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The Influence of Food on Induction of Males in Daphnia Pulex

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THE INFLUENCE OF FOOD ON INDUCTION OF MALES IN *DAPHNIA PULEX*

by

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INTRODUCTION

Daphnia pulex Leydig (Crustacea, Cladocera) is a common component of the zooplankton of freshwater lakes and ponds. Its importance in the aquatic trophic structure as food for small fish has long been recognized.

Typical populations of daphnia consist almost exclusively of females. Under favorable conditions this situation is perpetuated by parthenogenetic reproduction. Eggs produced in this manner are referred to as parthenogenetic or summer eggs. When stressed by falling temperatures, drought, overpopulation, or starvation, populations shift to sexual reproduction. Parthenogenetic eggs are produced which develop into males. Other eggs known as ephippial or winter eggs are also produced, which after fertilization survive the environmental stress inside cocoon-like ephippia. Upon return to favorable conditions, these eggs hatch into normal diploid females and help reestablish the population.

The cause of male induction is intriguing. Cytological (Mortimer, 1936; von Dehn, 1948; Ojima, 1958) and genetic (Banta and Wood, 1928) studies have shown that both sexes in daphnids are diploid and possess identical genomes. Thus, some (Mortimer, 1936; von Dehn, 1955) believe that sex determination is purely phenotypic. Furthermore, it is their contention that phenotype is under environmental control. This position has been succinctly stated by von Dehn (1955): "Die Geschlectsbestimmung kann ... nur modifikatorisch erfolgen unter der Wirkung von Aussenfaktoren." Whether this environmental control is absolute is open to debate, but its dominant role in the process is generally accepted

(Waterman, 1960).

Male and female differentiation in crustaceans proceed through the influence of hormones. Sex intergrades are believed to be products of the interaction of male and female hormones (Charniaux-Cotton, 1960; Sehnal, 1971). Thus, it is conceivable that the factors responsible for production of males also favor sex intergrades.

Due to the perennial appearance in late autumn of male daphnids in lakes in northern temperate latitudes, past investigations have examined the influence of environmental conditions present then on induction of males. Factors studied have included physical parameters, such as temperature and photoperiod, and biological parameters, such as crowding and starvation. In autumn lakes in these latitudes exhibit a slow, but progressive, drop in water temperature and a gradual shortening of the photoperiod. Dicyclic cladocerans (Hutchinson, 1967; Wetzel, 1975), including Daphnia galeata (Hall, 1964), manifest a second population maximum in late fall in conjunction with late phytoplankton blooms. They rapidly harvest the algae, soon resulting in a scarcity of food. Very little research, however, has been directed toward the influence of ingested chemical factors such as food quality. Some work in this area by von Dehn (1937, 1950, 1955) and Gilbert (1966, 1971, 1972; Gilbert and Thompson, 1968) suggests that the food quality of nonalgal foods may play an important role in male induction of zooplankton.

Although several studies (Edmondson, 1957; Saunders, 1969) have failed to determine the diet of daphnia, it is widely accepted that all suspended particles, living or detrital, in the range of 1-15 μm (Brooks and Dodson, 1965) are filtered indiscriminately. Algae constitute a

major food.

Changes in the food quality of algae can be expected in response to periodic and seasonal changes in the environment. One important parameter known to vary in concentration seasonally (Hutchinson, 1967; Wetzel, 1975) and to profoundly affect the chemical composition of algae (Spoehr and Milner, 1949) is nitrogen. Ammonium and nitrate, the forms of nitrogen utilized in nature by most phytoplankton, are generally available in sufficient concentrations following spring and fall overturn, but exist in low concentrations throughout most of the summer and autumn in the epilimnion.

It is interesting to note that periods of maximal male production coincide closely with annual maxima of inorganic nitrogen in lakes and, conversely, that male production is low when concentrations of inorganic nitrogen are low.

It was decided, therefore, to investigate the influence of algal food quality on induction of males in daphnia. Food quality will be varied by the presence or absence of inorganic nitrogen in the algal growth medium.

Daphnia pulex was chosen for this study for the following reasons: 1. Common occurrence in freshwater zooplankton populations, 2. Widespread distribution across North America (Brooks, 1957), 3. Amount of information available on the species, 4. Simple culture requirements, and 5. Rapid sexual maturation.

LITERATURE REVIEW

Sexing Criteria

Pennak (1953) and Edmondson (1957) separated male cladocerans from female cladocerans by their smaller size, larger antennules, and modified postabdomen and first legs. Banta, et al. (1939) distinguished between male and female Daphnia longispina on the basis of five sex characters: size, rostrum, antennules, ventral margin of the carapace, and first filtering appendages. The rostrum is rounded in males, but pointed in females; the ventral margin of the carapace is well rounded in females, but slightly concave and hairy in males; and the first filtering appendages are heavier in males than females, and bear a small hook for clasping the female. In a monograph on daphnid taxonomy, Brooks (1957) added the following sexing criteria for D. pulex: the second abdominal process is generally longer in males; the optic vesicle bulges from the head in males, but not in females; and the dorsal margin of the head is slightly concave or flat in males, but rounded in females.

Stimpfl (1971) relied heavily on the head and antennules in sexing D. pulex. Slobodkin (1954) focused on the antennules and breast margin with D. obtusa. According to Banta, et al. (1939) in sex intergrades the antennules and rostrum, respectively, are the first and second most frequently modified sex characters. The head region, therefore, seems to be a prime area in determination of gender and searching for tendencies toward sex intergradation.

Criteria for sex intergrades have been quite loose in order to accommodate all intermediate forms between males and females. "The vast majority of sex intergrades are not mosaics of female and male regions but appear as blends of female and male influence as expressed in a little less maleness here, a little more there, and perhaps none yonder" (Banta, et al., 1939).

Culture of Nitrogen-Deficient Algae

Nitrogen-deficient algae can be prepared by: 1. transferring the alga to a medium devoid of nitrogen, or 2. culturing the alga in a medium with a very limited amount of nitrogen (Syrett, 1962). Nitrogen-fixing species, needless to say, are exceptions. The two methods are hereafter referred to as the nitrogen starvation method and the nitrogen exhaustion method.

Ketchum and Redfield (1949) induced nitrogen starvation in Chlorella pyrenoidosa by transferring healthy cultures to a medium containing calcium chloride in lieu of the nitrogen source, calcium nitrate. Nitrogen-starved Scenedesmus obliquus was prepared by resuspending exponentially growing cells in a medium, wherein the nitrogen as potassium nitrate had been replaced by an equimolar amount of potassium chloride (Thomas and Krauss, 1955).

Nitrogen exhaustion has been produced in aging cultures of Chlorella (van Oorschot, 1955; Bongers, 1956) and C. pyrenoidosa (Spoehr and Milner, 1949; Aach, 1952) by simply allowing the algae to deplete the small supply of nitrogen.

Influence of Nitrogen Deficiency on Metabolism in Chlorophytes

Ketchum and Redfield (1949) found differences in the composition of Chlorella pyrenoidosa grown with or without nitrogen. Cells grown with nitrogen contained about 50% protein, while those grown without nitrogen contained about 15% protein. In both cell types, the remaining material was composed of approximately equal amounts of carbohydrate and fat.

An immediate exponential decrease in the total nitrogen content of Scenedesmus obliquus followed transfer to a nitrogen-deficient medium (Thomas and Krauss, 1955). No significant changes were detected in the proportions of the various nitrogen fractions, however. The growth rate declined sharply during the first 24 hours after transfer, but slowly rose again during the next few days.

Protein synthesis in Chlorella ceased soon after removal of nitrogen from the medium, but carbohydrate synthesis increased (van Oorschot, 1955). As nitrogen starvation progressed, lipid synthesis became more dominant.

Bongers (1956) discovered nitrogen starvation impaired photosynthesis in Chlorella. Within a few days, the rate of photosynthesis had fallen to about five per cent its initial rate. In a similar study with Chlorella, van Oorschot (1955) also noticed a fall in the quantum efficiency of photosynthesis.

Aach (1952) reported the fat content of nitrogen-depleted C. pyrenoidosa cells rose from 22% of the dry weight on the second day of culture to 70% of the dry weight on the 25th day. Upon depletion of the

nitrogen supply, protein synthesis halted and carbohydrate synthesis fell off; however, lipid synthesis continued on at the same rate resulting in an accumulation of fat. Bongers (1956) confirmed fat accumulation in nitrogen-exhausted Chlorella.

C. pyrenoidosa cultures, which depleted the nitrogen supply to under 0.001 M concentration, produced cells with a large amount of lipid; those, wherein the nitrogen concentration remained above 0.001 M, produced cells with a small amount of lipid (Spoehr and Milner, 1949). Nitrogen depletion also caused decreases in the chlorophyll and carotene contents. In extreme cases, the chlorophyll content dropped to 0.05-0.2% of its original value; falls in carotene content were less dramatic.

