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A model evaluating the contribution of environmental factors to the production of resting eggs in the rotifer *Brachionus plicatilis*

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Key words: rotifer, resting eggs, logistic model, poly-linear model, generalized linear model, salinity, food concentration

Abstract

The production of resting eggs by the rotifer *Brachionus plicatilis* was tested at four salinities (9, 18, 27 and 36‰) and six concentrations of the alga *Chlorella stigmatophora* (0.25, 0.5, 1.0, 2.0, 4.0 and 6.0×10^6 cells ml^{-1}). The results indicated that resting eggs were produced only at two salinities (9‰ and 18‰) and that their number was affected by the amount of food provided. A model consisting of two generalized linear sub-models was built to evaluate the contribution of each of the tested food concentrations at the two salinities. The sub-models were used to distinguish between two different components of resting egg production: one related to the presence or absence of resting egg production, and the other to the number of resting eggs produced, given that production had occurred. Besides indicating the best combination of salinity and food concentration for obtaining large numbers of resting eggs, they revealed the contribution of internal population factors that were not controlled in the course of the experiment. The model identified the positive contribution of the relative number of females to males, and the negative association between high rotifer densities and the production of resting eggs. The results of the present study help in defining the optimal conditions for mass production of resting eggs, which are of potential importance in aquaculture.

Introduction

Rotifers (*Brachionus plicatilis* O. F. Muller) now serve as the only food offered to larvae of several species of cultured marine fish during the first period of exogeneous feeding. The number of rotifers raised in a hatchery determines, to a large extent, the number of marine fish larvae that can be produced (Hirata, 1980). Today rotifers are obtained through mass culture systems that rely on constant harvesting. Unpredicted events may result in failure of rotifer cultures, leaving the aquaculturist without an immediate, available food source. The possible use of rotifer resting eggs as a source for live rotifers has been suggested in the past (Lubzens *et al.*, 1980; Lubzens, 1981). These resting eggs would have to be available in large quantities and at a relatively low price. They could then be used in a way that *Artemia* cysts are utilized today in aquaculture. The conditions for preservation and hatching of these eggs have been published (Lubzens, 1981; Minkoff *et al.*, 1983).

Unlike *Artemia* eggs, rotifer resting eggs are not

† Deceased, September 1991.

widely available from natural resources. Thus, in order to obtain them in large quantities, controlled methods for their mass production have to be devised. It is accepted in most cases (except those shown by Ruttner-Kolisko, 1983) that resting eggs are fertilized eggs resulting from the occurrence of sexual reproduction (Ruttner-Kolisko, 1972; see review by Pourriot & Snell, 1983).

The occurrence of sexual reproduction and its extent in *B. plicatilis* have been shown to depend on internal and environmental factors. Differences between strains were reported by Hino & Hirano (1977), Hagiwara *et al.* (1988a, b, 1989) and Lubzens (1989). Salinity, temperature, ammonia concentration and food concentration were shown to modulate the level of sexual reproduction (Hino & Hirano, 1985, 1988; Lubzens *et al.*, 1985; Snell, 1986; Snell & Boyer, 1988). The age of the algae fed to rotifers was also found to affect the pattern of expression of mixis in *B. plicatilis* (Lubzens & Minkoff, 1988). Density of rotifers was proposed to affect the appearance of mixis (Hino & Hirano, 1977), the encounter probabilities between male and female rotifers (Snell & Garman, 1986) and the threshold for mictic female reproduction (Snell & Boyer, 1988). Also, a computer simulation was used to explore the production of resting eggs as a result of the frequency of sexual reproduction and the proportion of a female's daughters reproducing sexually (Snell, 1987).

In the present paper we examine the effect of salinity and food concentration on the production of resting eggs in an experimental system, through a model with two different generalized linear submodels, with the aim of applying them later to facilitate the mass production of resting eggs. The model also aims at identifying factors, which were not experimentally controlled, that contribute significantly to promoting or preventing the production of resting eggs.

Materials and methods

Sea water (salinity 36‰) was collected 200 m offshore of the National Institute of Oceanography in Haifa and stored in 20 l plastic containers at 20 °C for periods of 1–14 days. Before being used in rotifer or algal cultures, the water was sieved through a membrane filter (0.45 μm). Sieved sea water was heat sterilized before use in algal cultures. Dilutions of sea water were carried out by adding deionized tap water, and salinity was measured by a refractometer (bioMarine, USA). *Chlorella stigmatophora* was cultured, harvested and used as described previously (Minkoff *et al.*, 1983). A laboratory culture of *Brachionus plicatilis* was used throughout the experiments. The culture originated from several individuals collected from sea-water fish ponds at Dor, 40 km south of Haifa, Israel. Rotifers were maintained, prior to the experiments, in batch cultures in 5 l Ehrlenmeyer flasks containing sea water at a salinity of 36‰. They were fed every 1–2 days with freshly prepared *C. stigmatophora* (see Minkoff *et al.*, 1983).

