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Effect of crowding on nauplii production during mating time in *Tisbe clodiensis* and *T. holothuriae* (Copepoda, Harpacticoida)

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ABSTRACT: *Tisbe clodiensis* and *T. holothuriae* females and males were maintained at different densities, from sexual maturity to the extrusion of the first egg sac, in order to study the effect of crowding on the number of nauplii produced by the first egg sac. They were tested in pure cultures at densities of 1, 2, 4, 6 and 8 couples per 20 ml of sea water, and in mixed cultures (half of the individuals belonging to each of the two species) with 2, 4, 6 and 8 couples per 20 ml, without food limitation. At the highest crowding level, and for *T. holothuriae* only, the effect of daily renewal of the culture water was examined. The mean number of nauplii per female decreases with increased density. A significant linear regression of number of nauplii on number of couples was found. The regression slopes appear to be the same for both species, regardless of whether they are cultured singly or together. The daily renewal of water diminishes the effect of crowding. The possibility that a complex chemical compound, produced by the animals, which enables the latter to perceive and to react to crowding, is discussed.

INTRODUCTION

The experiments on the copepod *Tisbe* reported in this paper are part of a series designed in our laboratory to study the relevance of the biotic components of the environment, such as the genetic structure, intra- and interspecific competition and crowding, on the population biology of these marine organisms.

Larval crowding has been found to play a role in modifying many vital functions in several experimental organisms: in particular it consistently depresses the number of offspring per female (Frank et al., 1957; Barker & Podger, 1970). A similar result was found by Hoppenheit (1976) in populations of *Tisbe holothuriae.* Moreover, earlier experiments in our laboratory have suggested the possibility that the number of nauplii produced might be dependent upon density at the time of mating.

In the present experiments we studied the effect on nauplii production of varying the density of sexually mature individuals. Investigations were carried out only over a short period of the life cycle of these organisms so as to estimate the significance of crowding and other biological factors at different stages of the life cycle.

To obtain data which would be more informative as to the relation between crowding and number of nauplii produced, two species which differ in several characteristics were tested. First they differ in respect to their intrinsic rate of natural increase (r_m): estimates made on *T. holothuriae* (Parise & Lazzaretto, 1966; see Volkmann-Rocco, 1971, for the true taxonomic identification) suggest a value of 0.323; for the *PP* genotype in *T. clodiensis* (Lazzaretto-Colombera & Polo, 1969) r_m is 0.215. This is reflected also in standard monospecific cultures of *T. holothuriae* which are much more crowded than the corresponding cultures of the other species.

Other differences exist with respect to the ecology of natural populations. *T. holothuriae* appears to cope with a wider range of ecological conditions (Fava & Volkmann, 1975) and to survive in highly variable environments (Fava & Volkmann, 1977). Generally, this species has a higher population density in nature.

Another reason for testing these two species arises from the fact that in competition experiments (Fava, 1972) *T. holothuriae* specimens appear to exert a strong inhibition on the growth of *T. clodiensis* individuals: the latter disappear from mixed cultures within less than one generation. This outcome also appears when the species are put together in a 1:1 ratio over a range of densities, water aeration and salinity (Fava & Crotti, unpublished). Thus, similar interactions between the two species are expected to occur when both coexist at the time of mating.

MATERIALS AND METHODS

The stocks utilized in these investigations came from animals collected in the lagoon of Venice in 1970, and reared in the laboratory for several years employing the culture method proposed by Battaglia (1970). For *T. clodiensis* the strain used was the dominant homozygote *PP*, in which the females are characterized by the presence of a red-violet pigment in some segments of the second, third and fourth pair of legs. This facilitated species identification.

All experiments were carried out at a constant temperature of 18 °C, using small glass bowls containing 20 ml of sea water ranging between 34 and 36 ‰ salinity and a small

Table 1

Numbers of vessels and individuals per vessel used. (A) *T. holothuriae* and *T. clodiensis*, tested singly; (B) both species tested together

	No. of vessels	♂♂	+	♀♀		No. of couples
(A)	24	1		1		1
	12	2		2		2
	6	4		4		4
	4	6		6		6
	3	8		8		8
	No. of vessels	*T.h.* + *T.c.* ♂♂		*T.h.* + *T.c.* ♀♀		No. of couples
(B)	12	1	1	1	1	2
	6	2	2	2	2	4
	4	3	3	3	3	6
	3	4	4	4	4	8

piece of *Ulva*. Every two days the animals were fed with fragments of boiled wheat; the amount was roughly proportional to the number of individuals. Food was always in excess.