Influence of Physical Factors on Male Induction in Cladocerans

Berg (1931) believed the autumnal decrease in lake temperature was the apparent cause for the simultaneous appearance of male cladocerans. He was unable, however, to account for males in the spring. Issakowitsch (1909) found Simocephalus males appeared at low temperatures. Von Dehn (1937) could not induce males in Moina rectirostris either at cold or hot temperatures. Banta, et al. (1939) established maximal male production in M. macrocopa at 14°-17°C and above 30°C and found minimal production at 22°-27°C and below 12°C.

Tauson (1930) reported that temperature influenced the sex ratio of Daphnia pulex offspring; von Scharfenberg (1911) found no such correlation in D. magna. Stimpfl (1971) found males in broods of D. pulex at 10°, 16°, and 22°C. The percentage of male offspring was highest at

16°C. Males were observed in D. magna broods as the culture temperature was successively lowered from the optimum for growth (Mortimer, 1936).

Stross and Hill (1965) discovered short photoperiods elevated percentages of male offspring in D. pulex. They established that the duration, not the quantity, of light was the crucial factor. Male offspring percentages at the various photoperiods were: L:D = 16:8 (0%), 14:10 (5.9%), 13:11 (53.8%), and 12:12 (48.6%).

In research on D. pulex, Stimpfl (1971) found photoperiod more significant than temperature in inducing males. At photoperiods of L:D = 12:12, 15:9, and 18:6, numbers of male offspring varied inversely with hours of daylight. Leary (1967) demonstrated that photoperiods equal to or less than L:D = 14:10 gave rise to males in populations of D. pulex.

At low food levels, male production in Moina was higher in constant darkness than constant light (von Dehn, 1937). Offspring sex ratios did not vary in Chydorus, Scapholeberis, or Daphnia, whether cultured in diffuse light, darkness, or at the window (Mortimer, 1936).

Influence of Biological and Chemical Factors on Male Induction in Cladocerans

Since biological and chemical factors are often closely interwoven in male production, they will be reviewed together.

Mortimer (1936) and Banta, et al. (1939) found overpopulation effective in inducing males in several species of cladocerans. Working with Moina macrocopa, Banta and Brown (1929a) observed males more fre-

quently in cultures containing several females rather than one female. Males were observed most frequently in crowded cultures. Crowding could successfully induce males either by decreasing the volume of medium or increasing the density of females.

Increasing the population density from 3 females/50 ml to 30 females/50 ml produced more male offspring in Daphnia pulex (Leary, 1967). Stimpfl (1971) whowed that the percentage of male offspring from D. pulex rose as the culture density was increased from 1 female/24 ml to 4 females/24 ml. A further increase to 8 females/24 ml was unsuccessful in elevating the percentage of males higher.

Mortimer (1936) found a reduction in the quantity of food resulted in the appearance of males in cultures of D. pulex, D. magna, D. cucullata, Chydorus sphaericus, and Scapholeberis mucronata. Males comprised a larger percentage of M. rectirostris offspring, when the populations were fed lower concentrations of algae (von Dehn, 1937). Hosseinie (1966) speculated that scarcity of food may have been partially responsible for production of nonfunctional D. middendorffiana males.

Stuart and Banta (1931) showed the sex ratio of M. macrocopa offspring could be influenced by the amount of bacteria present in the culture medium. High concentrations of bacteria yielded only female offspring, but a moderate decrease in the bacteria concentration produced males. Excessive reductions resulted again in only female progeny. Slobodkin (1954) observed emergence of males in starved D. obtusa cultures accompanied the initial decline in the reproductive rate. Wetzel (1975) comments: "Male induction is correlated with ... a rapid reduction of food supply, as opposed to a constant low food supply which sim-

ply inhibits reproduction."

Banta and Brown (1929a) attributed male induction in Moina primarily to an accumulation of excretory products due to crowding. Mortimer (1936) found food concentration unimportant, when he demonstrated the presence of males in well fed but overpopulated cultures of Daphnia, Chydorus, and Scapholeberis. Leary (1967) had similar results with D. pulex, when he held the food level per individual constant and obtained more male offspring at higher population densities. Mortimer (1936) and Leary (1967) feel the active principle in crowding is the buildup of excretory wastes.

Excretory products induced males in uncrowded M. macrocopa cultures placed in media recently vacated by crowded cultures (Banta and Brown, 1929b). Berg (1931) observed male production in crowded Daphnia cultures occurred before a large accumulation of wastes built up. This observation was verified in D. longispina by Banta, et al. (1939).

Banta and Brown (1929c) showed that the accumulation of excretory products in media with Moina females induced a general retardation of their physiological functions. They then proved that male production was directly proportional to the degree of maternal retardation. Berg (1934) contended that among cladocerans the common denominator in induction of males was a general metabolic depression in the female, brought on by unfavorable environmental conditions.

Some research seems to indicate that the quantity of food available to cladocerans may determine the quantity of food eventually incorporated. Richman (1958) fed D. pulex cultures four different food concentrations of Chlamydomonas reinhardii over a 40-day period. As the food

concentration was raised from 25,000 cells/ml/day to 100,000 cells/ml/day more food was consumed, but the percentage of food assimilated by mature females declined from 32% to 14%. Over the same range, the percentage of food assimilated by immature females declined from 24% to 7%. These changes were accompanied by five- to six-fold increases in the caloric value of the feces.

Schindler (1968) found that passage of algae through the gut of D. magna during periods of plentiful food left many algal cells intact or only partially digested. Feces of high nutritional value were collected.

Utilization of algae was more complete in daphnids fed sparingly rather than generously (von Dehn, 1955). Wastes were frequently reingested. Feeding leftover algae from old crowded M. rectirostris cultures to new cultures of variable density caused some production of males (von Dehn, 1937).

METHODS AND MATERIALS

Culture of Daphnia

Stock cultures of Daphnia pulex Leydig were procured commercially (Carolina Biological Supply Co., Burlington, NC) and maintained in autoclaved lake water in five liter glass aquaria. Taxonomic verification was made with Brooks' (1957) daphnia key. The water was obtained from nearby Twin Lakes (Kalamazoo County). Organic matter and living material present in the water was removed upon standing or killed during autoclaving.

All water was stored in glass vessels after discovery of significantly higher daphnia mortality in water previously stored in polyethylene containers. Buikema (1968) found some materials, such as polyethylene, leached residues which were toxic to D. pulex.

Growth and reproduction were excellent in the autoclaved lake water. Attempts at using a defined culture medium were unsuccessful. Use of tap water from the City of Kalamazoo water system, sodium thiosulfate-treated tap water, or solar-irradiated tap water were invariably fatal to the cultures. Synthetic media, such as Knop's solution (Kamemoto and Goodnight, 1956) or special daphnia media described by Taub and Dollar (1964), Frear and Boyd (1967), and Leary (1967), were suitable for only brief periods, during which reproduction was poor.

Stock cultures were kept at $20^{\circ} \pm 1^{\circ}C$ at a noninductive photoperiod of L:D = 15:9. This temperature is optimal for daphnid cultures (Mortimer, 1936; Kastal'skaia-Karzinkina, 1942). Gentle aeration was provided to assure longer retention of the food in suspension. Popu-

lation densities were kept low by subculturing.

Various nonalgal foods were examined: a calf's meal infusion (Shuba, 1974), lettuce (Hyman, 1937), egg yolk (Carolina Biological Supply Co., 1971), and yeast. Yeast was chosen as the food for the stock cultures, because it seemed to satisfy the nutritional requirements of the daphnia, was convenient to prepare, and was least prone to produce excess debris in the aquaria.

The yeast solution was prepared by dissolving 0.4 g powdered baker's yeast (Universal Foods Corp., Milwaukee, WI) in 100 ml autoclaved lake water. It was refrigerated and could be used for up to one week. The volume of yeast solution added to the cultures depended on the population density.

Handling and Preservation

For examination, the animals were concentrated on Nytex filters and gently washed into a small volume of water. They were transferred by eyedropper to clear glass spot plates (Sargent-Welch Scientific Co., Skokie, IL), where excess water was removed to restrict movement. This procedure usually left the animals lying on their side for convenient sexing. Examination and sexing were done under a dissecting microscope at 30-60X magnification. Irwin loops and insect mounting pins were useful in positioning the organisms for viewing.

Upon completion of both experiments, all specimens were preserved in a modified formalin solution (Haney and Hall, 1973). This preservative generally inhibited "ballooning" of the carapace, which renders sexing difficult.

