforts during the study

Sampling Date	^{14}C-leucine injected (μCi/oyster)	Incubation time (h)	Number assayed
08/16/90	2	10, 20, 30	15
09/26/90	1.5	1, 2, 5, 10	16
10/29/90	1.5	1, 2, 5, 10, 30	50
02/01/91	1.5	1, 2, 5, 10, 30	25
03/19/91	1.5	1, 2, 5, 10, 30	25
04/16/91	1.5	1, 2, 5, 10, 30	25
05/23/91	1.5	1, 2, 5, 10, 30	25
07/15/91	1.5	1, 2, 5, 10, 30	25

Laboratory procedures

The samples of mantle tissue were incubated in thioglycollate medium for 2 weeks (Ray, 1966), at which time the infection intensity of *P. marinus* was scored from 0 (uninfected) to 5 (heavily infected) according to Mackin (1962) as modified by Craig *et al.* (1989).

The frozen oyster samples were thawed and excess water was removed. First the total wet weight of each oyster was measured, then the body mass containing gonadal material was separated from the other somatic tissues and weighed. Wet weight was converted to dry weight using the empirical relation: dry weight = wet weight × 0.196 (Choi *et al.*, 1993). After a known volume of phosphate buffered saline (PBS; 0.15 *M* NaCl, 0.003 *M* KCl, 0.01 *M* phosphate, pH 7.3) was added, the body tissue was homogenized first in a glass syringe tissue grinder and then in an ultrasonicator. The homogenate was centrifuged at 1200 × *g* for 10 min, and the volume of supernatant recorded. The quantity of total protein in the supernatant was determined using the BCA Protein Assay (Pierce, Illinois) with bovine serum albumin as the protein standard.

The quantity of oyster eggs was measured using rabbit anti-oyster egg serum (Choi *et al.*, 1993) and a single-ring immunodiffusion assay according to Mancini *et al.* (1965) and Gallagher *et al.* (1988). A 1.5% (w/v) agarose gel was prepared in barbitone buffer (0.01 *M* sodium barbital, 0.02 *M* barbital, and 0.05% sodium azide as preservative, pH 8.6). A 2-ml aliquot of oyster egg-specific rabbit antiserum was mixed with 18 ml agarose gel and poured onto a 10 × 10 cm glass plate. The gel was solidified in a refrigerator for 10 min and 4-mm-diameter wells were made using a gel puncher. On each plate, 20-μl aliquots of various concentrations of oyster egg protein (positive controls), reproductively inactive female oyster homogenate (negative control), and the oyster homogenate to be analyzed were added to the wells and incubated for 72 h in a humid container at 4°C. After incubation, the plate was pressed, dried, stained with 1% Coomassie brilliant blue (w/v) (Bio-Rad, California) dissolved in 50% ethanol and 10% glacial acetic acid for 2 h, and destained in 50% ethanol and 10% glacial acetic acid for 2 h. After the plate was stained and destained, the diameter of the ring-shaped antigen-antibody precipitates was measured to 0.1 mm and converted to square millimeters. A nonlinear regression curve was constructed from the positive controls to quantify the amount of egg protein. Biomass of the eggs was then estimated from the single-ring immunodiffusion assay results and the following empirical relationship: total dry weight of egg = oyster egg protein (mg ml^{-1})/0.370 (percent weight of protein in oyster egg) × total homogenate volume (ml). A gonadal-somatic index (GSI) was calculated as the ratio of dry weight of oyster eggs to dry weight of total oyster.

^{14}C-labeled leucine used in the synthesis of gonadal protein and other somatic protein was analyzed using protein A immunoprecipitation and trichloroacetic acid (TCA) protein precipitation. For measuring the amount of ^{14}C-leucine incorporated into somatic protein, 1 ml of oyster homogenate was brought up to 10% TCA with 50% TCA to precipitate all proteins in the homogenate. The TCA-oyster homogenate mixture was placed in an ice-filled bucket, incubated for 30 min, and centrifuged. After centrifugation, the supernatant was saved for further analysis of free leucine. TCA-precipitated protein was then rinsed twice with 100% ethanol, dissolved using Solusol (National Diagnostics, New Jersey), and measured using a liquid scintillation counter. The counter readings were corrected for quench by internal standards (Gordon, 1980).

The amount of free leucine in oysters at the time of sacrifice was analyzed from the TCA supernatant by use of an amino acid analyzer with a lithium citrate buffer system and *o*-phthalaldehyde as the detecting agent. Norleucine was added as the internal standard. The eluent fraction containing leucine was collected from the amino acid analyzer with a fraction collector, mixed with Soluscint A (National Diagnostics, New Jersey) and counted

using a liquid scintillation counter. Quench was corrected for as previously described.

The incorporation of ^{14}C-leucine into oyster eggs was estimated using immunoprecipitation with staphylococcal protein A (*Staphylococcus aureus* Cowan I strain cell suspension, Sigma) as an immunoadsorbent (Kessler, 1975). Rabbit anti-oyster egg IgG (2 mg ml^{-1}) was reacted with 1 ml of oyster homogenate (containing up to 2 mg egg protein ml^{-1} as determined from the single-ring immunodiffusion assay) in a test tube and incubated for 1 h at room temperature. After incubation, 100 mg ml^{-1} protein A cell suspension was added according to the manufacturer's directions, incubated for 1 h, and centrifuged at 4000 $\times$ *g* for 10 min. The antibody-antigen-protein A complex was rinsed twice with 10 ml PBS and then dissolved in a liquid scintillation solution (Solusol—National Diagnostics, New Jersey). The amount of ^{14}C-leucine in the eggs was determined by liquid scintillation counting as previously described for the TCA precipitate.

Model

Perspective

Three important, but conservative, assumptions are required to use radiolabeled leucine as a tracer in this study. First, oysters do not distinguish ^{14}C-leucine from unlabeled leucine present in oysters, so both isotopes are used indiscriminately during protein synthesis and degradation. Second, the amount of ^{14}C-leucine injected into the oysters is small enough not to disturb the amino acid balance. Third, the labeled leucine does not exchange with somatic or gonadal components by any process other than protein synthesis and degradation.

In order to calculate the instantaneous rate of gonadal production (the amount of gonadal material produced per unit time), the measured rate of incorporation of labeled leucine must be corrected by the specific activity of the free leucine pool (Samarel, 1991). Stated mathematically,

$$dg/dt = [dg^*/dt]\,[f(t)/f^*(t)] \qquad (1)$$

where g is the amount of leucine incorporated (moles g dry wt^{-1}), g^* is the amount of labeled leucine incorporated (dpm g dry wt^{-1}), t is time, f is the amount of free leucine (moles g dry wt^{-1}), and f^* is the amount of labeled free leucine (dpm g dry wt^{-1}).

The experimental protocol requires that f^*, the amount of labeled free leucine, be a function of time because a constant perfusion technique was not used. Because most animals undergo a stress response to experimental manipulation which results in changes in the free amino acid pool, f, the amount of free leucine present, is also likely to be a function of time. As a result, evaluation of equation (1) first requires an evaluation of $f(t)$ and $f^*(t)$.

Most pulse-labeling experiments utilize a number of replicates to permit calculation of the mean effect and the variation about the mean. Such experiments are based on the assumption that all individuals are initially equivalent, to the extent permitted by the normal stochastic variation about the mean. Experiments on reproduction, however, frequently do not meet this assumption, particularly those run in the field where temperature cannot be manipulated to control the reproductive cycle. The individuals used in the experiments we describe could be expected to be in different stages of their reproductive cycle, as indeed they were. Accordingly, no true replicates exist in this set of experiments, and the mean value is not necessarily meaningful for all interpretations. Consequently, equation (1) ideally would be solved separately for each individual.

The problem posed as equation (1) cannot be solved separately for every individual, however. Nevertheless, components of it can be solved for single individuals, provided that the specific rates of some components can be assumed to be common to all individuals. The specific rates that are important in the solution of equation (1) are those controlling the loss of labeled leucine from the free leucine pool, the changes in concentration of the free leucine pool, the rate of uptake of free leucine into the gonadal protein pool, and the rate of loss of leucine from the gonadal protein pool. Because the rates of uptake and loss from the gonadal pool are unlikely to be equivalent in all individuals, we must assume that the rates of processes controlling the specific activity of labeled leucine $[f(t)/f^*(t)]$ are equivalent. This assumption is reasonable because the loss of labeled leucine probably involves diffusional and metabolic processes common to most individuals, and the change in the free leucine pool involves a stress response to a manipulation common to all individuals. In particular, the amount of labeled leucine used in the formation of gonadal tissue is small relative to the amount injected. Accordingly, variations in the metabolic processes involved in reproduction had little effect on the total available pool of labeled leucine.

Calculation of specific activity—the labeled free leucine pool

We assume that the loss of labeled leucine from the free leucine pool is a first-order process. Accordingly,

$$df^*/dt = -kf^* \qquad (2)$$

where k is the first-order rate constant (time^{-1}). Comparison of the results obtained by evaluating equation (2) with the measured values shows that equation (2) does not adequately describe the change in labeled free leucine in the free leucine pool over the experimental time course. Assuming that the labeled leucine exists in two separate pools is, however, much more satisfactory. Hence,

$$df_1^*/dt = -k_1 f_1^* \quad (3)$$

and

$$df_2^*/dt = -k_2 f_2^* \quad (4)$$

where $f_1^* + f_2^* = f_{\text{total}}^*$, the measured value.

The two-pool model does not necessarily imply that only two pools exist or that the pools are continuously discrete. Failure of equation (2) to predict the measured results requires a multipool model if the processes are first-order. Experience indicates that equations (3) and (4) are good curve-fitting routines and often adequately fit data from multiple pools. Accordingly, in using equations (3) and (4), we do not necessarily conclude anything about the processes determining the time course of labeled leucine except that a multiple pool model is required.

We solved equations (3) and (4) using the boundary conditions $t = t_\circ$ at $f^*(t) = f_\circ^*(t)$. We cannot generally set $t_\circ$ equal to zero, the time of injection, because the time course before the first sampling is unknown. Initially, the specific activity would be controlled by processes affecting the distribution of the label throughout the animal as well as by tissue-specific metabolism. Accordingly, the experimental protocol necessitates that $t_\circ$ be the time of the first sampling (usually 1 h). As a consequence, data from the first sampling cannot be used to evaluate any subsequent process rate. That sampling only defines the metabolic milieu at the beginning of the measured time course.

Solving equations (3) and (4) yields

$$f^*(t) = f_1^*(t) + f_2^*(t) = f_{\circ 1}^* e^{k_1(t_\circ - t)} + f_{\circ 2}^* e^{k_2(t_\circ - t)}. \quad (5)$$

We define $f_\circ^* = f_{\circ 1}^* + f_{\circ 2}^*$ as the mean amount of labeled leucine observed in the first sampling period. The two first-order rate constants, k_1 and k_2, and the fraction of the labeled free leucine in each pool, $f_{\circ 1}^*/f_\circ^*$, were obtained iteratively by computer by searching for the values

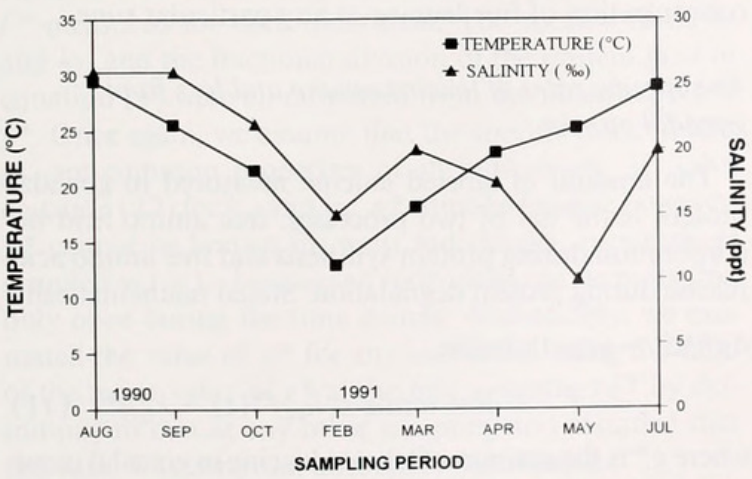

Figure 2. Water temperature (°C) and salinity (‰) at each sampling period.