Experimental design

The effect of six algal concentrations (0.25, 0.5, 1.0, 2.0, 4.0 and 6.0×10^6 cells ml^{-1}) and four sea water salinities (9, 18, 27 and 36‰) on the production of females, males and resting eggs was tested for 39 days in a factorial designed experiment. Each combination of food × salinity was tested in three replicates, and a total of 72 flasks was used in the experiment (Table 1).

Seven days prior to the onset of the experiment, rotifers were removed from batch cultures and placed at a density of 2 ml^{-1} in six flasks containing 100 ml sea water at a salinity of 36‰. One flask was used at each of the concentrations tested later (0.25, 0.5, 1.0, 2.0, 4.0 and 6.0×10^6 cells ml^{-1}). Flasks were placed on a rotating table (New Brunswick, USA) in a temperature controlled cabinet (25 ± 1 °C) under constant illumination.

On day 0 of the experiment, female rotifers, each carrying one amictic egg, were removed from each of the six flasks and distributed into 4 new flasks. Each flask contained sea water at a different salinity (9, 18, 27 and 36‰). Rotifers were

Table 1. The number of females and males (per 10 ml) in cultures at the indicated salinities provided with *Chlorella stigmatophora* at 6 food concentrations (0.25, 0.5, 1.0, 2.0, 4.0 and 6.0×10^6 ml^{-1}). The mean and standard deviation (SD) was calculated for 3 replicates at each experimental condition for observations made within each time period. Six observations were made during days 3–14 of the experiment, and four observations were performed during each of the other two periods (days 16–25 and 28–39). Total numbers of resting eggs (R.e.) are also indicated.

Time period (days)	Food conc.	Salinity 1 (9‰)					Salinity 2 (18‰)					Salinity 3 (27‰)[a]				Salinity 4 (36‰)[a, b]	
		Females		Males		Re	Females		Males		Re	Females		Males		Females	
		Mean	SD	Mean	SD		Mean	SD	Mean	SD		Mean	SD	Mean	SD	Mean	SD
3–14	1	122	49.9	15	8.7	3	131	35.9	11	7.5	0	148	64.4	0.2	0.5	52	35.1
	2	234	113.5	30	15.1	12	228	108.8	18	17.9	4	217	128.0	1.1	2.1	126	95.7
	3	301	143.1	73	66.5	8	449	257.6	46	46.2	2	312	270.5	1.9	3.5	168	115.4
	4	307	206.1	145	294.8	8	724	562.0	201	255.7	3	448	534.3	0	–[c]	82	71.2
	5	1526	1408.5	456	799.2	4	1184	1005.5	177	244.2	0	141	171.3	0	–[c]	58	37.7
	6	1968	1670.3	268	291.3	6	815	718.6	59	77.0	0	243	282.9	0	–[c]	31	12.7
16–25	1	96	39.0	27	40.1	17	108	24.7	3	0.6	6	133	67.7	0.3	0.7	119	61.9
	2	166	60.2	45	54.4	6	60	72.8	3	4.3	2	316	153.0	1.7	3.7	276	111.8
	3	232	84.7	93	82.5	16	325	117.7	30	9.3	11	628	252.4	3.7	7.0	251	117.1
	4	274	52.2	64	30.7	1	1169	250.7	184	74.8	34	1109	227.2	2.2	5.4	86	53.9
	5	1304	562.7	352	270.3	9	1836	269.8	117	21.1	13	712	673.9	5.9	14.7	54	32.7
	6	2292	1050.9	411	284.7	3	1474	345.4	72	77.9	3	1273	1188.6	37.8	84.9	2	6.7
28–39	1	188	26.7	15	7.1	7	106	111.2	0	0.5	1	65	47.1	0	–[c]	75	53.1
	2	94	104.9	9	15.6	4	0	0.0	0	0.0	0	199	140.0	0.4	1.1	90	103.7
	3	68	79.2	2	2.4	0	218	47.7	3	3.3	2	262	250.9	0.4	0.5	116	145.4
	4	120	104.1	12	19.5	1	495	392.2	6	7.4	10	632	795.2	2.5	8.8	4	0.7
	5	846	603.5	102	118.8	22	522	461.2	25	36.6	3	745	979.2	0.2	0.6	13	8.0
	6	827	587.1	162	187.3	1	800	751.3	36	65.0	1	1146	1508.9	0	–[c]	0	0