Sexing Criteria

Morphological distinctions between male (Figure 1) and female (Figure 2) daphnia are quite clear. Determination of sex was based entirely on secondary sex characteristics as described by Banta, et al. (1939) and Brooks (1957). These are summarized in Table 1. Initial examination focused on the head, rostrum, and antennules. The individual was further examined, if any of the above sex characters proved positive for maleness.

Each sex intergrade was described individually. Intergradation of their sex characters was limited to the head region.

Culture of Algae

Stock cultures of Chlorella sp. were grown in large Erlenmeyer flasks in Bold's (1967) inorganic medium (Table 2). The temperature was maintained at $17^{o} \pm 0.5^{o}C$ by placement inside a Sherer Controlled Environment Laboratory, model 4-4 (Sherer-Gillett Co., Marshall, MI). Light was provided by six 20 watt fluorescent tubes and two 75 watt incandescent bulbs, supplying the cultures with an average illuminance of 300 ± 40 ft-c. This is ample illumination for good algal growth (Starr, 1973). The illuminance was measured with an EG & G Radiometer/Photometer, model 550 (EG & G, Inc., Salem, MA). The photoperiod was set at L:D = 14:10.

Cultures were magnetically stirred to inhibit clumping, ensure uniform distribution of nutrients, and prevent localized buildup of wastes. The cultures were periodically gassed with carbon dioxide for faster growth. Sterile techniques were employed when possible; occasional

Figure 1.

Male Daphnia pulex

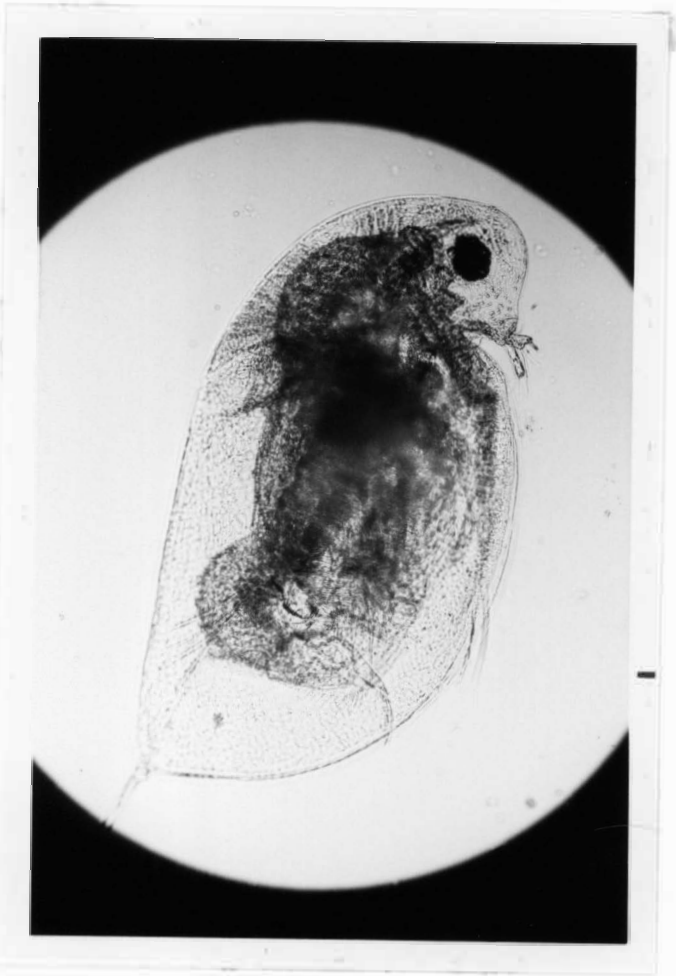


Figure 2.

Female Daphnia pulex

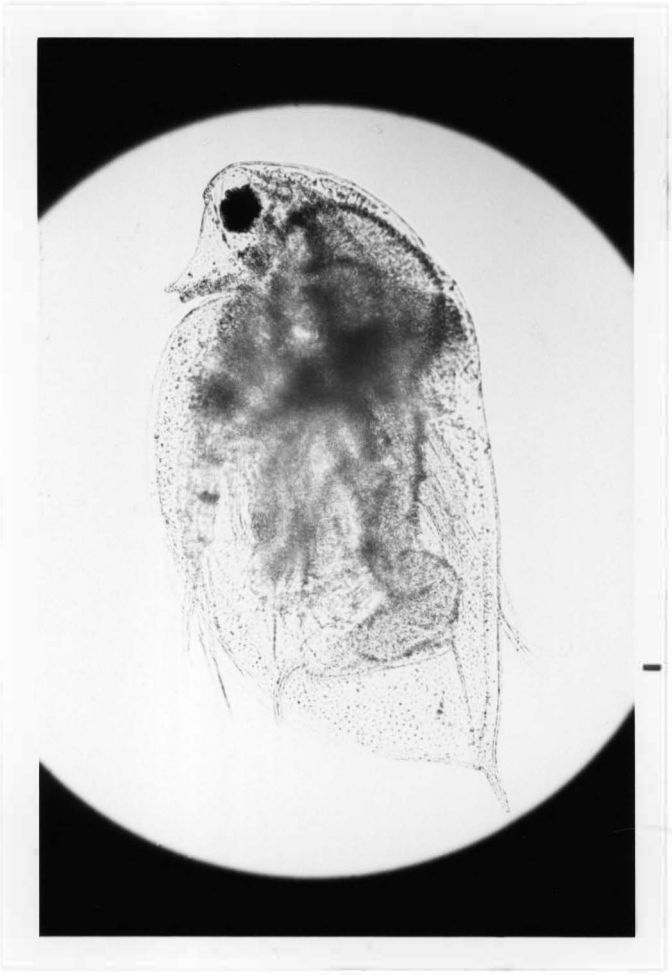


Table 1.

Daphnia Secondary Sex Characteristics
(from Banta, et al., 1939; Brooks, 1957)

Sex Character	Female	Male
Ventral margin of head	Concave	Straight
Rostrum	Pointed	Rounded
Antennules	Rudimentary	Enlarged
Dorsal margin of head	Rounded	Straight/Concave
Breast margin	Rounded	Concave, hairy
First filtering appendages	Slender	Stout, hooked
Second abdominal process	Short	Long

Table 2.

Bold's (1967) Basal Medium*

$NaNO_3$	2.94 mM	$ZnSO_4 \cdot 7H_2O$	30.7 µM
KH_2PO_4	1.29 mM	$FeSO_4 \cdot 7H_2O$	17.9 µM
KOH	0.552 mM	$MnCl_2 \cdot 4H_2O$	7.28 µM
K_2HPO_4	0.431 mM	$CuSO_4 \cdot 5H_2O$	6.29 µM
NaCl	0.428 mM	MoO_3	4.93 µM
$MgSO_4 \cdot 7H_2O$	0.304 mM	$Co(NO_3)_2 \cdot 6H_2O$	1.68 µM
H_3BO_3	0.185 mM		
EDTA	0.171 mM		
$CaCl_2 \cdot 2H_2O$	0.170 mM	H_2SO_4	3.60×10^{-4} N

* In the modified Bold's medium NaCl is substituted for $NaNO_3$, giving a total NaCl concentration of 3.37 mM.

microscopic examination of the algal cultures confirmed their integrity.

Algae fed the experimental daphnia were grown in a medium with or without inorganic nitrogen for a minimum of 20 days after transfer from the Bold's medium. The former, hereafter referred to as Chlorella A, were resuspended in fresh Bold's medium. The latter, hereafter referred to as Chlorella B, were resuspended in a modified Bold's medium, wherein the sodium nitrate was replaced by an equimolar concentration of sodium as sodium chloride.