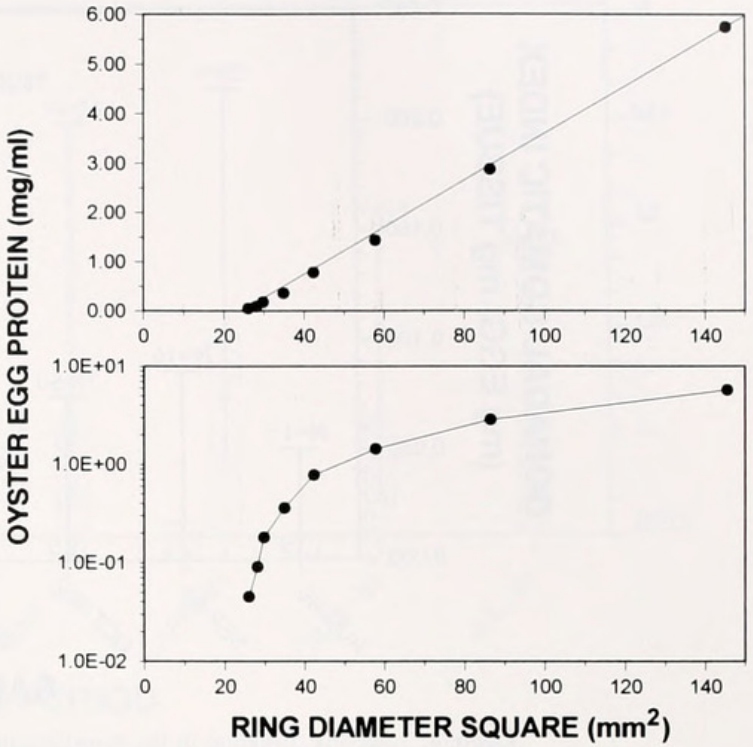

Figure 3. A typical standard curve for the single-ring immunodiffusion assay for rabbit anti-oyster egg serum.

yielding the best fit to the observations using a chi-square-type error term to evaluate the goodness-of-fit.

As stated earlier, if the specific rates are assumed equal among all individuals (that is, the specific rates describe a process common to all individuals), then the value for $f^*(t)$ for any individual can be obtained by solving equation (5) using the value of $f_\circ^*$ for that individual. In essence, this assumes that the variation between individuals is produced by the efficiency of injection—some animals received more label than others—rather than the processes controlling loss after injection. Comparing the data obtained during January, where metabolic processes were slowed by low temperatures, with all other observations, suggests that this assumption is valid. The success of injection is the primary determinant controlling the variation in the amount of labeled free leucine available for use in metabolism during the experiment.

Calculation of specific activity—the free leucine pool

The free leucine pool was also not stable during the experimental time course. Typically, free leucine rose in concentration during the first few hours and then declined. This transient rise in concentration was found in most months and probably represents a response to the stress of injection. The invertebrate stress response to experimental manipulation typically includes protein breakdown and a rise in the concentration of free amino acids.

The free amino acid pool is governed by a balance between the addition of amino acid from protein breakdown

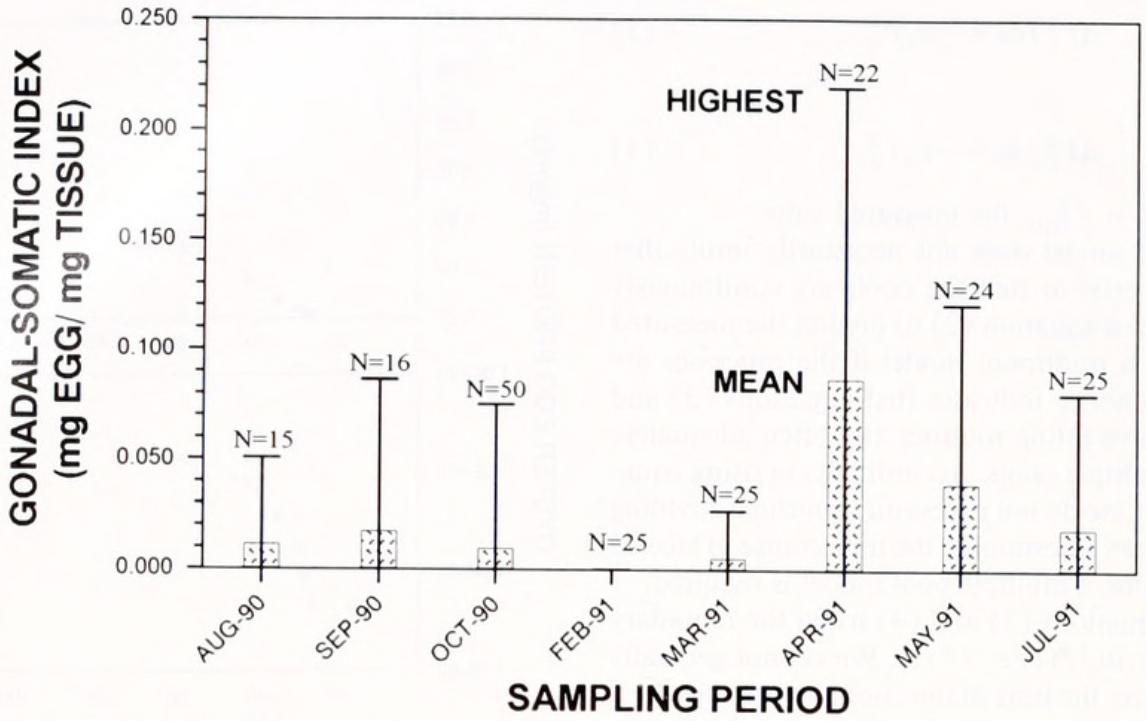

Figure 4. Seasonal variation in the gonadal-somatic index (GSI) of oysters. The mean (bar) and the range (highest and lowest values) are plotted.

or digestive processes and the loss of amino acid by anabolic and catabolic processes. Accordingly, and assuming that all processes are again of first-order,

$$df/dt = \text{production terms} - \text{loss terms} = k_3P(t) - k_4f \quad (6)$$

where $P(t)$ is the precursor pool and k_3 and k_4 are specific rates. $P(t)$ can be described as the result of a first-order reaction analogous to equation (2), where

$$dP/dt = -kP \quad (7)$$

Once again, evaluation of equation (6) generally showed that a one-pool model was inaccurate. A two-pool model generally provided an accurate description of the measured values:

$$df_1/dt = k_3P_1(t) - k_4f_1 \quad (8)$$

and

$$df_2/dt = k_5P_2(t) - k_6f_2 \quad (9)$$

where $f_1 + f_2 = f_{\text{total}}$, the measured value. We assume $f_1/f_2 = P_1/P_2$. Once again, the same caveats and assumptions apply as were discussed in the evaluation of f^*, and the equations for P_1 and P_2 are of the form of equations (3) and (4). Once again, we solved the equations using the boundary conditions $t = t_\circ$ at $f(t) = f_\circ(t)$ and set $t_\circ$ to be the time of the first sampling (usually 1 h). Equation (8) yields

$$f_1(t) = f_{\circ 1}e^{k_4(t_\circ - t)} + [k_3P_{\circ 1}/(k_4 - k_3)]\,[e^{k_3(t_\circ - t)} - e^{k_4(t_\circ - t)}]. \quad (10)$$

Equation (9) yields a similar solution. The four first-order rate constants (k_3, k_4, k_5, k_6) and the fraction of free leucine in each pool, $f_{\circ 1}/f_\circ$ and $f_{\circ 2}/f_\circ$, were obtained iteratively by computer by searching for the values yielding the best fit to the observations using a chi-square-type error term to evaluate the goodness-of-fit.

The values of P_1 and P_2 are unknown. We assume that $P_1 + P_2 = P_{\text{total}}$ is equivalent to the highest value of free leucine measured (f_{total}). To the extent that this is inaccurate, the specific rates calculated (k_3, k_4, k_5, k_6) will not reflect those rates actually present. These values, then, are relative to the estimated protein pool size. However, because these rates are obtained from observations of the free leucine pool and equations (8) and (9) are used solely to estimate f, the use of specific rates scaled to an unknown protein pool size still provides an accurate estimate of the concentration of free leucine at any particular time.

The specific rates of leucine uptake and loss from gonadal protein

The amount of labeled leucine measured in gonadal protein is the net of two processes: free amino acid incorporation during protein synthesis and free amino acid release during protein degradation. Stated mathematically

$$dg^*/dt = \text{growth terms} - \text{loss terms} = k_{gg}f^*(t) - k_{lg}g^* \quad (11)$$

where g^* is the amount of labeled leucine in gonadal tissue and k_{gg} and k_{lg} are the specific rates governing the rates of protein synthesis and degradation, respectively. We use

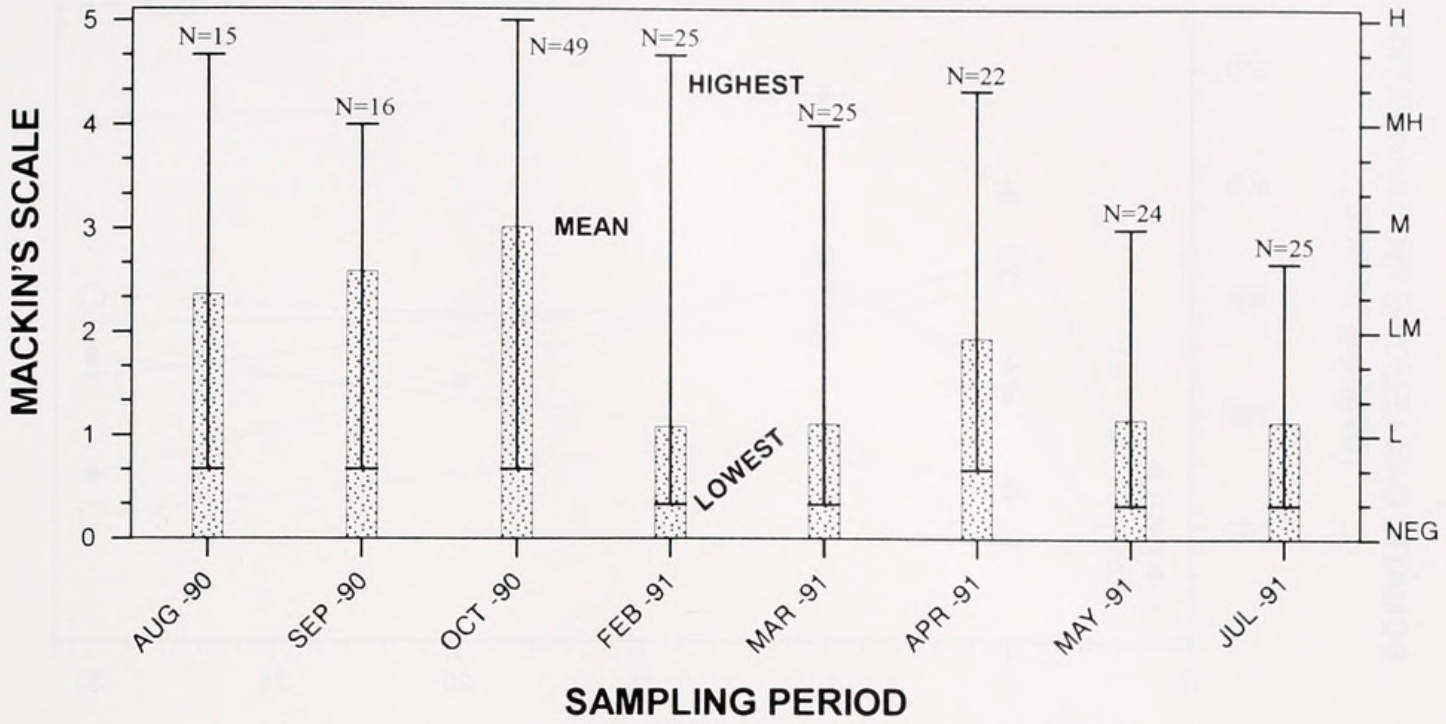

Figure 5. Seasonal fluctuation in infection intensity of *Perkinsus marinus*. The plot includes the mean (bar) and range (highest and lowest values) for each sampling month.

a first-order process for synthesis, rather than zero-order as modeled by Koehn (1991), assuming that the rate of synthesis depends upon substrate supply. Although these rates of reaction can be expected to vary with temperature, we assume that temperature was relatively constant within each experimental time course and so do not include temperature in the equations. Solving (11) yields

$$g^*(t) = g^*_\circ e^{k_{lg}(t_\circ - t)} + k_{gg}[[f^*_{\circ 1}/(k_{lg} - k_1)][e^{k_1(t_\circ - t)} - e^{k_{lg}(t_\circ - t)}] + [f^*_{\circ 2}/(k_{lg} - k_2)][e^{k_2(t_\circ - t)} - e^{k_{lg}(t_\circ - t)}]]. \quad (12)$$

Equation (12) was used to calculate the specific rates (k_{gg}, k_{lg}) for each individual. We obtained $f^*_{\circ 1}$ and $f^*_{\circ 2}$ for each individual using equation (5), and the specific f^* measured for each individual. The specific rates, k_1 and k_2, and the fractional division of the protein pool in equation (5) were all estimated from the mean values of f^*. Once again, we assume that the specific rates, k_1 and k_2, are common properties of all individuals. To solve equation (12) for k_{gg} and k_{lg}, g^* must be known; however, g^* cannot be known for most individuals, except those sampled at $t = t_\circ$, because an individual can be measured only once during the time course. Accordingly, we estimated the value of g^* for any individual using the ratio of the mean value of g^* at the first sampling ($\bar{g}^*$ by definition) to that at any other sampling and assumed that this ratio was common among all individuals.

$$g^*_{\circ i} = [\bar{g}^*(t = t_\circ)/\bar{g}^*(t = t_i)]\, g^*_i(t = t_i) \quad (13)$$

We further assumed that $g^*_\circ e^{k_{lg}(t_\circ - t)} \approx g^*_\circ$. We then obtained k_1 and k_2 using equation (11) by iterative search by computer using the measured value of $g^*(t)$.