Fifty ovigerous females were taken from the laboratory populations, for each test. They were isolated in single vessels to lay the nauplii of one egg sac. When the 4th or 5th copepodite stage was reached, males and females from each offspring were placed in different vessels; thus brother/sister mating and hence inbreeding depression was avoided.

To study the effect of crowding on nauplii production, the numbers of vessels and individuals were as listed in Table 1.

Each vessel was checked daily. As soon as the females became ovigerous, they were isolated in single vessels to allow them to lay the nauplii of the first egg sac. Such nauplii were counted within a few hours after birth. Six days following pair formation the remaining non-ovigerous females were discarded.

The effect of the renewal of the "medium" was tested with *T. holothuriae* only, at the highest crowding level. Ten vessels were used for the experiment and ten as control. The experimental animals were transferred daily into a freshly prepared vessel, except for already ovigerous females which were isolated singly, for counts of nauplii as described above. Control animals were removed daily and reintroduced into the same vessels, and ovigerous females isolated singly to count hatched nauplii. This process lasted six days, after which the remaining non-ovigerous females were discarded.

All the experiments were repeated, at an interval of about three months. Unfortunately, for practical reasons, it was impossible to count the number of nauplii from egg sacs later than the first one.

RESULTS

Effect of crowding

In all experiments the mean number of nauplii laid per female is strongly reduced by crowding, the value for eight couples per vessel being about half that obtained for one couple per vessel (Tables 2 and 3).

Before applying regression analysis, we tested whether differences might exist between vessels, in each crowding level for each experiment. In only two out of twenty-eight grouping for *Tisbe holothuriae* at the second crowding level and for *T. clodiensis* + *T. holothuriae* at the sixth level of crowding (both in the first experiment) was a significant difference ($P < 0.05$) found. Thus it appeared reasonable to assume homogeneity within groups, and the data were analysed without regard to the fact that the counts were made on females coming from different vessels.

The analysis of variance shows a highly significant effect of crowding (reduction of the mean number of nauplii laid per female) in all experiments ($P < 0.001$). To find the function relating nauplii production to crowding level, the linear regression of number of nauplii per female on $\log_{10}$ (number of couples) was calculated and significant regression was found in all experiments for both species except for *T. clodiensis* in mixed culture in the first experiment (Tables 2 and 3; Figs 1 and 2).

It is possible to obtain significant regressions even if the absolute number of couples, instead of their logarithm, is considered. However, the logarithmic relation appears to give

Table 2

Tisbe holothuriae. Mean numbers of nauplii per female ($\bar{Y}$), standard deviations (s) at the different crowding levels, and linear regression for each experiment (Y = number of nauplii; X = log number of couples)

No. of experiment	No. of couples per vessel	No. of ovigerous females	Nauplii per female $\bar{Y}$	s	Linear regression
Singly					
I	1	21	59.8	10.88	
	2	23	54.1	9.27	$Y = 63.29 - 42.86\ X$
	4	21	42.3	9.09	$s_b = 7.637$
	6	16	22.4	8.32	$P < 0.05$
	8	23	24.9	9.75	
II	1	23	52.8	5.95	
	2	22	44.9	5.88	$Y = 53.20 - 29.62\ X$
	4	24	35.5	5.60	$s_b = 0.608$
	6	23	30.0	5.22	$P < 0.001$
	8	22	26.2	4.78	
With *T. clodiensis*					
I	2	10	47.6	5.87	$Y = 58.37 - 33.21\ X$
	4	12	39.0	4.41	$s_b = 4.216$
	6	12	34.2	4.88	$P < 0.05$
	8	11	26.6	6.36	
II	2	12	84.3	8.42	$Y = 106.85 - 70.51\ X$
	4	12	68.3	10.30	$s_b = 9.173$
	6	9	47.7	5.66	$P < 0.05$
	8	10	43.9	5.13	

Table 3

Tisbe clodiensis. Mean numbers of nauplii per female ($\bar{Y}$), standard deviations (s) at the different crowding levels, and linear regression for each experiment (Y = number of nauplii; X = log number of couples)