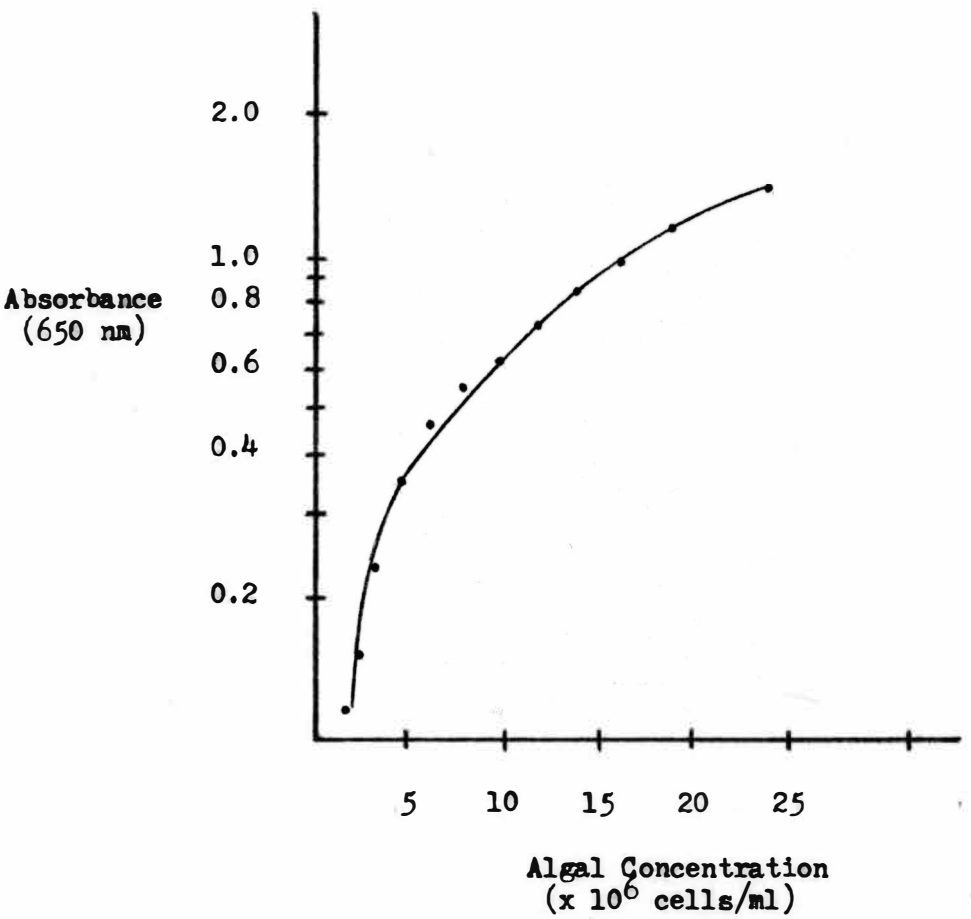
Transfers from one medium to another were made by centrifuging the algal culture down at 11,000 g for 20 min in a Sorvall Automatic Superspeed Centrifuge, model SS-3 (Ivan Sorvall, Inc., Norwalk, CT), pouring off the supernatant, and resuspending the algae in the new medium. Cultures of Chlorella A and Chlorella B were centrifuged down and resuspended in autoclaved lake water before being fed to the daphnia.

To facilitate preparation of the experimental algal solutions, a standard curve relating optical density to algal concentration was derived using a hemacytometer (Figure 3). Spectrophotometric determinations were performed at 650 nm (Taub and Dollar, 1968) with a Spectronic 20 (Bausch & Lomb, Rochester, NY). Algal suspensions of absorbance 0.270-0.330 were employed. Suspensions of Chlorella A and Chlorella B were of approximately equal concentration.

Cultures of Chlorella A and Chlorella B were grown for 21 days and analyzed for differences in composition of carbon, hydrogen, and nitrogen. Samples from the centrifuged cultures were transferred by spatula to metal planchets and dried thoroughly under heat lamps. Subsamples were then weighed on a Cahn Gram Electrobalance (Cahn In-

Figure 3.

Optical Density of Chlorella sp.
in Autoclaved Lake Water



strument Co., Paramount, CA) and analyzed by combustion-gas chromatography in an Elemental Analyzer, model 1104 (Sanda, Inc., Scientific Instruments, Philadelphia, PA).

Experimental Design

The experimental daphnia consisted of medium to large parthenogenetic females randomly drawn from the stock cultures. Females were placed in half-pint milk bottles containing 200 ml autoclaved lake water and transferred to a Sherer environmental chamber (Figure 4). The photoperiod was set at L:D =12:12 and the temperature at 15 ± 0.5°C, both of which are favorable to male production. The photoperiod (Nautical Almanac, 1972) and temperature (Miller and Thompson, 1970; Wetzel, et al., 1972) are typical in October for lakes at the present latitude.

Experiment 1 investigated the effects of food quality and crowding on male induction. Population densities of 2 females/20 ml and 5 females/20 ml were selected as being representative of less crowded and more crowded situations. Each culture was fed Chlorella A, Chlorella B, or yeast at concentrations of approximately 28.8-63.0 million algal cells/ml or 103.3 million yeast cells/ml.

Food was added in excess every 2-3 days to eliminate food quantity as a variable; a slow accumulation of food on the bottom of the cultures demonstrated that food was always available. The volume of food was increased during the experiment due to greater numbers of offspring. Animals at the lower density were fed 0.5-4.0 ml of the algal suspension or 0.5-0.75 ml of the yeast suspension per feeding. Animals at the higher density were fed a proportionally greater amount of food, viz.,

Figure 4.

Daphnia Cultures in the Environmental Chamber

1.25-10.0 ml of the algal suspension or 1.25-1.88 ml of the yeast suspension per feeding. Gentle aeration in the bottles assured longer retention of the food in suspension. The experiment was terminated after 18 days.

In Experiment 2 the effect of food quality on male induction was reinvestigated. Yeast was eliminated as a food. Population density was fixed at 5 females/20 ml. More replicates were used in an attempt to reduce the high sample variance from Experiment 1.

Chlorella A or Chlorella B was added in excess every 1-2 days at concentrations of approximately 29.3-39.4 million algal cells/ml. Animals were fed 1-2 ml of the algal suspension per feeding. Aeration was provided. The experiment was terminated after 30 days.

Statistical Analysis

All calculations were performed on an Olivetti Underwood Programma 101 (Olivetti Underwood Corp., New York, NY) desk top computer-calculator.

To avoid sampling error, all individuals were examined and sexed. Raw data from each culture were converted to per cent male offspring and per cent intergrade offspring. Treatment means and standard errors were calculated.

Data from Experiment 1 and Experiment 2 were analyzed by two-way, and one-way, analyses of variance, respectively. Assumptions of normality and homogeneity of variance could not be satisfied with the data from either experiment. Percentage data typically follow a binomial distribution (Zar, 1974).

Correction of percentage data with an arcsin transformation usually results in data of nearly normal distribution and stable variance (Zar, 1974). Other daphnid studies (Leary, 1967; Stimpfl, 1971) have applied this transformation to their percentage data prior to analyses of variance.

Our data were corrected by the arcsin transformation according to the following formula (Zar, 1974): $x' = \sqrt{x}$, where x is the original data expressed as a percentage and x' is the transformed data expressed as an angle in degrees. The transformation achieved good success as measured by a chi-square goodness-of-fit test of normality and Bartlett's homogeneity of variance. Bartlett's (1947) suggestion for improving zero percentage transformations by replacing zero with 1/4 n, where n equals the sample size, was followed.

Significant differences in the analyses of variance were examined by Newman-Keuls multiple range test.

A correlation analysis was run on the male and intergrade offspring data from Experiment 2.

Composition differences between Chlorella A and Chlorella B were analyzed by Student's t test.

All hypotheses were tested at the five per cent ($\alpha = 0.05$) significance level. Most tests employed one-tailed decisions; the correlation analysis was a two-tailed decision.

RESULTS

Elemental (CHN) Analysis

To confirm that growth in the modified algal medium altered the nitrogen composition of the experimental alga, samples of Chlorella A and Chlorella B were combusted and analyzed by gas chromatography for carbon, hydrogen, and nitrogen.

Differences between algal qualities in carbon and hydrogen content appeared negligible (Table 3). Mean carbon percentages for Chlorella A and Chlorella B were 52.3% and 52.4%, respectively. Mean hydrogen percentages for Chlorella A and Chlorella B were 10.8% and 10.5%, respectively. A slightly larger difference in nitrogen content was noted (Table 3). Mean nitrogen percentages for Chlorella A and Chlorella B were 8.40% and 7.60%, respectively.

These differences in elemental composition were analyzed by Student's t test. Null hypotheses were formulated that: a) There is no difference between Chlorella A and Chlorella B in carbon content, b) There is no difference between Chlorella A and Chlorella B in hydrogen content, and c) There is no difference between Chlorella A and Chlorella B in nitrogen content.

The t test found no significant difference between algal qualities in either carbon or hydrogen content (Table 4). The difference in nitrogen content was significant ($p < 0.05$) as seen in Table 4.

Table 3.

Elemental (CHN) Analysis of 21-Day Old Cultures of Chlorella A and Chlorella B

Food	Sample	Element (%)		
		Carbon	Hydrogen	Nitrogen
Chlorella A	1	52.0	10.7	7.78
	2	52.0	10.7	8.44
	3	52.9	10.8	9.08
	4	52.5	11.5	8.47
	5	51.9	10.5	8.21
Mean ± S.E.		52.3 0.2	10.8 0.2	8.40 0.21
Chlorella B	1	51.8	10.2	6.96
	2	53.0	10.7	7.93
	3	52.0	10.6	7.00
	4	52.6	11.0	7.62
	5	52.4	9.98	8.51
Mean ± S.E.		52.4 0.2	10.5 0.2	7.60 0.29

Table 4.