The amount of gonadal protein production (g)

Following the arguments for equation (11),

$$dg/dt = k_{gg} f(t) - k_{lg} g \quad (14)$$

Solving equation (14), and recalling that $f(t)$ is the product of two pools, yields

$$g(t) = g_\circ e^{k_{lg}(t_\circ - t)} + k_{gg}[f_{\circ 1}/(k_{lg} - k_4)[e^{k_4(t_\circ - t)} - e^{k_{lg}(t_\circ - t)}] + f_{\circ 2}/(k_{lg} - k_6)[e^{k_6(t_\circ - t)} - e^{k_{lg}(t_\circ - t)}] + k_3 P_{\circ 1}/(k_4 - k_3)]\{[1/(k_{lg} - k_3)(e^{k_3(t_\circ - t)} - e^{k_{lg}(t_\circ - t)})] + [1/(k_{lg} - k_4)(e^{k_4(t_\circ - t)} - e^{k_{lg}(t_\circ - t)})]\} + [k_5 P_{\circ 2}/(k_6 - k_5)]\{[1/(k_{lg} - k_5)(e^{k_5(t_\circ - t)} - e^{k_{lg}(t_\circ - t)})] + [1/(k_{lg} - k_6)(e^{k_6(t_\circ - t)} - e^{k_{lg}(t_\circ - t)})]\}]. \quad (15)$$

Because we are interested in the amount of gonadal material made since the first sampling ($t_\circ$), the first term in equation (15) ($g_\circ e^{k_{lg}(t_\circ - t)}$) can be discarded. The remaining terms include the specific rates previously calculated and the values for the pool sizes $f_\circ$ and $P_\circ$. The latter are determined for individuals as described previously for $f^*_\circ$.

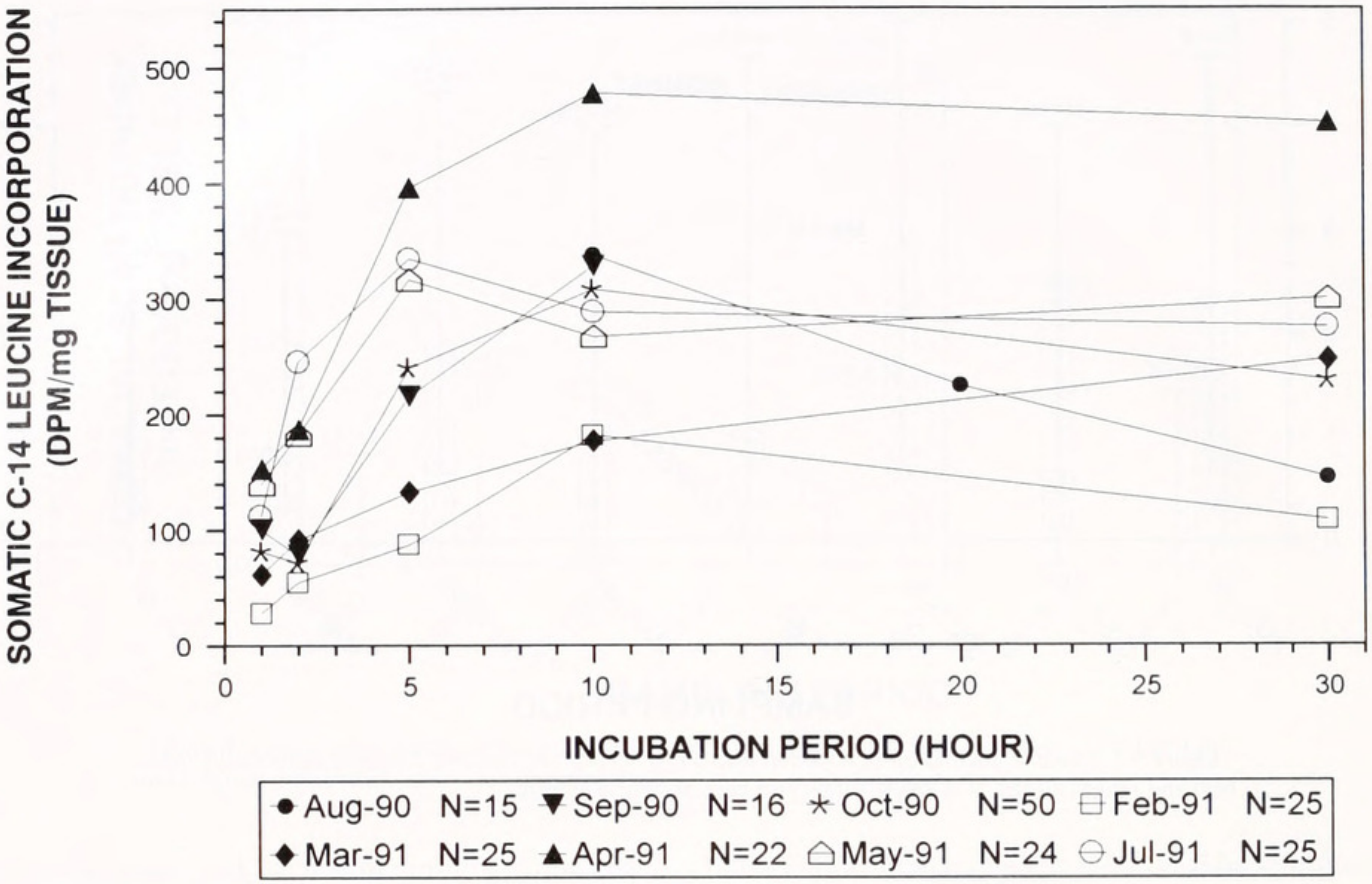

Figure 6. Incorporation of ^{14}C-leucine into somatic protein (dpm mg dry wt^{-1}).

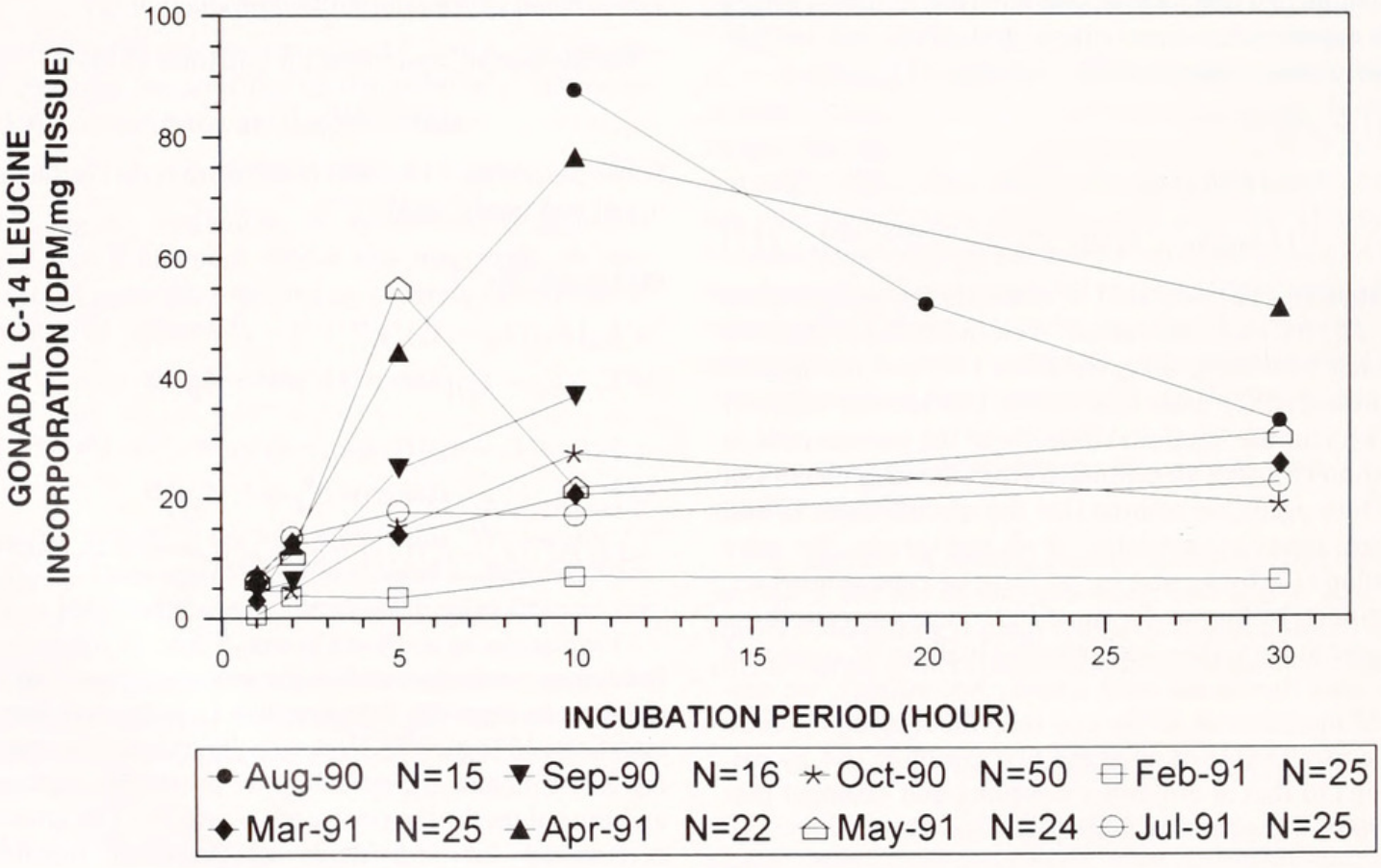

Figure 7. Incorporation of ^{14}C-leucine into egg protein (dpm mg dry wt^{-1}).

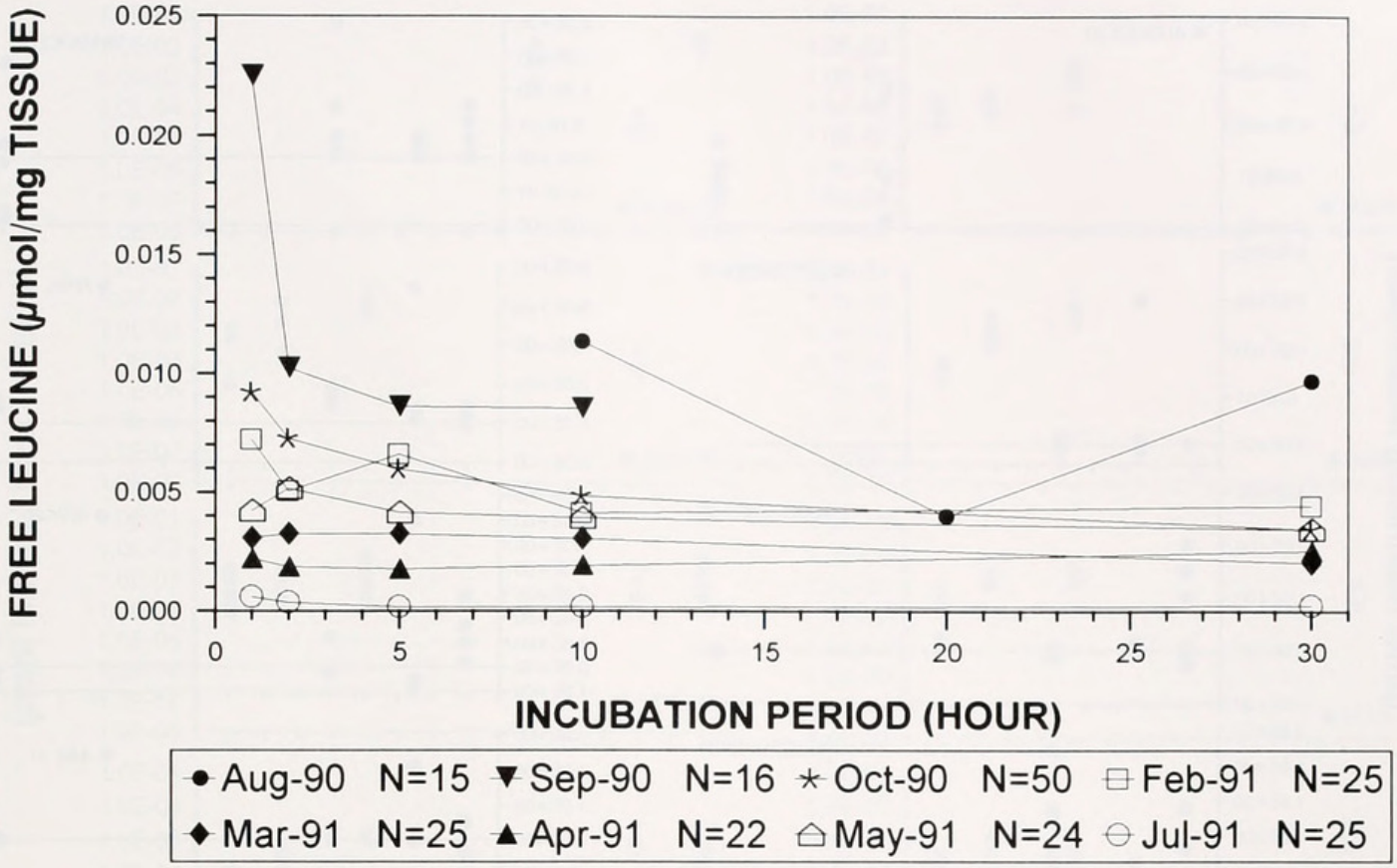

Figure 8. Concentration of free leucine in oysters at the time of sacrifice (μmol mg dry wt^{-1}).