No. of experiment	No. of couples per vessel	No. of ovigerous females	Nauplii per female $\bar{Y}$	s	Linear regression
Singly					
I	1	20	43.7	6.74	
	2	21	38.8	4.95	$Y = 44.92 - 30.16\ X$
	4	22	25.1	4.52	$s_b = 3.329$
	6	23	20.2	3.66	$P < 0.01$
	8	18	19.3	4.01	
II	1	23	46.2	5.26	
	2	21	40.5	3.94	$Y = 47.25 - 27.37\ X$
	4	22	31.5	5.04	$s_b = 1.643$
	6	21	25.2	5.49	$P < 0.001$
	8	21	22.2	5.47	
With *T. holothuriae*					
I	2	8	32.8	5.63	
	4	9	32.6	5.00	n. s.
	6	10	20.2	4.26	
	8	9	16.2	3.56	
II	2	10	60.2	6.27	$Y = 73.49 - 39.62\ X$
	4	11	53.0	7.90	$s_b = 6.859$
	6	10	40.3	3.53	$P < 0.05$
	8	11	37.7	4.67	

a better description of the phenomenon for the following reasons: (1) with the log scale the deviations from regression are significant in two out of seven cases (*T. holothuriae* singly, first experiment: $P < 0.001$; *T. clodiensis* singly, first experiment: $P < 0.01$), while with the linear scale they are significant in five out of seven cases. That is, with the log scale the residual variation around regression lines is at a minimum. (2) From the seven regression lines we could estimate that, if the linear scale is considered, no nauplii will be produced by the females when there are 12 to 17 couples per vessel; while with the logarithmic relation, the zero point is expected for crowding conditions ranging from 30 to 72 couples per vessel. From our laboratory experience we know that at 20–30 couples per vessel the number of nauplii produced per egg sac is far from zero. And this is in contradiction to the linear scale model, but not to the logarithmic one. But it is likely that for wider crowding intervals the relation will no longer be a linear one within either scale.

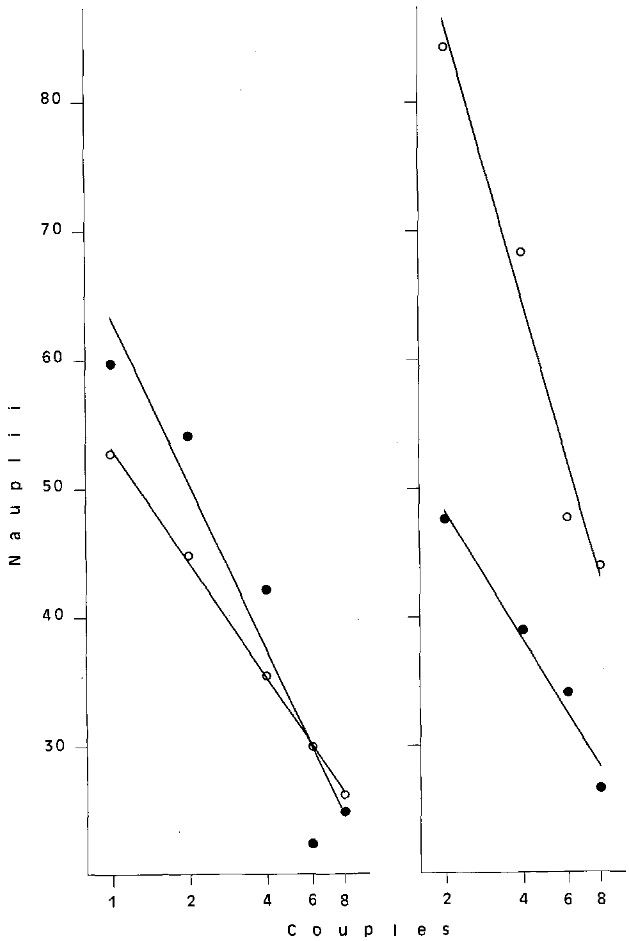

Fig. 1: *T. holothuriae.* Mean number of nauplii per female at the different crowding levels. Significant regression lines are drawn. Left: pure cultures. Right: mixed cultures. (●) First experiment. (o) Second experiment

The slopes of the regression lines do not differ, except for that calculated for *T. holothuriae* + *T. clodiensis* in the second experiment (the STP test suggested by Sokal & Rohlf, 1969, was applied). Thus, crowding appears to operate in the same way in both species, whether it is determined by animals belonging to the same species or to different, although closely related species.