Student's t Test of Differences in Elemental (CHN) Composition of 21-Day Old Cultures of Chlorella A and Chlorella B

Element	$\lvert\bar{X}_a-\bar{X}_b\rvert$	S^2_{pooled}	d.f.	t	
Carbon	0.1	0.206	8	0.35	n.s.
Hydrogen	0.3	0.157	8	1.20	n.s.
Nitrogen	0.8	0.325	8	2.22	*

n.s. = not significant
* = $p < 0.05$

Experiment 1

The offspring from Experiment 1 are shown in Tables 5A and 5B. Animals were differentiated only as male or female. Sex intergrades were mistakenly considered aberrant females and recorded with the females. Surviving females of the original 20 or 50 placed in each culture were also counted and included in the total population. Badly decomposed bodies were generally few and were ignored in the tabulations.

Female offspring outnumbered male offspring by ratios of 10:1 to 100:1 in all cultures (Tables 5A and 5B). Several cultures contained no male offspring. Cultures fed Chlorella A or Chlorella B contained more males than cultures fed yeast (Tables 5A and 5B). In general, cultures fed Chlorella A contained more males than cultures fed Chlorella B (Tables 5A and 5B). Cultures at the higher density generally had more males than those at the lower density (Tables 5A and 5B).

Offspring production in all algal cultures was approximately equal and greater than in cultures fed yeast (Tables 5A and 5B). More offspring were produced in the cultures at the higher population density (Tables 5A and 5B).

Mortality of original females was appreciably higher in the cultures fed yeast (Tables 5A and 5B). Original female mortality was, on the whole, the same for both algal qualities (Tables 5A and 5B).

In order to quantify the data, the raw numbers of male offspring were converted into per cent male offspring. Means and standard errors were calculated.

Percentages of male offspring for the six groups ranged from zero to 5.71%. Means were highest (3.50% and 2.42%) in the two groups fed

Table 5A.

Offspring from Parthenogenetic Female Daphnia
Fed Various Food Qualities at
2 Females/20 ml Population Density

Food	Sample	Offspring			Original Female Survivors	Total Pop.
		Male	Female	Total		
Chlorella A	1	0	305	305	9	314
	2	5	258	263	13	276
	3	15	292	307	19	326
	4	9	254	263	20	283
	5	4	210	214	17	231
Chlorella B	1	6	232	238	18	256
	2	6	99	105	4	109
	3	1	335	336	15	351
	4	0	186	186	20	206
	5	0	246	246	17	263
Yeast	1	0	78	78	11	89
	2	(lost)	-	-	-	-
	3	1	28	29	4	33
	4	2	124	126	16	142
	5	0	12	12	5	17

Table 5B.

Offspring from Parthenogenetic Female Daphnia
Fed Various Food Qualities at
5 Females/20 ml Population Density

Food	Sample	Offspring			Original Female Survivors	Total Pop.
		Male	Female	Total		
Chlorella A	1	16	399	415	14	429
	2	22	414	436	42	478
	3	9	595	604	45	649
	4	17	517	534	47	581
	5	15	370	385	41	426
Chlorella B	1	22	550	572	36	608
	2	7	595	602	45	647
	3	0	440	440	39	479
	4	3	565	568	42	610
	5	3	438	441	45	486
Yeast	1	3	246	249	32	281
	2	1	271	272	44	316
	3	0	39	39	6	45
	4	2	111	113	8	121
	5	0	61	61	7	68

Chlorella A and lowest (0.67%) in one of the groups fed yeast (Table 6). Other means were between one and two per cent: means in the groups fed Chlorella B were 1.71% and 1.24%; the mean in the other group fed yeast was 1.26% (Table 6). Figure 5 shows that the mean percentage of male offspring for the Chlorella A group at the higher density was much larger than all other means.

The male offspring percentages were corrected by an arcsin transformation before use in a two-way analysis of variance. Transformed percentages, means, and standard errors are found in Table 7.

The transformed data were examined by a two-way analysis of variance to determine whether food quality or crowding induced males in Experiment 1. Null hypotheses were formulated that: a) There is no difference between food qualities in induction of male offspring, b) There is no difference between levels of crowding in induction of male offspring, and c) There is no interaction between food quality and crowding in induction of male offspring.

The analysis of variance indicated (Table 8) there was a significant ($p < 0.05$) difference between the three food qualities in induction of males; however, no significant difference was detected between the two levels of crowding. Interaction between food quality and crowding was absent.

To learn whether differences between food qualities existed at both levels of crowding, two one-way analyses of variance were performed. Null hypotheses were formulated that: a) There is no difference between food qualities at the lower population density, and b) There is no difference between food qualities at the higher population density.

Table 6.

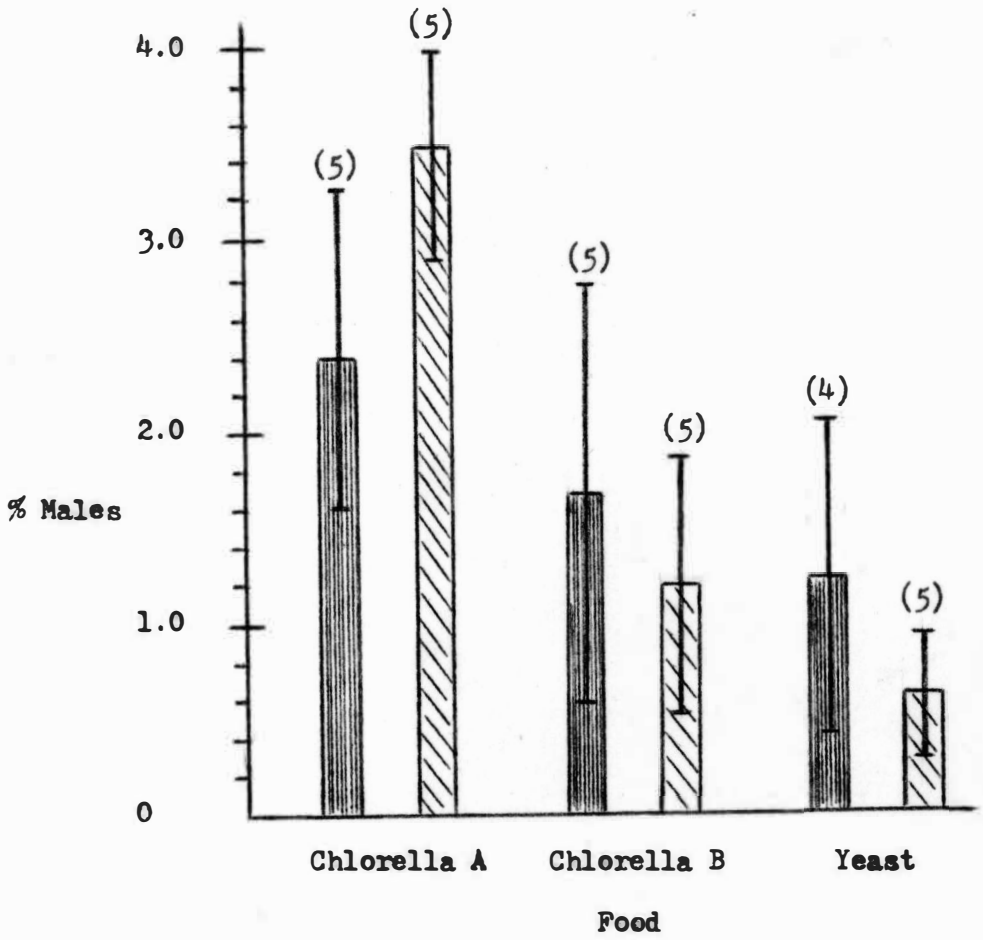
Percentages of Male Offspring from Parthenogenetic Female Daphnia Fed Various Food Qualities at Two Levels of Crowding

Food	Sample	2/20 ml Density		5/20 ml Density	
		Male Offspring (%)	Mean ± S.E.	Male Offspring (%)	Mean ± S.E.
Chlorella A	1	0		3.86	
	2	1.90		5.05	
	3	4.89		1.49	
	4	3.42		3.18	
	5	1.87	2.42 ± 0.82	3.90	3.50 ± 0.59
Chlorella B	1	2.52		3.85	
	2	5.71		1.16	
	3	0.30		0	
	4	0		0.53	
	5	0	1.71 ± 1.10	0.68	1.24 ± 0.68
Yeast	1	0		1.20	
	2	(lost)		0.37	
	3	3.45		0	
	4	1.59		1.77	
	5	0	1.26 ± 0.82	0	0.67 ± 0.35

Figure 5.