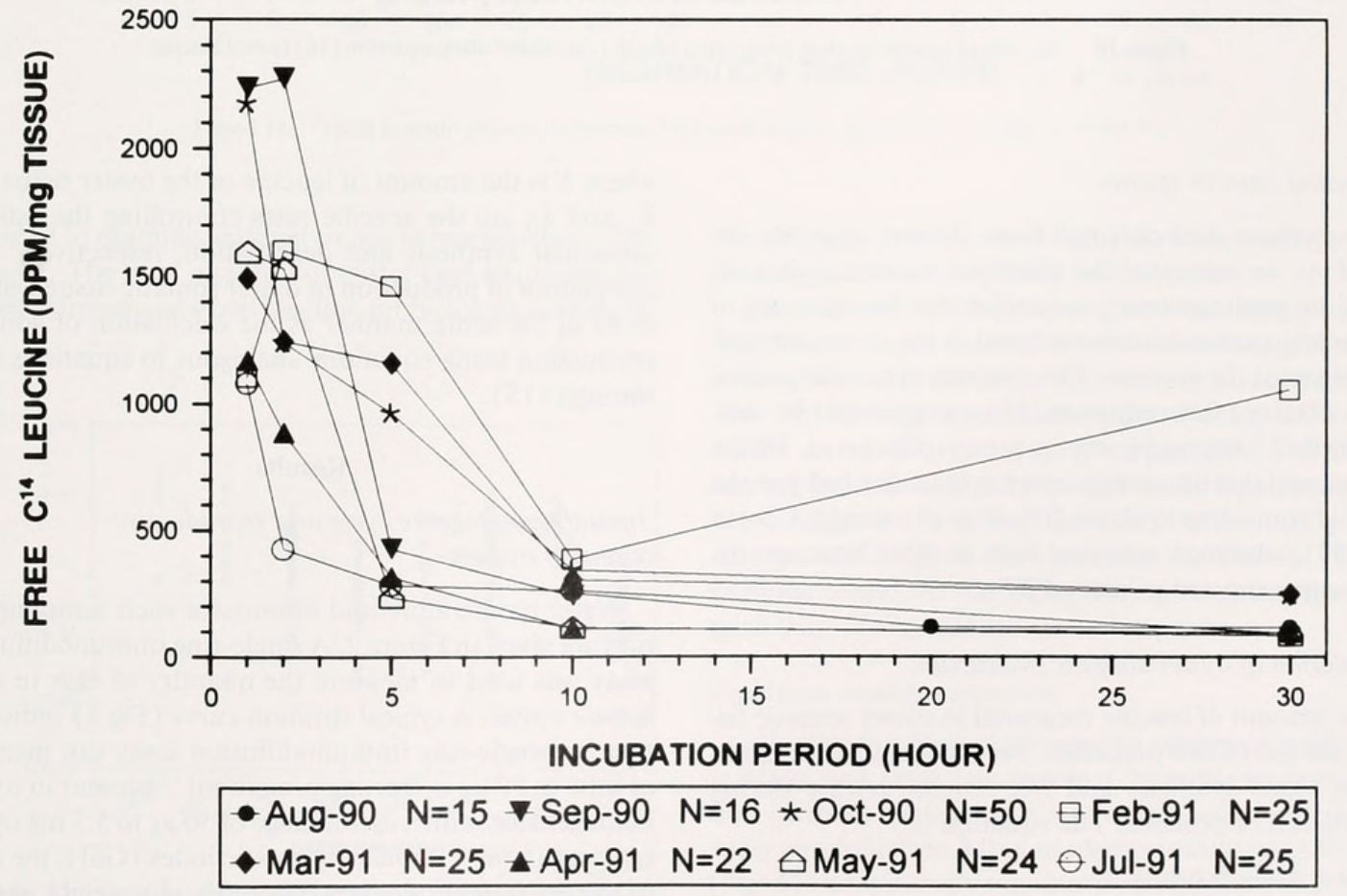

Figure 9. Concentration of free ^{14}C-leucine in oysters at the time of sacrifice (dpm mg dry wt^{-1}).

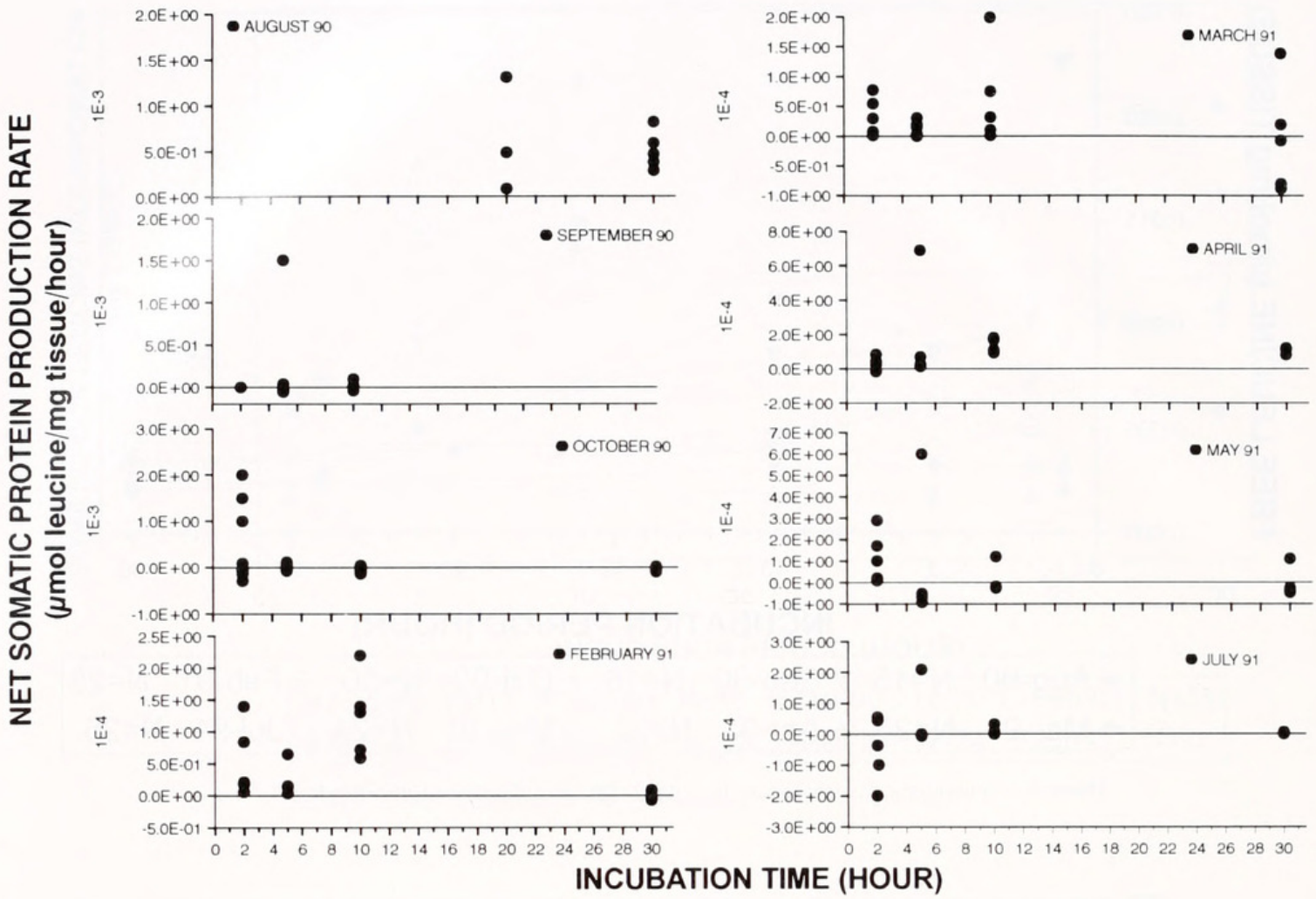

Figure 10. Net rate of somatic protein production (ds/dt) calculated using equation (16) (μmol leucine mg dry wt^{-1} h^{-1}).

Estimated days to spawn

To compare data obtained from different monthly observations, we calculated the estimated number of days required to reach spawning, assuming that the synthesis of egg protein continued uninterrupted at the measured rate. We converted the estimate of the amount of gonadal protein made, obtained from equation (15), to egg weight by multiplying by 2.7 mg egg per mg egg protein (Choi *et al.*, 1993). We assumed that the average oyster at spawning had gonadal material equivalent to about 20% of its dry weight. Choi *et al.* (1993) observed values as high as 40%; however, the mean approximated a value of 20%.

Calculation of oyster somatic production

The amount of leucine measured in oyster somatic tissue is the net of two processes: free leucine incorporation during tissue synthesis and free leucine release during degradation of proteins. The equation is

$$dS/dt = \text{growth terms} - \text{loss terms} = k_{gs} f(t) - k_{ls} S \quad (16)$$

where S is the amount of leucine in the oyster tissue and k_{gs} and k_{ls} are the specific rates controlling the rates of molecular synthesis and degradation, respectively. The calculation of production of oyster somatic tissue was derived in the same manner as the calculation of gonadal production using equations analogous to equations (11) through (15).

Results

Annual gametogenic cycle and reproductive output of oysters

Water temperature and salinity for each sampling period are given in Figure 2. A single-ring immunodiffusion assay was used to measure the quantity of eggs in each female oyster. A typical titration curve (Fig 3) indicated that the single-ring immunodiffusion assay can measure as little as 50 μg oyster egg protein ml^{-1} present in oyster homogenates, with a useful range of 50 μg to 5.5 mg oyster egg protein ml^{-1}. Gonadal-somatic index (GSI), the ratio of the dry weight of oyster eggs to the dry weight of total tissue computed from single-ring immunodiffusion assays,