[a] Resting eggs were not detected.
[b] Males were not detected.
[c] No males were found.

suspended in 50 ml (salinities 18 and 27‰) or 100 ml (9 and 36‰) at a density of 2 ml^{-1}. Algae were provided at the concentration to which the rotifers were previously exposed. For example, rotifers cultured for 7 days at 36‰ and receiving algae at a concentration of 0.25×10^6 cells ml^{-1} were now divided into 4 flasks, each at a different salinity, but continued to receive 0.25×10^6 cells ml^{-1}. Thus, at this stage 24 flasks were present. After 24 hours (day 1 of the experiment) of incubation, 60 neonates were removed from each of the 24 flasks and placed in three replicate 50 ml flasks containing 10 ml sea water and algae (initial rotifer density: 2 neonates ml^{-1}). Thus, we obtained 72 50-ml flasks, which were divided into four salinities and six food concentrations.

Every 2–3 days, counts were made of the number of amictic females, mictic females, males and resting eggs by using the following procedure: (1) all the rotifers were counted in flasks containing less than 100 individuals (*i.e.* less than 10 ind ml^{-1}). (2) three subsamples were randomly removed by glass pipettes from each flask containing more than 100 rotifers; each subsample was replaced before the next one was removed from the flask, and the contents of the flask were mixed gently. In the latter case, subsample volume, ranging from 0.1 to 5.0 ml, depended on the rotifer density in the flask; higher volumes were used for lower densities, and thus at least 20 rotifers were counted in each subsample. The contents of each flask were poured onto a small sieve (45 μm mesh) on which neonates, females and males were collected and resuspended in a small volume of sea water of the appropriate salinity in a small glass petri dish. The presence of males and resting eggs was recorded under a binocular microscope, and resting eggs were counted and removed. Rotifers were then sieved again, and resuspended in 10 ml of freshly prepared culture media of the appropriate salinity and algal concentration. Every week the culture medium of each flask was changed four times (on Sunday, Tuesday, Thursday and Friday). Counts were performed three times a week (on Sunday, Tuesday and Thursday). During the first week of the experiment, some deaths occurred in six flasks, and these flasks were replaced with identically pretreated rotifers. The age of the rotifer cultures in these flasks was calculated from the day of the replacement.

The data

The data presented in Table 1 show the number of females, males and resting eggs produced at four different salinities and six food concentrations on 14 observation periods carried out during 34–39 days of the experiment. The results are shown for three periods during the experiment: (1) days 3–14 (6 observations); (2) days 14–25 (4 observations); (3) days 28–39 (4 observations). The numbers of males and females at each salinity and food concentration changed during the experiment. These changes do not appear to have a simple pattern. The standard deviations reflect the large differences between the replicates within each observation period at every food concentration and salinity tested during the experiment. No males were observed in cultures of 36‰. Also, resting eggs were produced only in 9‰ and 18‰ cultures. In general, the number of females and males was higher at the lowest salinities (9‰ and 18‰) and tend to increase with food concentration. The numbers of resting eggs were variable (Table 1), and the factors associated with their appearance were examined in the model presented below. The first resting eggs were observed on day 4 of the experiment.

Statistical analysis

The model

The purpose of the data analysis is to suggest a model that would identify the factors which contribute significantly to the production of resting eggs on each day. An important characteristic in the resting-egg production process is that only in less than a third of the experimental days was the production of resting eggs greater than zero. The proposed model distinguishes between two dif-

ferent mechanisms, one related to the occurrence of production and the other to the variables which affect the number of resting eggs produced, given that production had occurred. Thus, two sub-models were proposed.

Let RE_{ijkt} denote the number of resting eggs in day t ($t = 4, 6, \ldots, 39$), replicate k ($k = 1, 2, 3$), for an experiment with a food concentration j ($j = 1, 2, 3, 4, 5, 6$) and salinity i ($i = 1, 2$; *i.e.* 9‰ and 18‰, the only two salinities at which resting eggs were found), and let X_{ijkt} denote the vector of factors which are assumed to affect the production of resting eggs in time t for replicate k with food concentration j and salinity i. The vector **X** represents the effects of the experimental conditions (salinity and food concentration) as well as factors such as the rotifer density and the corresponding ratio of males to females just before time t.