In this experiment, the last to be carried out, we obtained an exceptionally high number of nauplii in both species (Tables 2 and 3). This was probably due to the water quality as we use natural sea water collected monthly in the open sea; thus its composition cannot be maintained constant.

Effect of renewal of the medium

In these experiments we have not obtained clear evidence to enable us to exclude an added variance due to differences between vessels. Thus we applied a nested anova to the data.

Because of the complications arising from unequal sample sizes, we decided to equalize them as follows: (1) only vessels which gave more than 5 ovigerous females were considered, and (2) for each of these vessels only 6 females were included in the samples with the aid of a random number table. (In both experiments the maximum number of

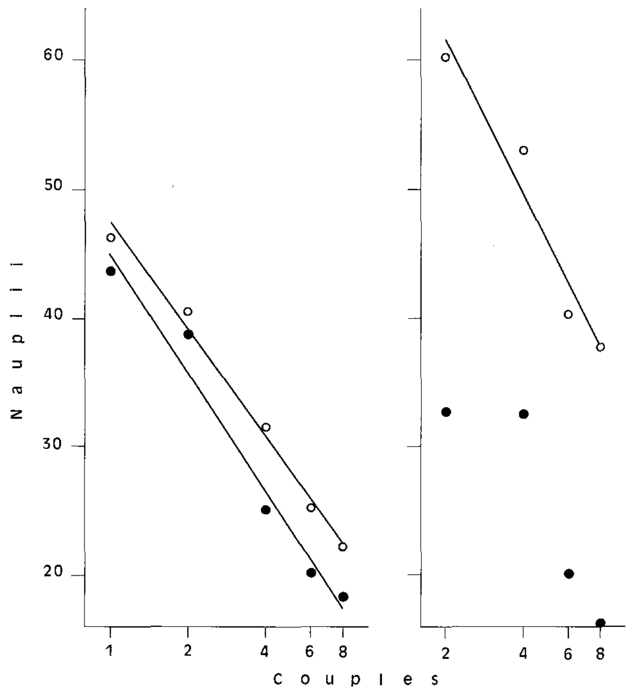

Fig 2: *T. clodiensis.* Mean number of nauplii per female at the different crowding levels. Significant regression lines are drawn. Left: pure cultures. Right: mixed cultures. (●) First experiment. (o) Second experiment

ovigerous females per vessel never exceeded eight. Thus, with this random procedure not much information was lost.) We discarded the data only from those vessels in which 50 % (or fewer) females became ovigerous in order to limit the bias that could be due to damage suffered by the animals during transfer.

Table 4

Tisbe holothuriae. Mean numbers of nauplii per female ($\bar{x}$) and standard deviations (s) for the renewal of the medium experiments

No. of experiment	Control $\bar{x}$	Control s	Water renewal $\bar{x}$	Water renewal s	N
I	29.1	6.05	32.9	6.37	48
II	25.4	6.07	30.6	6.50	42

The means and variances, however, do not differ in original and the rearranged data; the latter are summarized in Table 4. An analysis of variance was applied to the data. In both replicas of the experiment, the mean number of nauplii produced per female was significantly higher in the "treated" animals than in the "controls" ($P < 0.05$ in the first, and $P < 0.01$ in the second replica). In the first replica only, a significant difference was found between vessels ($P < 0.05$). No satisfactory explanation of this finding can be presented.

Nevertheless, the hypothesis that renewal of the culture water diminishes the effect of crowding is confirmed.

DISCUSSION AND CONCLUSION

The results of the experiments reported above show that the mean number of nauplii, produced by the first egg sac, is inversely correlated with the logarithm of the crowding level at which the females are exposed, during the relatively short life span, from sexual maturity to the extrusion of the first egg sac. That is, proportional increases in crowding produce linear decrements in the mean number of nauplii per female. At present we do not know if this effect is extended to the egg sacs following the first one; nevertheless the above results can be regarded as a confirmation that the period of the life cycle considered is a crucial one.