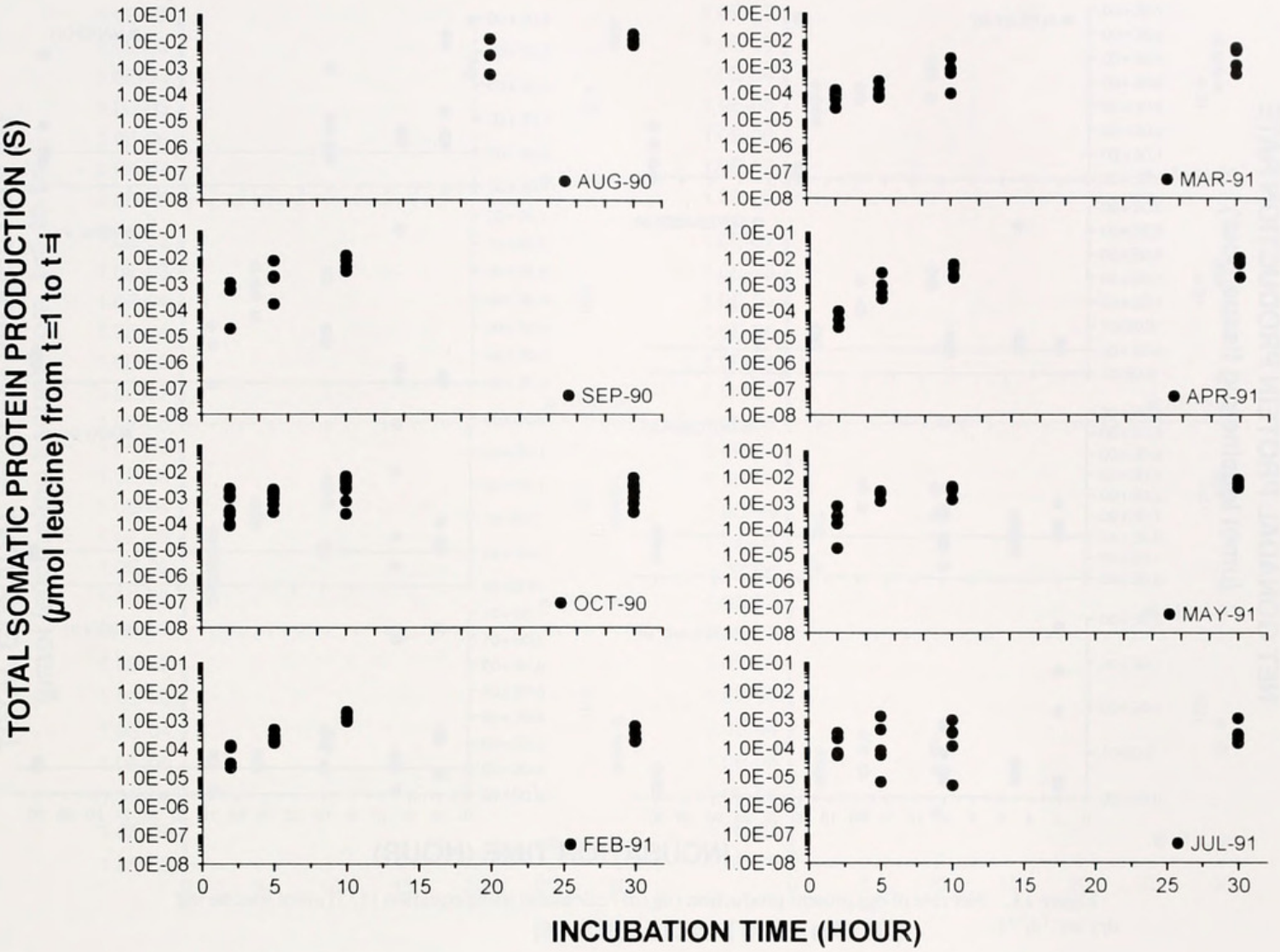

Figure 11. Total somatic protein production (s) (μmol leucine mg dry wt^{-1}) from $t = 1$ to $t = i$.

was used to examine seasonal trends in reproductive effort (Fig. 4). The total quantity of oyster eggs estimated was biased by removing a body section for histological analysis; thus the reported measures of gonadal quantity are not true measures of completely intact oysters.

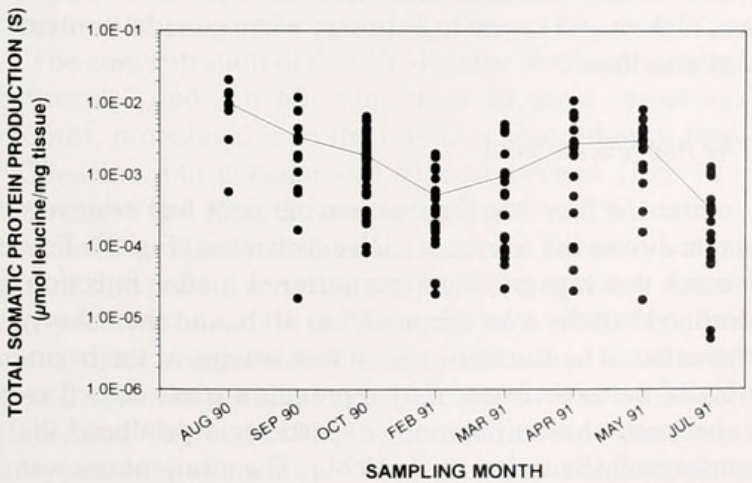

Figure 12. Seasonal fluctuation of somatic protein production (s) (μmol leucine mg dry wt^{-1}). All oysters analyzed are plotted.

Monthly mean GSI increased from mid-August to mid-September, then decreased in late October, indicating that a fall spawning pulse occurred during the intervening period. None of the oysters collected in February 1991 contained a detectable quantity of egg proteins. The GSI increased from mid-March to mid-April, then declined in May, suggesting a spring spawning pulse during those months. GSI dropped from late May to mid-July. Overall, monthly mean GSI was higher during the spring spawning peak than during the fall spawning peak.

Perkinsus marinus *infection*

All oysters collected during the course of study were infected with *Perkinsus marinus*. Monthly median infection intensity varied from 1.1 (a light infection) in February and March to 3.0 (a moderate infection) in October (Fig. 5). A statistically significant correlation was observed between salinity and *P. marinus* infection intensity; oysters

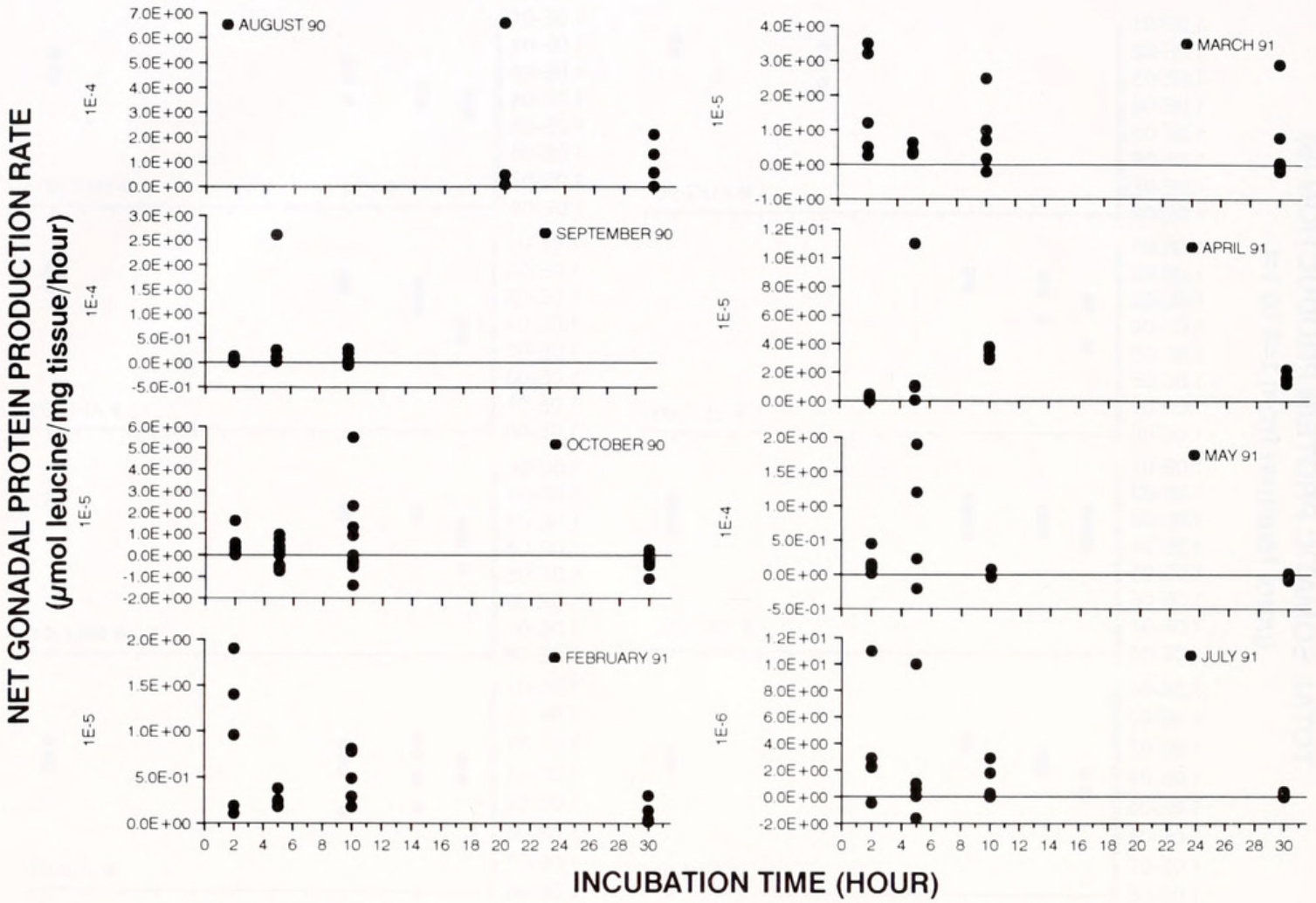

Figure 13. Net rate of egg protein production (dg/dt) calculated using equation (13) (μmol leucine mg dry wt^{-1} h^{-1}).

collected during higher salinity months (August to October 1990, 22 to 26‰) exhibited higher infection intensities than oysters collected during lower salinity months (February to July 1991, 10 to 20‰) (ANOVA, $P < 0.0001$). Multivariate analysis of variance (MANOVA) failed to detect a statistically significant effect of *P. marinus* parasitism on oyster GSI.

Time course of ^{14}C-leucine incorporation

^{14}C-leucine incorporation into somatic protein rose over the first 10 h after injection (Fig. 6) and remained stable or slightly decreased thereafter. The amount of ^{14}C-leucine incorporated into somatic protein was highest in April, moderately high during most of the spring and fall months when tissue growth occurs, and lowest in the winter when oysters typically have a net negative energy balance (Powell *et al.*, 1992).

^{14}C-leucine incorporation into gonadal protein was measured using the rabbit anti-oyster egg IgG and protein A immunoprecipitation technique. A maximum incorporation of ^{14}C-leucine into gonadal protein occurred between 5 and 10 h after injection (Fig. 7). Between 10 and 30 h after injection, ^{14}C-leucine content tended to plateau or decrease. The extent of ^{14}C-leucine incorporation into gonadal protein paralleled the gonadal/somatic index; values were highest in April when gonadal content was highest and lowest in February when gonadal content was also low.

The free leucine pool

After the first 2 h, the free leucine pool was relatively stable during the course of each experiment (Fig. 8). Free leucine was highest in concentration 1 h after injection, declined rapidly over the next 5 to 10 h, and then slowly thereafter. The transient rise in free leucine at the beginning of the experiment may represent a stress caused by experimental manipulation. Aspartic acid produced the same result (Saunders *et al.*, 1994). The transient rise was not due to the amount of amino acid injected, which was small compared to the amino acid pool. Yearly variation

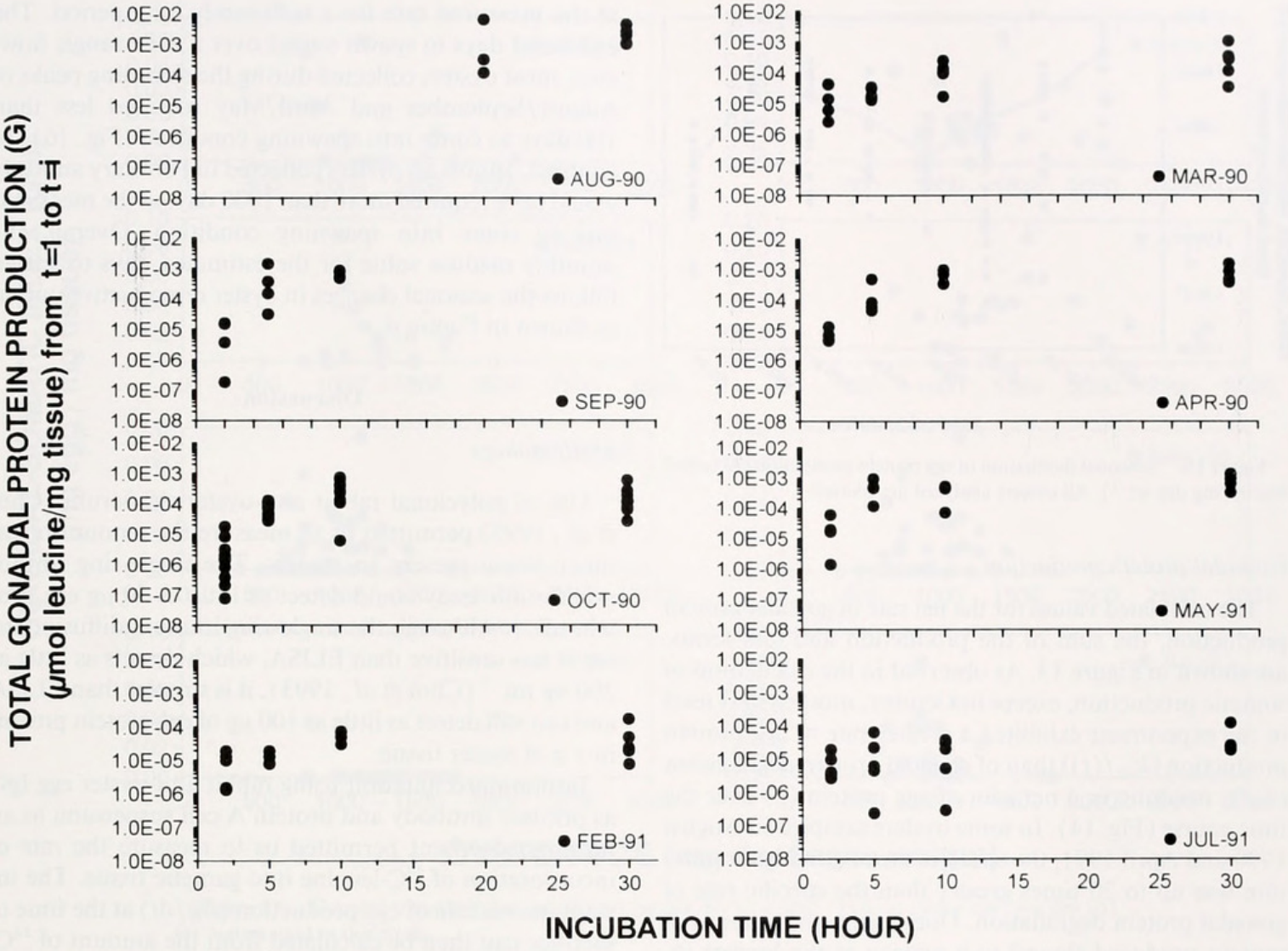

Figure 14. Total egg protein production [g from equation (15)] (μmol leucine mg dry wt^{-1}) from $t = 1$ to $t = i$.