Submodel for the first mechanism: production vs. non-production

The probability of resting egg production in a culture is given by

$$P_{ijkt} = P(RE_{ijkt} > 0 \mid \mathbf{X}_{ijkt}) .$$

We assumed that this probability is greater than zero from the third day of the experiment, and that it is related to a set of factors through the following logistic model (Hosmer & Lemeshaw, 1989):

$$\log \frac{P_{ijkt}}{1 - P_{ijkt}} = \mu + a_i + \delta_j + \lambda_{ij} + \beta_1 U_{ijkt} + \beta_2 \sqrt{U_{ijkt}} + \beta_3 SR_{ijkt} + \beta_4 \sqrt{SR_{ijkt}} + \beta_5 TL1_{ijkt} + \beta_7 TL2_{ijkt} + \beta_8 TQ2_{ijkt} + \beta_9 MISS_{ijkt} , \quad (1)$$

where

μ the baseline level.

α_i the effect of salinity i.

δ_j the effect of food concentration j.

λ_{ij} the interaction term between salinity i and food concentration j.

$\beta_{1,2\ldots9)}$ coefficients for the effects described by U_{ijkt}, SR_{ijkt}, $TL1_{ijkt}$, $TQ1_{ijkt}$, $TL2_{ijkt}$ and $TQ2_{ijkt}$.

U_{ijkt} the mean of the rotifer density at days $t-2$ and $t-4$ for replicate k of an experiment at salinity i and food concentration j, given that at least once the two sexes were present, in days $t-2$, $t-4$. Values of days without males were not included in this term.

SR_{ijkt} The mean of the sex ratios (M/F) at days $t-2$ and $t-4$, for replicate k of an experiment at salinity i and food concentration j.

$$TL1_{ijkt} \begin{cases} t & \text{Salinity} = 9‰ \\ 0 & \text{else} \end{cases}$$ Indicates the changes in production of resting eggs during the time course of the experiment at salinity 9‰.

$$TQ1_{ijkt} \begin{cases} (t-16)^2 & \text{Salinity} = 9‰ \\ 0 & \text{else} \end{cases}$$ Indicates the square of TL1, where 16 is taken as a midpoint during the time of the duration of the experiment.

$$TL2_{ijkt} \begin{cases} t & \text{Salinity} = 18‰ \\ 0 & \text{else} \end{cases}$$ Same as TL1 above, except for salinity 18‰.

$$TQ2_{ijkt} \begin{cases} (t-16)^2 & \text{Salinity} = 18‰ \\ 0 & \text{else} \end{cases}$$ Same as TQ1 above, except for salinity 18‰.

The binary variable (0, 1) is equal to 1 for those experimental days where no male rotifers were counted at days $t-2$ and $t-4$. On the occasions where no males were recorded on 3 consecutive observations, the variable MISS was introduced to account for the possibility of the contribution to the production of resting eggs from previous days in the course of the experiment.

The rationale behind the sub-model is that the rotifer density and the ratio of males to females affect the probability of mating between males and females in each of the experimental conditions. These matings may produce a fertilized egg which develops into an egg that can be distinguished as a resting egg within 1–3 days. The square root transformation of the density and sex ratio were used as the basic explanatory variable

in the model, because their distributions are closer to Gaussian. Such a transformation leads to a logistic model which fits the data better.

The sub-model allows for a non-monotonic relationship between the resting egg production at time t and the density and sex ratio at the days preceding t, since the original scales of these variables were considered. It also assumes (by using TL1, TQ1, TL2 and TQ2) that sexual reproduction as well as resting egg production may change during the experiment as a response to changing environment.

The sub-model coefficients were estimated by a standard logistic regression program (BMDPLR, see Dixon, 1983) which assumes that the events (production – nonproduction) at adjacent times, when the effects of the considered factors are removed, are uncorrelated. The small value of the serial correlation between the residuals justifies this assumption.

Sub-model for the second mechanism: the number of resting eggs produced

The relationship between the number of resting eggs at those days of the experiment where production was observed (125 days) and the above set of factors was assessed by a log-linear model (McCullogh & Nelder, 1983) which assumes that:

1. The variation in the non-zero number of resting eggs is proportional to the square of its average value.
2. The log of the expected number of resting eggs, given that production had occurred (N_{ijkt}) for time t, replicate k of an experiment at salinity i and food concentration j, is related to the same 10 studied factors by:

$$\begin{aligned}\log N_{ijkt} = {} & \mu + \alpha_i + \delta_j + \lambda_{ij} + \beta_1 U_{ijkt} \\ & + \beta_2 \sqrt{U_{ijkt}} + \beta_3 SR_{ijkt} \\ & + \beta_4 \sqrt{SR_{ijkt}} + \beta_5 TL1_{ijkt} \\ & + \beta_6 TQ1_{ijkt} + \beta_7 TL2_{ijkt} \\ & + \beta_8 TQ2_{ijkt} . \end{aligned} \quad (2)$$

Note that the parameters of this submodel account for the same factors that are included in the logistic submodel. However, the values in both submodels need not be the same. Sub-model coefficients were estimated by using the BMDP3R program (see Dixon, 1983).