Mean Percentages of Male Offspring from Parthenogenetic Female Daphnia Fed Various Food Qualities at Two Levels of Crowding

Densities: ▒ = 2/20 ml, ▨ = 5/20 ml



Horizontal bars represent 1 S.E.
Sample sizes in parentheses.

Table 7.

Arcsin Transformed Percentages of Male Offspring from Parthenogenetic Female Daphnia Fed Various Food Qualities at Two Levels of Crowding

Food	Sample	2/20 ml Density		5/20 ml Density	
		Male Offspring (°)	Mean ± S.E.	Male Offspring (°)	Mean ± S.E.
Chlorella A	1	1.28		11.33	
	2	7.92		12.99	
	3	12.78		7.02	
	4	10.66		10.28	
	5	7.86	8.10 ± 1.94	11.39	10.60 ± 0.99
Chlorella B	1	9.14		11.32	
	2	13.82		6.18	
	3	3.14		1.28	
	4	1.28		4.17	
	5	1.28	5.73 ± 2.48	4.73	5.54 ± 1.65
Yeast	1	1.40		6.29	
	2	(lost)		3.49	
	3	10.71		1.28	
	4	7.25		7.64	
	5	1.40	5.19 ± 2.30	1.28	4.00 ± 1.30

Table 8.

Two-Way Analysis of Variance of the Effects of Food Quality and Crowding on Arcsin Transformed Percentages of Male Offspring in Daphnia

Source of Variation	S.S.	d.f.	M.S.	F	
Total	512.32	28			
Treatments					
Food Quality	123.68	2	61.84	3.85	*
Crowding	0.60	1	0.60	0.04	n.s.
Interaction	18.31	2	9.16	0.57	n.s.
Error	369.73	23	16.08		

n.s. = not significant
* = $p < 0.05$

The first analysis of variance (Table 9) found no significant difference between food qualities in male induction at the lower density. The difference between food qualities at the higher density, therefore, must be highly significant for a significant F-value to appear in the two-way analysis of variance. At the higher density, the difference between food qualities was, indeed, significant ($p < 0.025$) (Table 10).

The next step was to resolve which food qualities at the higher density were capable of inducing male offspring. The three transformed mean percentages were examined by the Newman-Keuls multiple range test. Null hypotheses were formulated that: a) There is no difference in male-inducing potential between Chlorella A and Chlorella B, b) There is no difference in male-inducing potential between Chlorella A and yeast, and c) There is no difference in male-inducing potential between Chlorella B and yeast.

The multiple range test (Table 11) declared that significant ($p < 0.025$) differences were present between Chlorella A and Chlorella B and between Chlorella A and yeast. The difference between Chlorella B and yeast was insignificant.

Experiment 2

Offspring produced in Experiment 2 are summarized in Tables 12A and 12B. Animals were classified as male, female, or sex intergrade. The length (as measured from the top of the head to the base of the tail spine) of each male was also taken. Original female survivors were counted. Badly decomposed bodies were again ignored in the tabulations.

Table 9.

One-Way Analysis of Variance of the Effects of Food Quality on Arcsin Transformed Percentages of Male Offspring in Daphnia at 2 Females/20 ml Population Density

Source of Variation	S.S.	d.f.	M.S.	F	
Total	284.42	13			
Treatment					
Food Quality	22.53	2	11.27	0.47	n.s.
Error	261.89	11	23.81		

n.s. = not significant

Table 10.

One-Way Analysis of Variance of the Effects of Food Quality on Arcsin Transformed Percentages of Male Offspring in Daphnia at 5 Females/20 ml Population Density

Source of Variation	S.S.	d.f.	M.S.	F	
Total	227.30	14			
Treatment					
Food Quality	119.46	2	59.73	6.65	*
Error	107.84	12	8.99		

* = $p < 0.05$

Table 11.

Newman-Keuls Multiple Range Test of the Effects of Food Quality on Arcsin Transformed Percentages of Male Offspring in Daphnia at 5 Females/20 ml Population Density

Comparison	$\lvert\bar{X}_a-\bar{X}_b\rvert$	S.E.	d.f.	q	
Chlorella A vs. Yeast	6.60	1.34	12	4.92	*
Chlorella A vs. Chlorella B	5.06	1.34	12	3.77	*
Chlorella B vs. Yeast	1.54	1.34	12	1.15	n.s.

n.s. = not significant
* = $p < 0.05$

Line Separation Summary**

Ordered Rank	1	2	3
Food	Yeast	Chlorella B	Chlorella A
Mean	4.00	5.54	10.60

** Means underlined by a common line do not differ from one another; Means not underlined by a common line differ from one another.

Table 12A.

Offspring from Parthenogenetic Female Daphnia
Fed Chlorella A or Chlorella B
at 5 Females/20 ml Population Density

Food	Sample	Offspring				Original Female Survivors	Total Pop.
		Male	Int.	Female	Total		
Chlorella A	1	15	3	322	340	28	368
	2	0	7	215	222	15	237
	3	3	2	269	274	6	280
	4	13	4	817	834	6	840
	5	4	2	622	628	10	638
	6	5	5	671	681	11	692
	7	6	4	722	732	11	743
	8	13	1	523	537	7	544
	9	10	2	580	592	19	611
	10	21	13	540	574	7	581
	11	1	1	582	584	8	592
	12	8	1	488	497	11	508
	13	5	18	614	637	17	654
	14	0	13	627	640	21	661

Table 12B.

Offspring from Parthenogenetic Female Daphnia
Fed Chlorella A or Chlorella B
at 5 Females/20 ml Population Density

Food	Sample	Offspring				Original Female Survivors	Total Pop.
		Male	Int.	Female	Total		
Chlorella B	1	4	2	555	561	24	585
	2	0	0	579	579	9	588
	3	0	0	767	767	25	792
	4	5	5	690	700	21	721
	5	1	1	650	652	22	674
	6	0	1	237	238	13	251
	7	1	1	673	675	19	694
	8	1	0	503	504	25	529
	9	3	2	494	499	10	509
	10	9	7	704	720	26	746
	11	4	0	743	747	29	776
	12	4	4	608	616	14	630
	13	3	2	604	609	19	628
	14	3	6	518	527	18	545

Female offspring outnumbered male offspring by ratios of 25:1 to 500:1 (Tables 12A and 12B). Females led intergrade offspring generally by the same wide margins (Tables 12A and 12B). Many cultures, especially in the Chlorella B group, contained no males and/or intergrades. On the whole, Chlorella A cultures contained more male and intergrade offspring (Tables 12A and 12B). The vast majority of males were small (Table 13).

Offspring production was about the same for both algal qualities (Tables 12A and 12B).

The mortality rate of original females was higher in the Chlorella A group (Tables 12A and 12B).

Three varieties of sex intergrade offspring were identified, hereafter designated by the letters P, Q, and R.

The most striking characteristic of P-intergrades (Figure 6) was a greatly reduced rostrum. The rostrum usually appeared to be flattened against the head, resulting in a head profile reminiscent of the male. Also the head appeared to be tilted slightly forward, obscuring the normally clear demarcation between head and valves.

Q-intergrades (Figure 7) exhibited a triangular-shaped rostrum. The unusual shape was due to the abrupt angle at which the rostrum projected from the head. It contrasts sharply with the gentle slope of the female rostrum.

R-intergrades (Figure 8) were characterized by a spear-like rostrum. The angle between their head and rostrum was much sharper (90^{o}) than in Q-intergrades (135^{o}), and the upper surface of the rostrum was more scalloped out.

Table 13.

Length of Male Offspring from Parthenogenetic Female Daphnia Fed Chlorella A or Chlorella B at 5 Females/20 ml Population Density

	Chlorella A			Chlorella B		
Sample	Length (mm)			Length (mm)		
	< 1.0	1.0-1.3	> 1.3	< 1.0	1.0-1.3	> 1.3
1	7	3	5	4	0	0
2	0	0	0	0	0	0
3	1	2	0	0	0	0
4	12	1	0	5	0	0
5	4	0	0	1	0	0
6	5	0	0	0	0	0
7	4	2	0	1	0	0
8	1	12	0	1	0	0
9	3	2	5	0	2	1
10	6	14	1	9	0	0
11	0	0	1	4	0	0
12	2	6	0	4	0	0
13	1	3	1	2	1	0
14	0	0	0	3	0	0
Total	46	45	13	34	3	1

Figure 6.