in free leucine did not follow salinity, nor did it follow the reproductive cycle. Values were lowest in July and highest in August and September. Lynch and Wood (1966) also found leucine to be stable over a broad salinity range.

The concentration of free ^{14}C-leucine declined rapidly between 1 and 5 h after injection in most sampling months, probably due to the rapid incorporation of free ^{14}C-leucine into gonadal and somatic protein (Fig. 9). Between 10 and 30 h, the concentration of free ^{14}C-leucine plateaued or slowly declined, except in February, suggesting that protein recycling was buffering the pool. Rates of protein turnover are on the order of 1 to 20 days for many tissue components (Koehn, 1991) and were estimated at 16% of the basal metabolic rate in *Mytilus edulis* (Sibly and Calow, 1989). The amount of incorporation into somatic protein would have provided a source adequate to maintain the free amino acid pool at the measured specific activity at these turnover rates. The higher specific activity in February suggests lower somatic tissue incorporation, which was observed.

Somatic tissue production

The rate of somatic tissue production, the net of the production and degradation terms, was relatively stable over the time course in each sampling month. Most oysters exhibited positive somatic tissue growth during most months and throughout the experimental time course (Fig. 10). The amount of somatic tissue produced increased over the time course, but at a slower and slower rate, in all months (Fig. 11). Either growth rate dropped, the accuracy of our calculations declined as specific activity decreased, or ^{14}C-leucine was lost from the rapidly overturning component of the somatic tissue pool. The latter is the more likely case.

Somatic production varied seasonally. Rates were low during midwinter and midsummer (Fig. 12). These seasonal fluctuations were significant (ANOVA, $P < 0.0001$).

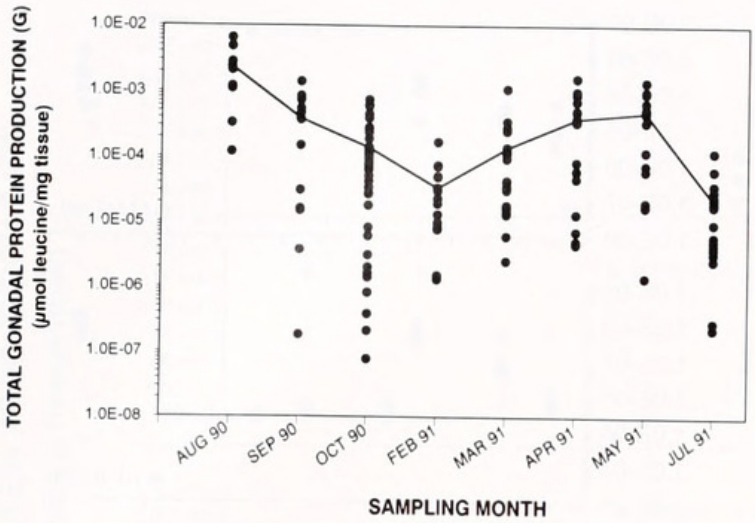

Figure 15. Seasonal fluctuation of egg protein production (g) (μmol leucine mg dry wt^{-1}). All oysters analyzed are plotted.

Gonadal protein production

The calculated values for the net rate of gonadal protein production, the sum of the production and loss terms, are shown in Figure 13. As observed in the calculation of somatic production, except in October, most oysters used in the experiment exhibited a greater rate of egg protein production ($k_{gg}f(t)$) than of gonadal protein degradation ($k_{lg}{}^{g}$), resulting in a net gain of egg protein (g) over the time course (Fig. 14). In some oysters sampled in August 1990 and April 1991, the specific rate of gonadal production was up to 20 times greater than the specific rate of gonadal protein degradation. Once again, the amount of protein produced slowed to a plateau at the longest incubation times in some months. Either the rate of production slowed, the calculation of specific activity became more inaccurate, or protein recycling resulted in a net loss of ^{14}C-leucine from the more rapidly overturning portion of the tissue pool.

Overall, the observed seasonal pattern in gonadal synthesis (Fig. 15) coincides with the annual cycle of reproductive effort in oysters shown in Figure 4. Two discrete peaks occur, one in August/September and the other in April/May. The values for gonadal synthesis obtained during the spawning peaks are significantly higher than the values obtained during the intervening periods ($P < 0.0001$). However, gonadal production and gonadal/somatic index (*i.e.*, rate versus quantity) did not correspond exactly. April and May were similar in synthesis rate, but oysters sampled in April had higher GSI (more eggs). The rate of egg synthesis was higher in August/September than in April/May; the opposite was true for the number of eggs present.

Estimated days to spawn

We estimated the number of days required for an animal to become ready to spawn if egg synthesis continued at the measured rate for a sufficiently long period. The estimated days to spawn varied over a wide range; however, most oysters collected during the spawning peaks of August/September and April/May required less than 100 days to come into spawning condition (Fig. 16). In contrast, almost all oysters collected in February and July would have required more than 1000 days at the measured rate to come into spawning condition. Overall, the monthly median value for the estimated days to spawn follows the seasonal changes in oyster reproductive output as shown in Figure 4.

Discussion

Methodology

Use of polyclonal rabbit anti-oyster egg serum (Choi *et al.*, 1993) permitted us to measure the amount of gametic tissue present in oysters. The single-ring immunodiffusion assay could detect as little as 50 μg egg protein ml^{-1}. Although the single-ring immunodiffusion assay is less sensitive than ELISA, which detects as little as 200 ng ml^{-1} (Choi *et al.*, 1993), it is simpler than ELISA and can still detect as little as 100 μg of egg protein present in 1 g of oyster tissue.

Immunoprecipitation using rabbit anti-oyster egg IgG as primary antibody and protein A cell suspension as an immunoadsorbent permitted us to measure the rate of incorporation of ^{14}C-leucine into gametic tissue. The instantaneous rate of egg production (dg/dt) at the time of sacrifice can then be calculated from the amount of ^{14}C-leucine incorporated and the specific activity of leucine measured by amino acid analysis. From the cumulative amount of leucine incorporated, we could calculate the instantaneous rate of gamete synthesis and the days re-

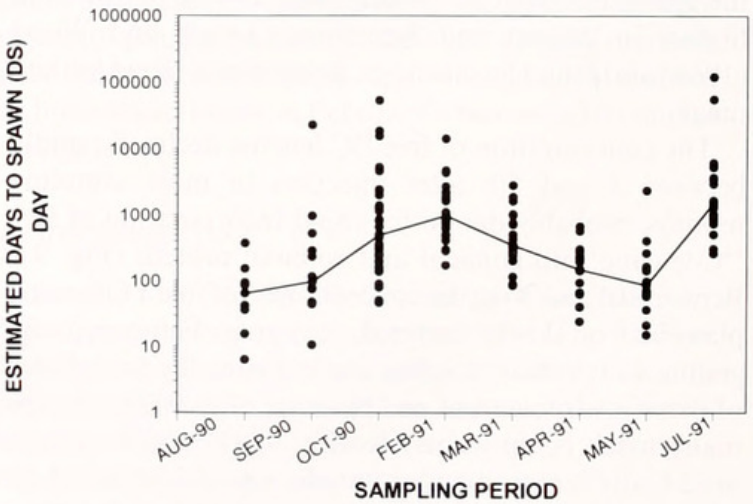

Figure 16. Seasonal fluctuation in the estimated number of days required for an individual to become ready to spawn if the measured rate of egg synthesis were to continue unaltered. All oysters analyzed are plotted.

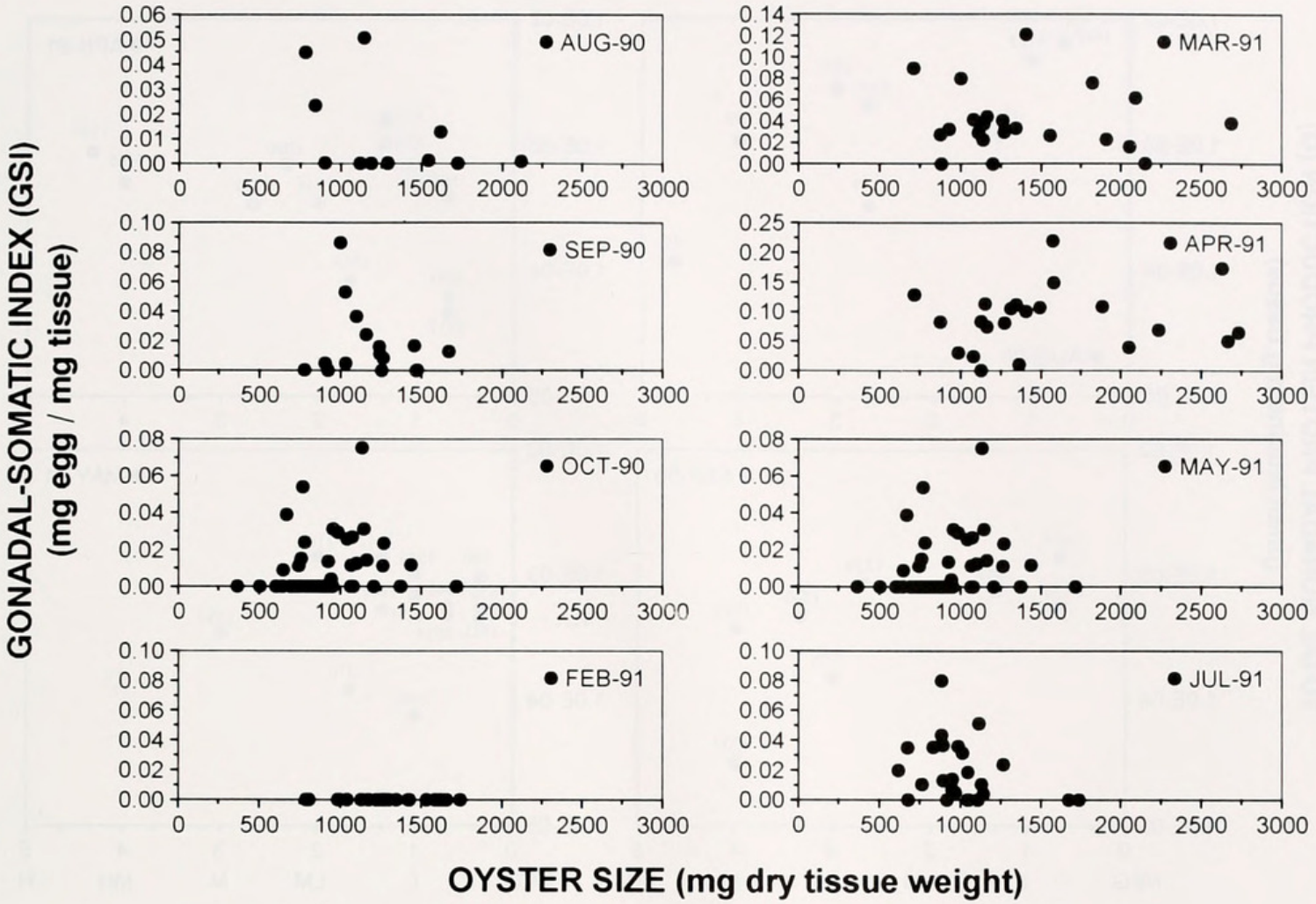

Figure 17. Gonadal-somatic index (mg dry wt egg mg dry wt oyster^{-1}) *versus* oyster size (mg dry wt) for oysters used in this study.

quired to reach spawning condition should that rate be maintained.