Contribution of the factors to the total production of resting eggs

The expected number of resting eggs in time t of replicate k of an experiment with salinity i and food concentration j can be expressed as

$$V_{ijkt} = P_{ijkt} N_{ijkt} ,$$

where P_{ijkt} is the corresponding probability of production, and N_{ijkt} is the expected number, given that production had occurred. The contribution of a factor x (i.e. a component of the variable vector X) to $\log (V_{ijkt})$ is given by

$$\frac{d \log V_{ijkt}}{dx} = (1 - P_{ijkt}) \beta_{LX} + \beta_{GX} ,$$

where β_{LX} and β_{GX} denote the coefficients of factor x in the logistic model for P_{ijkt} and the generalized linear model for $\log (N_{ijkt})$.

An estimate for the average contribution of factor x is

$$\beta_X = (1 - P...) \beta_{LX} + \beta_{GX} , \quad (3)$$

where β_{LX} and β_{GX} are the corresponding regression estimates, and $P....$ is the probability at a specific time, reflecting the average experimental conditions. Its estimated variance is given by

$$\begin{aligned}\mathrm{Var}(\beta_X) = {} & \mathrm{Var}(\beta_{LX}) (1 - P...)^2 \\ & + \frac{P...(1 - P...)}{n} \beta_{LX}^2 \\ & + \mathrm{Var}(\beta_{GX}) ,\end{aligned}$$

where $\mathrm{Var}(\beta_{GX})$ and $\mathrm{Var}(\beta_{GX})$ are the corresponding estimated variances.

Results

A sequence of events led to the production of resting eggs. At the beginning of the experiment, only amictic females were present; later, mictic females appeared in cultures maintained at 9, 18 and 27‰ salinity. This was followed by the appearance of males and later of resting eggs. Resting eggs were produced only at two salinities, 9 and 18‰.

The logistic regression sub-model was successfully fitted to the data. Predicted probabilities of resting egg production were plotted into histograms (not shown) for two groups: the production group and the non production group. These histograms show a small amount of overlap. The relative difference between the log likelihood statistics for the fitted model and the model without explanatory variables (0.37) indicates a strong association between the resting egg production and the factors included in the model (Haberman, 1982).

Analysis of the data by the two sub-models (the first and second mechanisms) and their combined effect are given in Table 2. Figs 1–3 show the analysis for the overall combined effect. The main effects of salinity and food concentration on the odds of production of resting egg and the coefficients of all the other factors in the first mechanism are described in Table 2, columns 1 and 2. Similarly, columns 3 and 4 show the analysis for the second mechanism. The salinity and food concentration coefficients represent their contributions apart from their indirect effects through density and sex ratio conditions.

For the first mechanism, both salinity and food concentration are significantly associated with the occurrence of resting egg production. The odds for the production at identical density and sex ratio are higher in salinity 9‰ compared to salinity 18‰, and they tend to decrease as food concentration increases.

Since the average densities in the 12 fixed conditions (2 salinities × 6 food concentrations)

Table 2. Coefficients and standard errors of the parameters accounting for salinity (SAL 1, SAL 2), food concentration (1–6), time (TL1, TLQ1, TL2, TLQ2), density (U, $\sqrt{U}$) and sex ratio (SR, $\sqrt{SR}$) for the logistic model (first mechanism), the poly-linear model (second mechanism) and the overall effect (combined model) ($z = \pm 1.96$ for $\alpha = 5\%$).

	First mechanism		Second mechanism		Combined model		
	Coefficient	S.E.	Coefficient	S.E.	Coefficient	S.E.	z value
Constant condition	−9.950	7.79300	−0.4688	0.9850	−7.457	5.56600	−1.3398
SAL 1	1.617	0.53550	0.7660	0.2980	1.896	0.48122	3.9399
SAL 2	−1.617	0.53550	−0.7600	0.2980	−1.896	0.48122	−3.9399
CONC 1	2.413	1.01700	−0.0773	0.4855	1.618	0.86537	1.8692
CONC 2	1.076	0.45650	−0.2840	0.2570	0.472	0.41162	1.1458
CONC 3	0.557	0.34210	−0.2300	0.1830	0.161	0.30229	0.5333
CONC 4	−0.174	0.40230	0.3120	0.2380	0.190	0.36946	0.5137
CONC 5	−1.280	0.46580	0.3680	0.2390	−0.531	0.40617	−1.3083
CONC 6	−2.592	0.57650	−0.0890	0.3310	−1.909	0.52618	−3.6287
Variable							
TL1	0.027	0.035	0.007	0.023	0.026	0.034	0.7714
TQ1	0.024	0.007	−0.008	0.005	−0.025	0.007	−3.4908
TL2	0.211	0.057	0.094	0.031	0.242	0.051	4.7830
TQ2	0.037	0.009	−0.027	0.006	−0.053	0.009	−6.0869
U	−0.002	0.00080	−0.0010	0.0004	−0.003	0.00069	−3.7815
$\sqrt{U}$	0.256	0.07043	0.0600	0.0400	0.240	0.06387	3.7533
SR	11.813	4.40100	−3.3320	2.1840	−11.629	3.79403	−3.0651
$\sqrt{SR}$	10.693	4.18300	4.4610	2.1110	11.972	3.62566	3.3019