Sang (1950), quoted by Barker and Podger (1970), outlined the importance that larval nutritional histories may assume in controlling fecundity. This cannot constitute a source of bias for the present data because individuals from different progenies were distributed among all tested densities. Furthermore, food does not appear to have any influence in our experiments because, as previously stated, it was always in excess.

Our results are in general agreement with the findings of Hoppenheit (1976), but the effect of crowding appears to be weaker in the present experiments. However, more precise comparison is impossible because the experimental methods are different.

The estimates of Parise & Lazzaretto (1966) and Lazzaretto-Colombera & Polo (1969) indicate that in absolute numbers, and for each crowding level, *T. holothuriae* produces more offspring than *T. clodiensis.*

Nevertheless, the regression coefficients appear to be the same for both species, whether they are tested singly or together. Some doubt could arise for this second kind of experiment. We obtained no evidence for regression, but a significant effect of crowding for *T. clodiensis* in the first replica, and a regression coefficient significantly different from others for *T. holothuriae* in the second replica. However, these different outcomes are not in the same direction, and do not appear to contradict the equality of the regression slopes. In fact, for *T. clodiensis*, the lack of regression could be due to chance. In the case of *T. holothuriae*, as stated above, the difference is most likely due to water quality. This outcome might suggest the possibility that the depression due to crowding is higher when the absolute number of nauplii produced is increased by better environmental conditions. But, at present, this is a working hypothesis only.

A conclusion drawn from our findings is that the differences between the two species, found in natural as well as laboratory populations, are not dependent upon, and do not reflect, a differential effect of crowding on offspring production. For both species, the reaction to density represents an equally strong feedback mechanism tending to keep the population size constant.

An unresolved problem is the understanding of the mechanism that enables the animals to perceive crowding level and to respond to it. This does not appear to be based on food shortage. On the other hand, the daily renewal of sea water was found to depress the effect of crowding. Thus, two possibilities can be suggested: the mechanism is based on "something" that animals (1) subtract to the environment, and/or (2) add to the environment. In the first case dissolved oxygen could be involved; considering the water surface of 20 cm^2 exposed to air in the vessels used and the presence of a small fragment of living *Ulva*, it appears unlikely that oxygen concentration could be strongly reduced. It is hard to suppose that other subtracted substances could be the mediators because we were able to maintain, in the same volume of water, more than 5 times crowded cultures for longer periods. It was also possible to rear *T. holothuriae* and *T. clodiensis* in the same water and in bigger vessels (1 or 2 l) without aeration for more than six months at crowding levels, which were not lower than the maximum tested in the present experiments.

Thus, the second possibility appears to be the more likely. In this case the "something" produced by animals could be a "non-specific" simple chemical compound, such as carbon dioxide or ammonia, or a more complex and "specific" biotic residue. The mediation of biotic residues was suggested for similar kinds of interactions in some *Drosophila* species (Weisbrot, 1966; Dawood & Strickberger, 1969; Budnik & Brncic, 1975). For the rotifer *Brachionus calyciflorus*, Pourriot and Rougier (1977) proposed a mechanism based on substances secreted by females to explain effects of grouping and population density on the production of mictic females. Thomas et al. (1975) studied the effect of chemical conditioning of the water by adult snails on the growth of juveniles. The importance of interactions based on chemicals produced by marine organisms was recently outlined by Levandowsky (1977).

A mechanism based on the mediation of a "specific" and complex chemical compound produced by the animals appears, in our opinion, the most likely to explain the results obtained. Such a compound could determine, directly or indirectly, a modification of the "physiological status" of the individuals, leading to a regular decrement of the fertility with increasing crowding. The present results appear to contradict those obtained from previous competition experiments between the two species (Fava, 1972), and for which the mediation of a metabolic product was also proposed. In fact, in those experiments only one species *(T. holothuriae)* survived after a very short time, while in the above described experiments, the depression of the nauplii number is the same, whether the two species are put together or not. However this may be only an apparent contradiction. In fact, the suggested compound need not necessarily affect adults and larval stages in the same way. It is neither contradictory, nor unreasonable, to suppose that it could be toxic for nauplii and harmless for adults in which it operates as a chemical signal. Thus both kinds of interaction could be due to the same compound produced.

The present results do not give unequivocal experimental evidence as to the validity of this hypothesis; however, considering all the experiments related to this problem in *Tisbe*, it appears, for the moment, the most probable.

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