Spawning season and GSI

The gonadal-somatic index (Fig. 4) indicates that at least two spawning peaks occurred during the study, one in spring and the other in fall. Choi *et al.* (1993) observed a similar bimodal spawning pulse in an annual gametogenic cycle of female oysters on Confederate Reef. The higher reproductive effort observed in both studies in the spring might be associated with higher food supplies in the spring, as reported by Hopkins (1935) and Soniat and Ray (1985), or with a greater degree of synchrony in gonadal development in the spring population, as suggested by Hofmann *et al.* (1992). In the fall, fewer oysters would be ready to spawn at any one time, reducing the overall mean and the likelihood of collecting an egg-heavy individual (Cox and Mann, 1992; Hofmann *et al.*, 1992). The absence of a statistically significant relationship between size, GSI, and season and the presence of apparently recently spawned individuals in every month of the spawning season (Fig. 17) indicate that portions of the population are spawning during most months, as suggested by the Hofmann *et al.* (1992) model and numerous references to continuous or dribble spawning (Hopkins, 1935; MacKenzie, 1977; Cox and Mann, 1992).

Parasitism by Perkinsus marinus

Perkinsus marinus, like other parasites (*e.g.,* Kabat, 1986), can significantly affect oyster reproduction and growth; however, unlike many parasites, the effect of *P. marinus* on oyster reproduction has been difficult to quantify predictably (Wilson *et al.,* 1988; White *et al.,* 1989; Cox and Mann, 1992; Ellis *et al.,* 1993). Theoretically, *P. marinus* could exert an effect in one of two ways: by reducing the number of eggs per spawn or by reducing the number of spawns per year by slowing the rate of egg protein production. White *et al.* (1988b) and Wilson *et al.* (1988) reported circumstantial evidence that higher infection intensity increases the time required for an individual to attain spawning condition. Choi *et al.* (1993) were unable to observe a relationship between the number

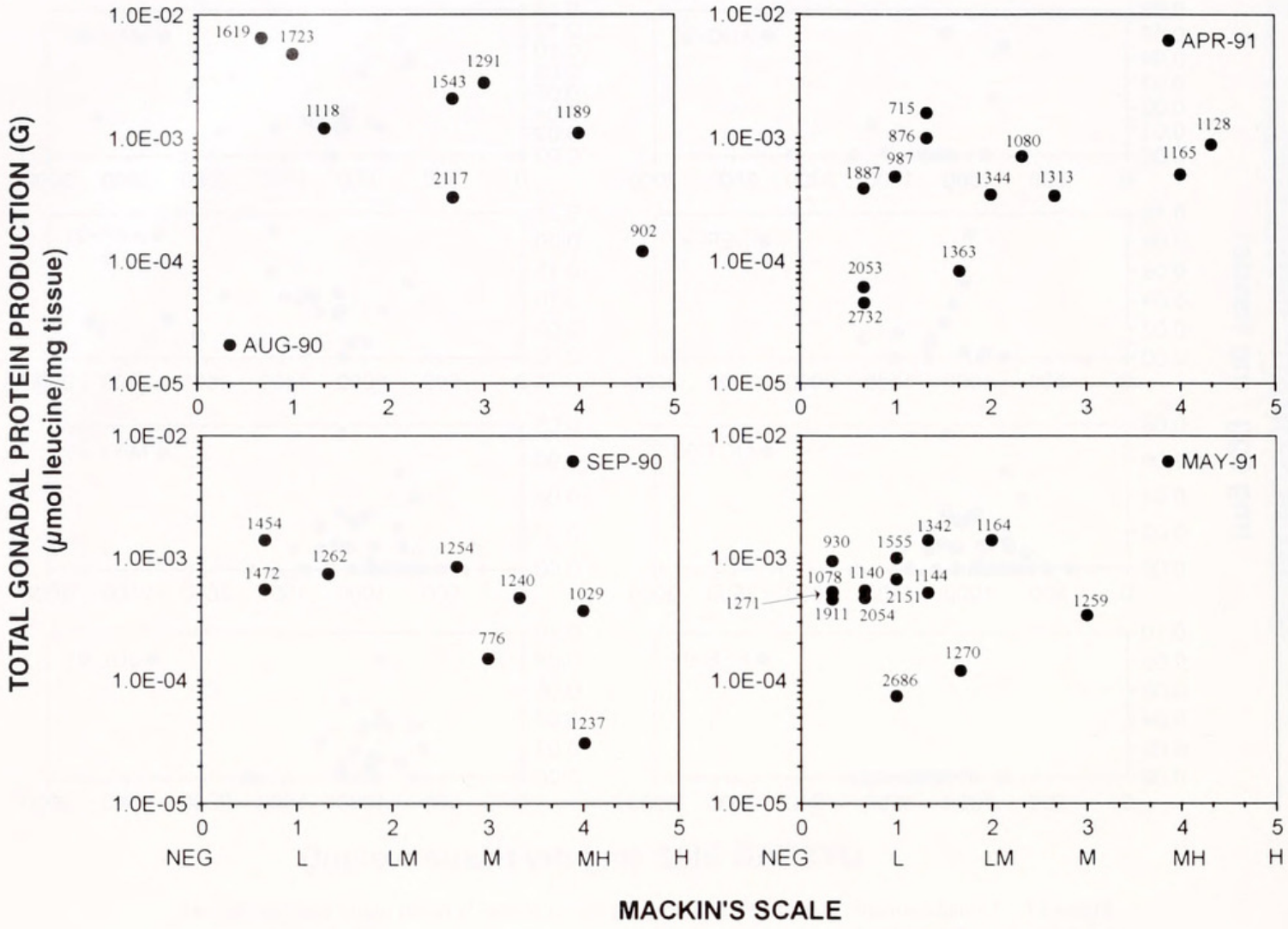

Figure 18. Infection intensity of *Perkinsus marinus* versus the amount of egg protein produced during the time course (g) (μmol leucine mg dry wt^{-1}). The analysis uses oysters collected during the fall and spring spawning season and only oysters sacrificed beyond 2 h after injection. Numbers indicate specimen tissue dry weight in milligrams.

of eggs and *P. marinus* infection intensity and suggested that *P. marinus* probably slows the rate of reproductive development rather than varying the size of an individual spawn. The asynchrony in spawning under Galveston Bay conditions suggests that such an effect will be difficult to demonstrate unequivocally. However, the effect, if present, might be most noticeable during periods of restricted food supply and at high infection intensities, which typically occur during the summer and fall. In addition, the cumulative impact over the spawning season might be noticeable at that time.

Figure 18 relates gonadal production with the infection intensity of *P. marinus* during the spring and fall peaks in spawning activity. A negative correlation between infection intensity and the rate of egg production is noticeable during the fall spawning season, but not during the spring spawning season. Figure 19 relates infection intensity with the number of days required to come into spawning condition. Again, more heavily infected oysters require more days to prepare for spawning in August and September. During the spring, when no significant trend exists, most infection intensities are too low to exert an impact and the cumulative effect of *P. marinus* parasitism is less.

The gametogenic cycle and the rate of egg protein production

One of the important observations of this study is that the seasonal cycle of gamete production as observed by direct measurements of egg protein production is not equivalent to that observed by standard GSI examination, except in a very general way. Gonadal production is lower in the winter and in midsummer when GSI is also low. Low winter rates are certainly a product of lower filtration rates, which restrict food supply in the winter (Powell *et al.*, 1992). The lower rates in July suggest a pause between the two major spawning events or, equally likely, a reduced

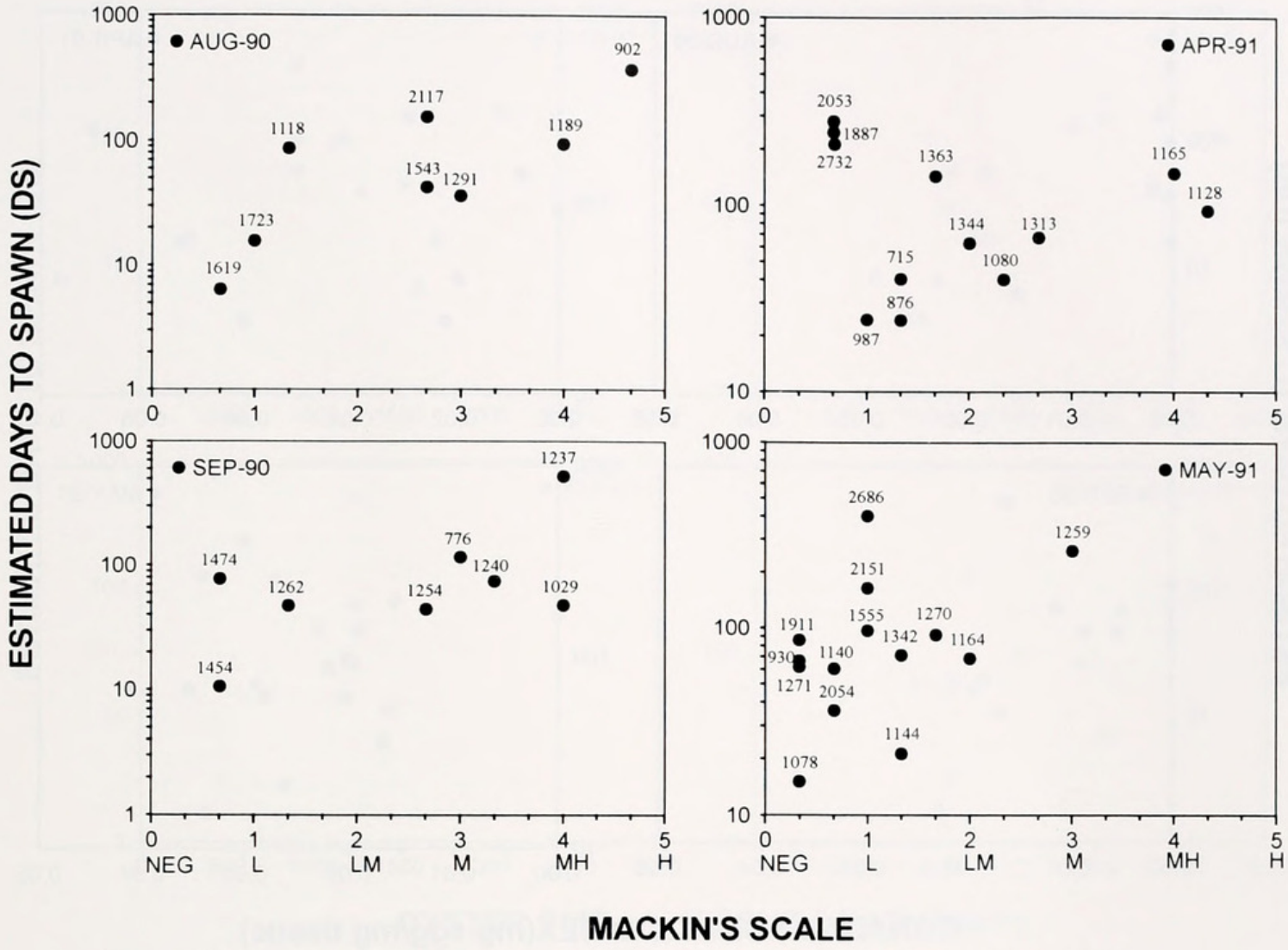

Figure 19. Infection intensity of *Perkinsus marinus* and the estimated days required to become ready to spawn at the egg production rate measured. The analysis uses oysters collected during the fall and spring spawning season and only oysters sacrificed beyond 2 h after injection. Numbers indicate specimen tissue dry weight in milligrams.

food supply during the summer months (our unpublished data show that food supply can drop to low levels during midsummer). Thus, at the most basic level, agreement exists between rate measurements and standing crop measurements (GSI).

Nevertheless, a closer examination shows that the lowest rates of gamete production were observed in July and October rather than in midwinter. Midsummer and fall are typically times of gonadal resorption following spawning, and a higher fraction of oysters was collected in this condition during this time. A perusal of Figures 10 and 12 shows that a smaller fraction of the population was growing in July and October than in other months, suggesting a restricted energy supply at both times as well. Restricted food supply and the temperature-dependent allocation of assimilated energy to reproduction would slow growth in these months. Figures 13 and 15 show that individuals displaying no net growth or gonadal resorption were also found in most months, but a much larger fraction of the population was in this condition in October, as the fall spawning season ceased and gonad was resorbed, rather than in midwinter. Furthermore, highest egg production rates were obtained in August, whereas GSI was highest in April, and a comparison of April and May reveals that egg production rates were essentially equal, whereas April's mean GSI was nearly double the May value. Accordingly, the two types of measurements, rates *versus* standing crop, reveal a substantially different picture about the details of the spawning season.