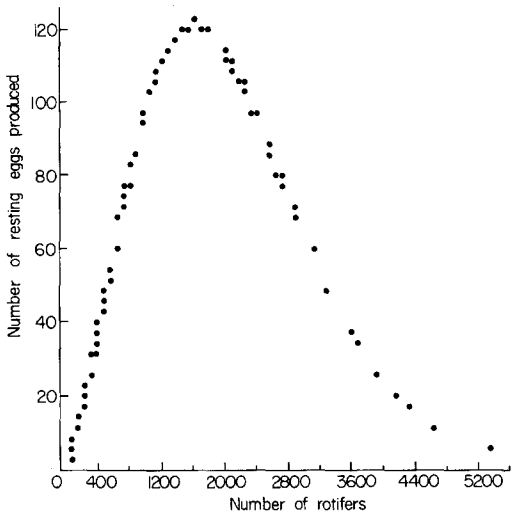

Fig. 1. The expected number of produced resting eggs (according to the combined model) relative to the rotifer density during 34–39 days. The X-axis indicates the number of rotifers (*Brachionus plicatilis*) in 10 ml cultuer media.

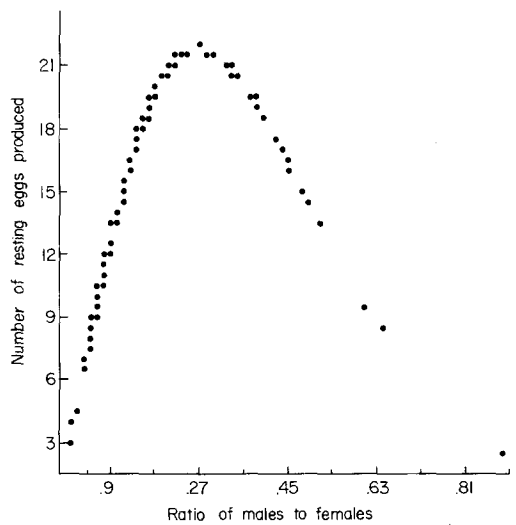

Fig. 2. The expected number of produced resting eggs relative to the ratio of males to females according to the combined model described in the text. Rotifers (*Brachionus plicatilis*) were cultured for 34–39 days.

were not similar (Table 1), we assessed also the odds of production in each fixed condition at its specific high density (= average plus standard deviation of the densities observed in each condition). Table 3 presents the ratio between these odds vs those in a baseline condition, namely

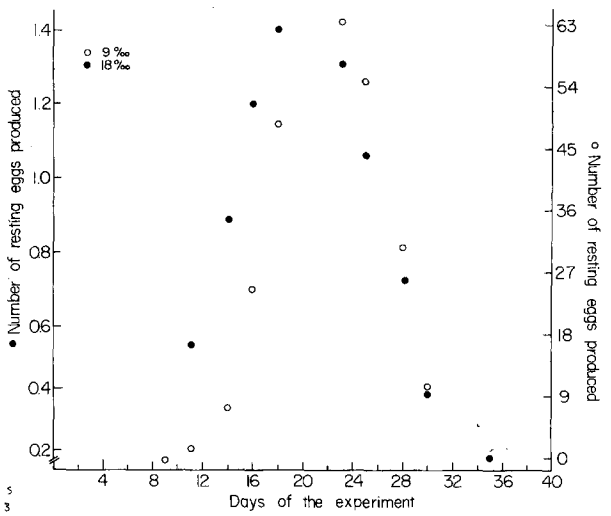

Fig. 3. The expected number of produced rotifer resting eggs at two salinities (9‰ and 18‰) during 34–39 days of the experiment, according to the combined model described in the text. The resting eggs were produced by *Brachionus plicatilis*.

those of salinity 18‰ and the lowest food concentration. Formally, these odds ratios are defined as

$$\mathrm{OR}_{ij} = \frac{P^*_{ij}/(1 - P^*_{ij})}{P^*_{2,1}/(1 - P^*_{2,1})},$$

where P^*_{ij} is the probability of production of resting eggs in salinity i and food concentration $j(ij)$ at its specific high density (baseline conditions: $i = 2$; $j = 1$).