Daphnia pulex P-Intergrade

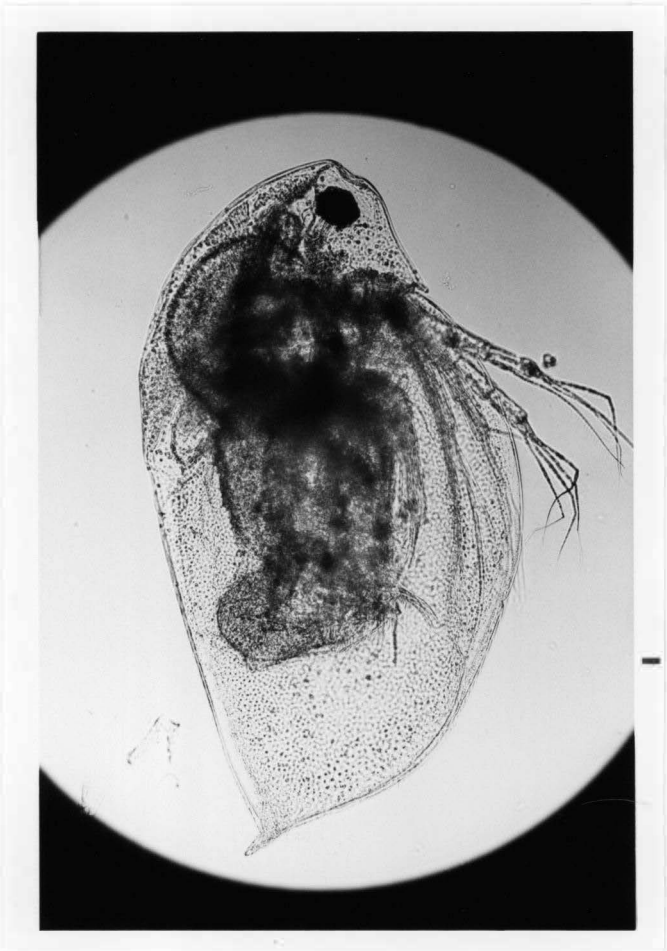


Figure 7.

Daphnia pulex Q-Intergrade

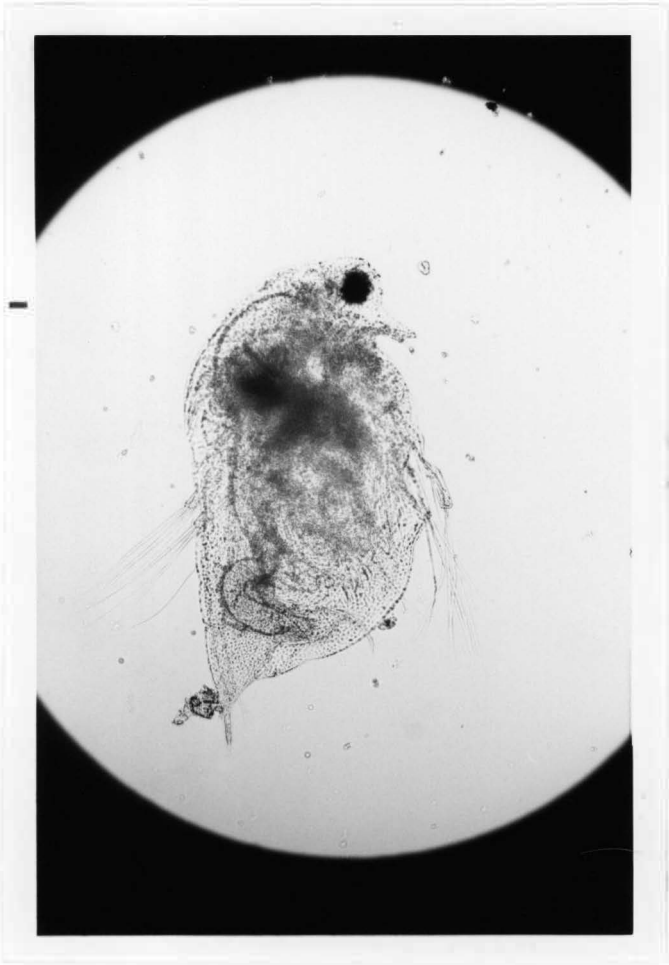
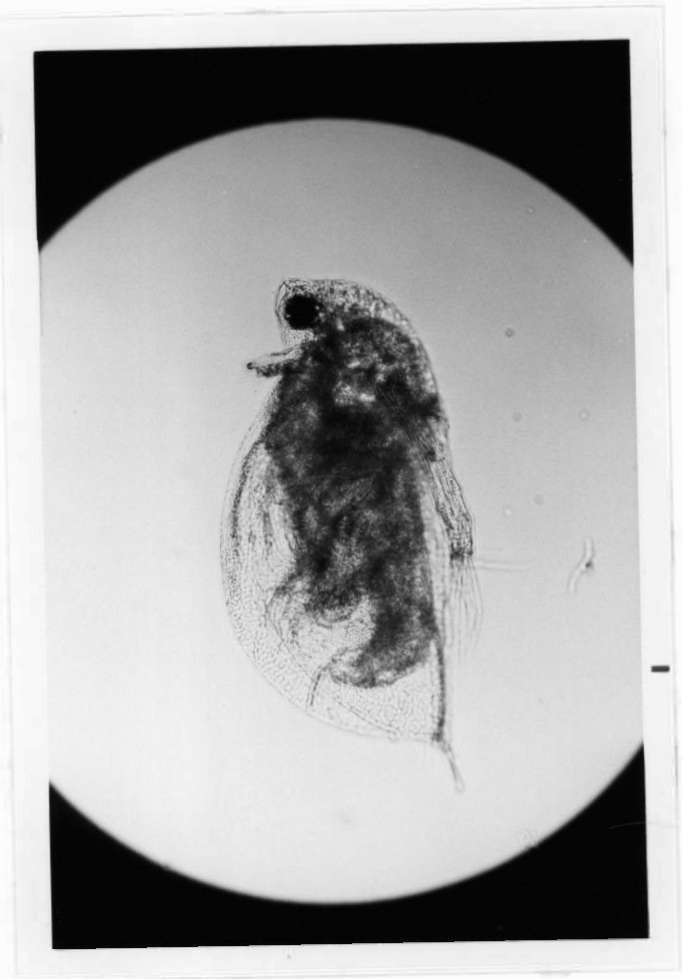


Figure 8.

Daphnia pulex R-Intergrade



R-intergrades were the most common variety, accounting for nearly one-half of all intergrades. They were followed, in decreasing frequency, by the P- and Q-intergrades (Table 14). The group fed Chlorella A possessed more of each sex intergrade variety than the group fed Chlorella B (Table 14).

For quantitative purposes, raw numbers of male offspring and sex intergrade offspring were converted into per cent male offspring and per cent sex intergrade offspring. Means and standard errors were calculated.

Percentages of male offspring and intergrade offspring were perceptibly higher in the cultures fed Chlorella A (Table 15). In the Chlorella A group percentages of male offspring ranged from zero to 4.41%, while percentages of male offspring in the Chlorella B group ranged from zero to 1.25%. Only a single sample percentage exceeded one per cent in the Chlorella B group (Table 15). Means for the two algal qualities reflected higher male production among the Chlorella A cultures (Figure 9). Mean male offspring percentages for Chlorella A and Chlorella B were 1.40% and 0.43%, respectively (Table 15).

The Chlorella A cultures, in general, also produced more intergrade offspring (Figure 9). Percentages varied between 0.17% and 3.15% in the Chlorella A cultures and between zero and 1.14% in the Chlorella B cultures. Intergrade means for Chlorella A and Chlorella B were 1.06% and 0.38%, respectively (Table 15).

The male offspring and intergrade offspring percentages were corrected by an arcsin transformation before use in a one-way analysis of variance. Transformed percentages, means, and standard errors are

Table 14.

Varieties of Intergrade Offspring from Parthenogenetic Female Daphnia Fed Chlorella A or Chlorella B at 5 Females/20 ml Population Density

	Chlorella A			Chlorella B		
Sample	Variety			Variety		
	P	Q	R	P	Q	R
1	0	0	3	0	0	2
2	6	0	1	0	0	0
3	0	0	2	0	0	0
4	0	3	1	0	2	3
5	0	0	2	0	0	1
6	0	2	3	0	0	1
7	0	1	3	0	0	1
8	1	0	0	0	0	0
9	0	1	1	0	1	1
10	5	2	6	0	1	6
11	0	0	1	0	0	0
12	0	0	1	1	0	3
13	13	1	4	0	1	1
14	7	1	5	5	0	1
Total	32	11	33	6	5	20

Table 15.