Days to reach spawning condition

The time required to reach spawning condition is a function of the daily rate of production of gametic tissue. Our estimates of the time required to reach spawning condition of as little as several weeks to 1 or 2 months agree well with estimates reported in the literature (Loosanoff and Davis, 1953; Kaufman, 1979). Accordingly, the

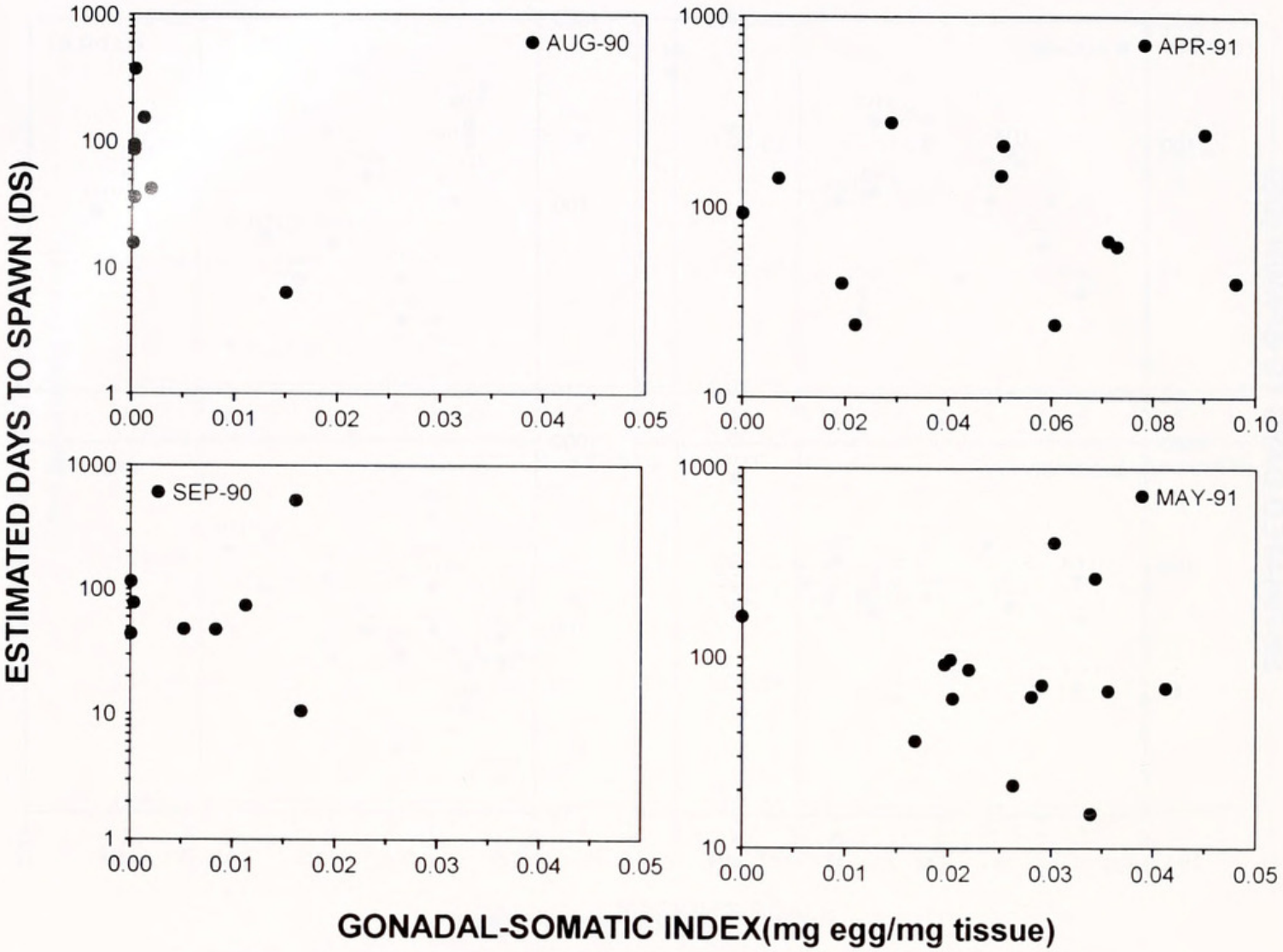

Figure 20. The relationship between estimated days to spawn and gonadal-somatic index (mg dry wt egg mg dry wt oyster^{-1}) during the two primary spawning periods, August/September and April/May. Oysters collected 2 h after injection were not included.

direct rate measurements we have made would seem to accurately reflect the true rate at which gametic tissue is produced in the field. One might expect that oysters with higher GSI would exhibit higher values for the estimated time required to reach spawning condition as we have calculated it, because the rate of gametic tissue production might decline as spawning condition was reached. We found no significant relationship between GSI and the number of days required to reach spawning condition (Fig. 20). Thus the rate of production of gametic tissue apparently does not intrinsically increase or decrease during gonadal maturation, even as spawning nears.

Oyster size is positively correlated with the estimated days to spawn only during the spring spawning peak in April/May, when larger oysters require more days to prepare to spawn (Fig. 21). By the fall, this relationship no longer exists. The significant relationship in the spring probably stems from the spring temperature signal (Loosanoff, 1942; Barber and Mann, 1991), which initiates gonadal development in all adults regardless of size, and from the fact that the spawning trigger would appear to include the requirement that gonadal tissue make up at least 20% of the oyster's biomass. As suggested by Hofmann *et al.* (in press, 1992), smaller oysters have higher food acquisition rates than larger ones and allocate more of the net energy gained to reproduction; thus smaller individuals should reach the 20% level sooner and spawn earlier. The longer time to spawn in larger oysters indicates the importance of the relative capabilities of different oyster size classes to obtain energy and allocate it to reproduction. The absence of a correlation between oyster size and days to spawn in the fall may be due to the gradual decay in the synchrony of reproductive development that accompanied the spring temperature signal. Synchrony declines for two reasons. (1) As the various size classes spawn during the spring and summer, the synchrony of reproduction in the population gradually degrades. The smaller size classes will almost certainly spawn more fre-

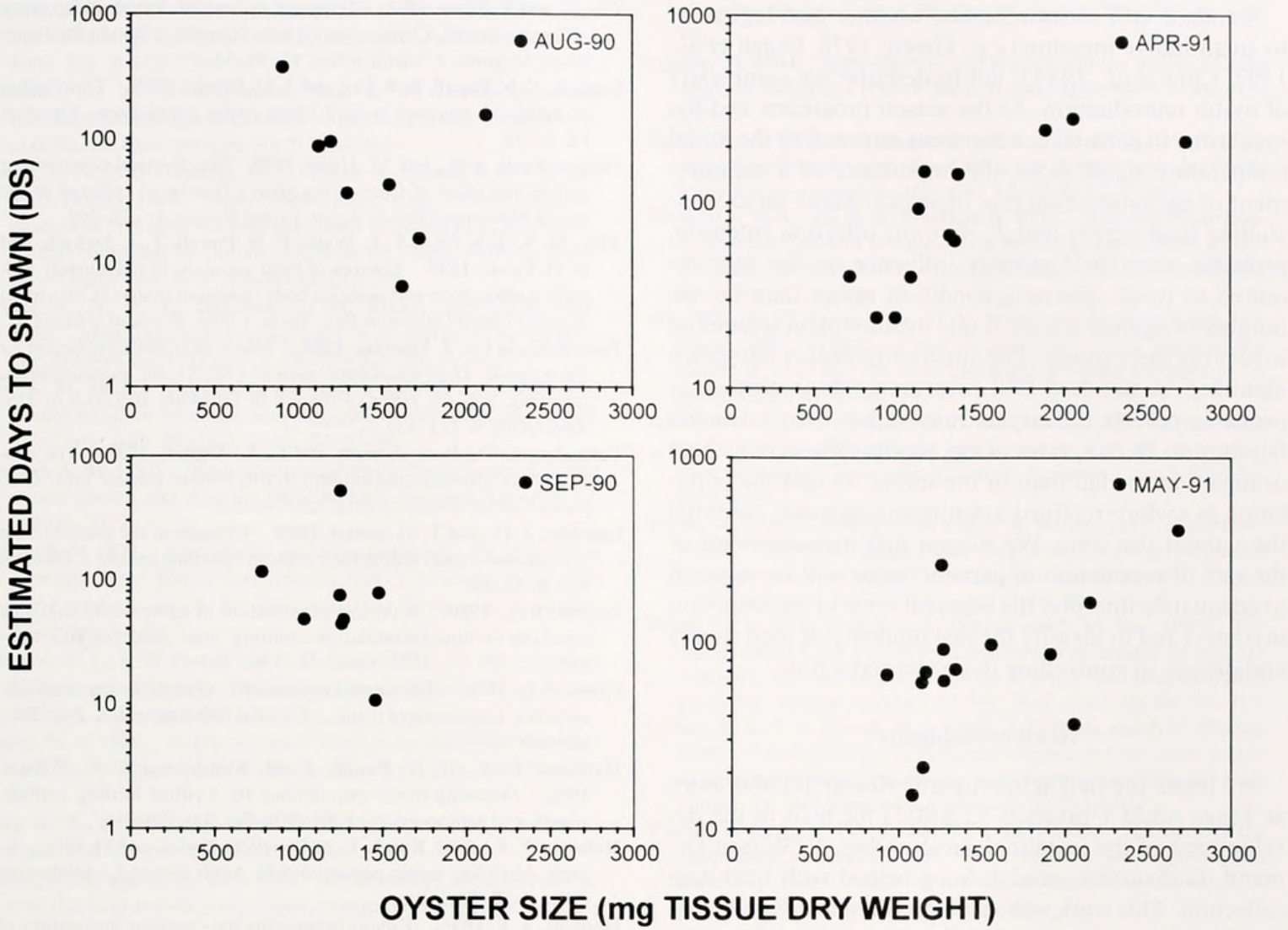

Figure 21. The relationship between oyster dry weight (mg) and the estimated number of days to reach spawning condition. Only oysters collected during the spring and fall spawning peaks are included. Oysters collected 2 h after injection not included.

quently than large oysters over the year. (2) The increase in *P. marinus* infection intensity gradually retards spawning in highly infected individuals. Less heavily infected individuals will spawn sooner (Fig. 19). By the fall, these two factors should result in asynchronously spawning individuals within and between size classes. Probably, the increased infection intensity of *P. marinus* is primarily responsible for the lack of a correlation between days to spawn and size in the fall.

Conclusion

This study represents the first examination of the *in situ* instantaneous rate of reproduction in oysters. Although a general relationship exists between a standard GSI measurement and the rate of production of gametic tissue, upon closer examination, the two differ markedly in detail. The suggestion is that oysters can rapidly produce gametic tissue and can maintain it for extended periods of time without loss or growth, particularly when food supply is low. A high GSI does not necessarily mean a simultaneously high rate of production of gametic tissue.

The data support the overriding importance of food supply and the important role of disease in controlling gametic production. Some of the lowest rates (in July) probably resulted from low food supply. Furthermore, the range in observed somatic and gametic growth emphasizes the conservatism of somatic growth and the volatility of gonadal growth that is borne out by the results of population dynamics models of oysters (Hofmann *et al.*, 1992, in press). Somatic production rates varied by a factor of 10 over the year; for gonadal production, this range was 100. Thus somatic growth was more conservative, and the data support the suggestion that the added assimilation that occurs in the spring with increased filtration rate and higher temperature is largely diverted into the production of gonadal tissue.

Standard GSI measurements, whether histological or by quantitative measure (*e.g.*, Green, 1978; Dolah *et al.*, 1992; Choi *et al.*, 1989), fail to describe the complexity of oyster reproduction. As the season progresses and the synchrony in gonadal development imposed by the initial temperature signal fades, the importance of a measurement of egg production rate increases. Many factors, including food supply and *P. marinus* infection intensity, probably exert their primary influence on the time required to reach spawning condition rather than on the number of eggs per spawn. A rate measurement is required to identify these trends. The apparently weaker fall spawn identified in standard GSI measurements in this study probably reflects this asynchrony rather than a smaller fall output. In fact, rates of egg production were as high or higher in the fall than in the spring, so that the population as a whole retained a significant spawning potential throughout this time. We suggest that measurements of the rate of production of gametic tissue will be required to adequately interpret the seasonal cycle of reproduction in oysters and to identify the real influence of food supply and disease in controlling oyster reproduction.

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