The results indicate that the density-adjusted odds of production in each food concentration are still higher at salinity 9‰. The highest probability of production in salinity 9‰ is achieved at food concentration 5. The highly significant interaction between salinity and food concentration in this model is reflected in Table 3.

The effect of the studied factors on the number of resting eggs produced, given that production had occurred (i.e. the second mechanism), is summarized in Table 2, columns 3 and 4. The results indicate that the number of resting eggs is not strongly related to the studied factors. The number of resting eggs produced is still larger in salinity 9‰. However, no effect of food concentration is observed. Table 3 shows the specific

density adjusted ratios of the averages of the number of resting eggs produced at five food concentrations (2–5) *vs.* the lowest food concentration (1). Here, the interaction between the salinity and food concentration was conspicuously insignificant and therefore was not included in the model; thus only the combined results for the two salinities are shown in Table 3.

The overall effect of the studied factors on the expected number of resting eggs is summarized in Table 2, columns 5, 6 and 7, and in Figs. 1–3. The statistical significance of salinity and food concentration effects are provided by the *z* values, which are approximately Gaussian statistics. They are defined as:

$$z \text{ value} = \text{coefficient/S.E.}$$

The *z* values for the combined model are shown in Table 2. The effects of salinity, sex ratio and time are similar to those found in the two separate analyses, as expected from equation (3). Although not all the effects of food concentrations seem to be significantly different from each other (as reflected by the *z* values in column 7, where the ratio of coefficient to standard error is smaller than ± 2, in a test using chi-square values), a statistically significant food concentration effect was found (results not shown). The overall food concentration effect at identical density and sex ratio conditions reflects better the estimated effect described for the logistic model.

The analysis of the first mechanism shows that the odds tend to increase with the density as long as the density is not larger than 3000 (per 10 ml), but decrease at higher densities (not shown). A similar phenomenon (but to a lesser extent) is observed for the male-to-female ratio. The odds increase rapidly with sex ratio as long as the number of males to females is smaller than 1/4. A larger sex ratio tends to decrease the odds for production of resting eggs. The odds of resting egg production for both salinities seemed to change during the experiment. It was found that the odds increase for the first 14–20 days of the experiment and decrease later on. It is worth noting that a similar pattern is found in the two salinities, but the scale of production is much higher at 9‰, as reflected in the magnitude of the coefficients (Table 2; TL2, TL1, TQ2, TQ1 – columns 1 and 2).

In the second mechanism, the effect of density and sex ratio on the number of resting eggs produced is similar to their effect on the odds of production (Table 2). This means that a similar phenomenon is described by the first and second mechanism. However, in the latter, a lower density and a higher sex ratio are required to obtain an optimal result. The number of resting eggs produced also was found to change during the experiment.

The density specific adjusted ratios of the average of the number of resting eggs produced (Table 3) are not strongly affected by the food concentration. In this overall analysis, the expected number of resting eggs tends to increase until the density reaches 1,800 and decreases later on (Fig. 1). The expected number of resting eggs tends to increase as long as the number of males to females is smaller than 0.27 (Fig. 2). In terms of the effect of time, the expected number of resting eggs increases until days 18–23 and decreases later on (Fig. 3).

Discussion

Rotifer resting eggs offer a possibility for a reasonably stable source of *Brachionus plicatilis* as live food in rearing fish larvae (Lubzens, 1981; Snell & Hoff, 1986; Hagiwara, 1988a, b, 1989). Food concentration and salinity have been shown to affect the appearance of mictic females and males (Lubzens *et al.*, 1980, 1985; Lubzens, 1981; Snell, 1986; Hagiwara *et al.*, 1988a, b, 1989). Resting egg formation was also found to be affected by salinity (Lubzens *et al.*, 1980; Lubzens, 1981; Hagiwara *et al.*, 1988a, b, 1989; Hino & Hirano, 1988) and temperature (Hagiwara *et al.*, 1988a). Hagiwara *et al.* (1988a, 1989) examined the effect of salinity on the production of resting eggs in L and S type rotifers. They reported that while in L type rotifers the numbers of resting eggs decreased with an increase in salinity, the reverse relationship was found with S type roti-

fers. The rotifers used in the present study fit the description for L type rotifers (Fukusho, 1983; Okauchi & Fukusho, 1985).

In the present study we examined the effect of food concentration and salinity on the production of resting eggs in 14 observations made during a period lasting 34–39 days. The sequence of events that led to the production of resting eggs was similar to that described by Hagiwara *et al.* (1988a). At the beginning of the experiment, only amictic females were present; later, mictic females appeared in cultures maintained at 9, 18 and 27‰ salinity. This was followed by the appearance of males and later of resting eggs. Resting eggs were produced only at two salinities: 9‰ and 18‰. The amount of food provided dictated the maximum number of individuals at each of the tested concentrations, but did not fully control it.

In sampling from a population, four types of females can be distinguished: (a) females that do not carry eggs; (b) females that carry unfertilized amictic eggs that will hatch into females; (c) mictic females that carry one or more unfertilized male eggs which are about half the size of amictic eggs; (d) mictic females that carry fertilized eggs that will develop into resting eggs. Females that do not carry eggs may belong to any one of the other three categories. Also fertilized eggs cannot be distinguished from amictic eggs during the first 24 hours following their extrusion. This may introduce some error if mixis is evaluated by females alone. Since unfertilized mictic females produce only males, it is possible to evaluate the level of mixis by counting males only. Snell (1986) reported a correlation (0.64 ± 0.03) between the number of mictic females and the number of males in a population, and this was also used here. However, Hagiwara *et al.* (1989) showed that the appearance of males did not ensure the production of resting eggs (Fig. 1; Hagiwara *et al.*, 1989). Similarly, males appeared in rotifers cultured at 27‰ in the present work but no resting eggs were produced.

The increase in production of resting eggs up to day 18 (in 9‰ and 18‰ cultures) and its decline later on are probably a reflection of an interplay between several factors. Food was provided at a pre-set concentration. This led to an increase in the rotifer population up to a maximum level, after which the total number of females fluctuated and was reduced, due to low production of eggs (see Table 1a). This in turn included also lower production of mictic females, which resulted in a lower proportion of the males in the population. Eventually no resting eggs were produced. The total number of females at the peak depended on the food concentration provided and varied between the different salinities. This peak was reached faster at 9‰ and 18‰ than at the higher salinities.

Two types of effect were examined: in the first one, the factors that contribute to the appearance of resting eggs were identified. In the second one, the factors that promote production of resting eggs, once it occurred, were analyzed. As expected, it was found that the relative number of males and females contributes significantly to resting egg production. This relationship may reflect the possible number of pairs that can be formed for fertilization. However, this bell-shaped relationship (Fig. 2) clearly indicated that ratios exceeding 0.27 (of males to females) resulted in a reduced production of resting eggs. A similar bell-shaped relationship was observed in relating the number of resting eggs produced to the density of females in the culture. Production of resting eggs increased up to a density of 180 females per ml (Fig. 1) and decreased at higher densities. It is possible that some of this density effect was due to partial starvation of females in high density cultures, as mentioned above.

Both sub-models showed a significant effect of food concentration on the production of resting eggs (Table 3) only if the specific high density at each considered condition is taken into account. In the first mechanism, the highest probability for production is expected for rotifers cultured in 9‰ salinity, at a food concentration of 4×10^6 cells ml^{-1}. In the second mechanism, where no significant interaction between salinity and food concentration was observed, the highest production is achieved at 2×10^6 cells ml^{-1}. In the overall combined model, the food concentration does

not exhibit a strong effect on the production of resting eggs.

A theoretical model proposed by Snell (1987), describing the relationship between the patterns of sexual reproduction and the production of resting eggs, was based on the following assumptions: (a) an intrinsic rate of population increase (r) of 0.75; (b) the percent of sexually fertilized females at a constant of 25%; (c) the number of resting eggs produced per fertilized female is constant at 3 resting eggs. The model did not take into account initially the population density. When population density was considered (Fig. 4; Snell, 1987), these assumptions were also applied to high density populations. In the present work, r values ranged from 0.09 to 0.50 in the various cultures (detailed results not shown). Also, in the results reported here, the percentage of fertilized females was variable and never reached 25%. In practice, population density and the relative number of males in the population were found to contribute significantly to the production of resting eggs. These factors, and several others, are not fully controllable, and the instantaneous changes that occur probably contribute to the large variations between replicate cultures. Although some of the variation reported may have been related to the fact that the rotifers stemmed from several individuals (not cloned), a similar trend was also observed in experiments performed on a cloned population (Lubzens, unpublished). Similarly, a high standard deviation was observed by Hagiwara *et al.* (1989; Tables 2 and 3) in the production of resting eggs.

The present work suggests that an increase in the production of resting eggs could be achieved by increasing the amount of food as the number of rotifers in the population increases. Similarly, the density of rotifers in culture could be partially maintained by increasing the volume according to the increased density. It is possible that, by using this strategy, the production of resting eggs would be a continuous process, leading to a steady harvesting of resting eggs.

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