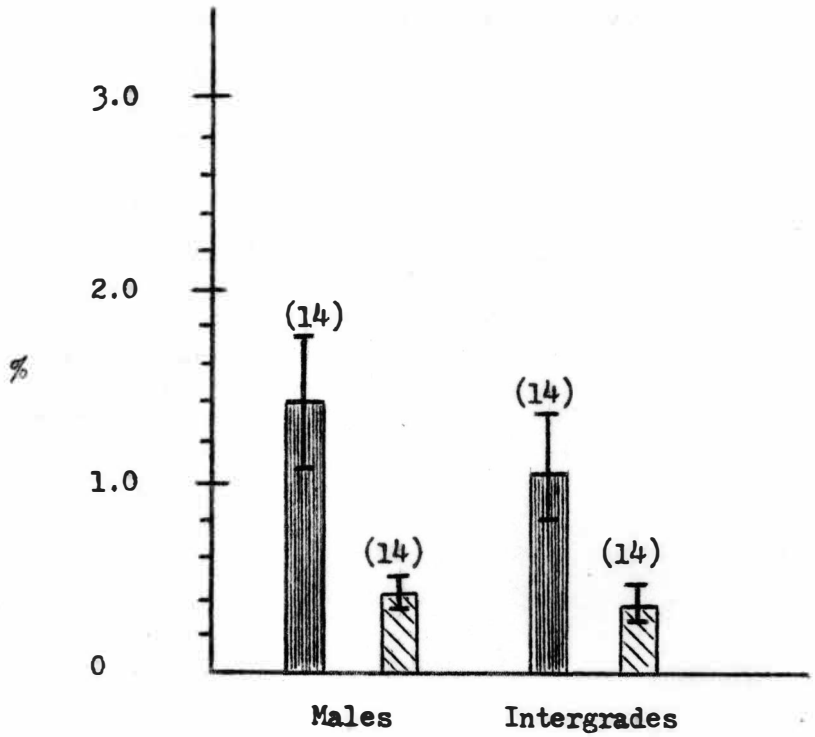
Percentages of Male and Sex Intergrade Offspring
from Parthenogenetic Female Daphnia Fed
Chlorella A or Chlorella B
at 5 Females/20 ml Population Density

	Chlorella A		Chlorella B	
Sample	Offspring (%)		Offspring (%)	
	Male	Int.	Male	Int.
1	4.41	0.88	0.71	0.36
2	0	3.15	0	0
3	1.09	0.73	0	0
4	1.56	0.48	0.71	0.71
5	0.64	0.32	0.15	0.15
6	0.73	0.73	0	0.42
7	0.82	0.55	0.15	0.15
8	2.42	0.19	0.20	0
9	1.69	0.34	0.60	0.40
10	3.66	2.26	1.25	0.97
11	0.17	0.17	0.54	0
12	1.61	0.20	0.65	0.65
13	0.78	2.83	0.49	0.33
14	0	2.03	0.57	1.14
Mean ±	1.40	1.06	0.43	0.38
S.E.	0.35	0.28	0.10	0.10

Figure 9.

Mean Percentages of Male and Sex Intergrade Offspring from Parthenogenetic Female Daphnia Fed Chlorella A or Chlorella B at 5 Females/20 ml Population Density

Food Quality: ▨ = Chlorella A, ▧ = Chlorella B



Horizontal bars represent 1 S.E.
Sample sizes in parentheses.

listed in Table 16.

To ascertain whether food quality induced males and/or intergrades in Experiment 2, the transformed data were examined by two one-way analyses of variance. Null hypotheses were formulated that: a) There is no difference in male-inducing potential between Chlorella A and Chlorella B, and b) There is no difference in intergrade-inducing potential between Chlorella A and Chlorella B.

The analyses found significant ($p < 0.025$) differences between Chlorella A and Chlorella B in induction of male offspring (Table 17) and intergrade offspring (Table 18).

The methods of induction of male and intergrade offspring may be similar. Examining this relationship more closely, a correlation analysis was run on the transformed percentages of male offspring and intergrade offspring. Correlation coefficients for the offspring from Chlorella A and Chlorella B were -0.24 and 0.66, respectively (Table 19). The latter correlation is significant ($p < 0.02$), while the former one is not (Table 19).

Table 16.

Arcsin Transformed Percentages of Male and Sex Intergrade Offspring from Parthenogenetic Female Daphnia Fed Chlorella A or Chlorella B at 5 Females/20 ml Population Density

	Chlorella A		Chlorella B	
Sample	Offspring (°)		Offspring (°)	
	Male	Int.	Male	Int.
1	12.12	5.38	4.83	3.44
2	0.81	10.23	0.81	0.81
3	5.99	4.90	0.81	0.81
4	7.18	3.97	4.83	4.83
5	4.59	3.24	2.22	2.22
6	4.90	4.90	0.81	3.72
7	5.20	4.25	2.22	2.22
8	8.95	2.50	2.56	0.81
9	7.47	3.34	4.44	3.63
10	11.03	8.63	6.42	5.65
11	2.36	2.36	4.21	0.81
12	7.29	2.56	4.62	4.62
13	5.07	9.68	4.01	3.29
14	0.81	8.19	4.33	6.13
Mean ±	5.98	5.30	3.37	3.07
S.E.	0.90	0.73	0.48	0.49

Table 17.

One-Way Analysis of Variance of the Effects of Food Quality on Arcsin Transformed Percentages of Male Offspring in Daphnia

Source of Variation	S.S.	d.f.	M.S.	F
Total	237.74	27		
Treatment				
Food Quality	47.97	1	47.97	6.57 *
Error	189.77	26	7.30	

* = $p < 0.05$

Table 18.

One-Way Analysis of Variance of the Effects of Food Quality on Arcsin Transformed Percentages of Sex Intergrade Offspring in Daphnia

Source of Variation	S.S.	d.f.	M.S.	F
Total	177.15	27		
Treatment				
Food Quality	34.63	1	34.63	6.32 *
Error	142.52	26	5.48	

* = $p < 0.05$

Table 19.

Correlation Analysis of Arcsin Transformed Percentages of Male and Sex Intergrade Offspring

Food	Cov.	S^2_{male}	$S^2_{int.}$	d.f.	r	
Chlorella A	-2.23	11.42	7.55	12	-0.24	n.s.
Chlorella B	2.17	3.17	3.41	12	0.66	*

n.s. = not significant
* = $p < 0.05$

DISCUSSION

Influence of Crowding on Induction of Males

Crowding was studied in Experiment 1. Experimental densities of 2 females/20 ml and 5 females/20 ml were selected as representative of less crowded and more crowded conditions. Leary's (1967) densities for Daphnia pulex served as a guide in determining appropriate levels of crowding.

Although production of male offspring was generally higher in cultures at the higher density (Tables 5A and 5B), on a proportional basis there was no difference between densities.

Sample variance of male offspring percentages was large in some groups; some percentages varied as much as two or three per cent (Table 6). About one-fourth of the cultures contained no males, but these appeared to be randomly distributed among the groups (Tables 5A and 5B). Examination of the mean male offspring percentages may permit better comparisons between the densities.

At the higher and lower densities, mean percentages for Chlorella A were 3.50% and 2.42%, respectively. At the same densities, means for Chlorella B were 1.24% and 1.71%, respectively. At the same densities, means for yeast were 0.67% and 1.26%, respectively. Figure 5 shows no consistent pattern in the mean data between the densities: male production was higher at the higher level of crowding in the Chlorella A cultures, but lower at the higher level of crowding in the Chlorella B and yeast cultures. Leary (1967) found male production rose as the culture density increased from 3 females/50 ml to 30 fe-

males/50 ml. The high sample variance among the Chlorella B and yeast cultures may account for these inverted results.

Offspring produced during the experiment were not removed from the cultures, resulting in a steady rise in population density. Final culture densities indicate this increase was quite substantial in most cultures (Tables 5A and 5B). Most cultures became overcrowded. Distinctions between the two levels of crowding were probably erased; hence, no significant difference in the two-way analysis of variance.

Periodic removal of offspring without disrupting the continuity of the cultures is an arduous task without elaborate equipment. Future studies should consider this, if constant population densities are necessary.

Influence of Nitrogen on Algal Food Quality

Elemental analysis of the experimental algae revealed few differences (Table 3). Mean carbon values for Chlorella A and Chlorella B were 52.3% and 52.4%, respectively. Mean hydrogen values for the same algae were 10.8% and 10.5%, respectively. The difference in nitrogen content was somewhat larger: means for Chlorella A and Chlorella B were 8.40% and 7.60%, respectively. These percentages are consistent with accepted algal values (Spoehr and Milner, 1949; Syrett, 1962).

Examination of these differences by Student's t test uncovered no significant difference in either carbon or hydrogen content. The difference in nitrogen content, though small, was found to be significant (Table 4). Culture of Chlorella sp. in the modified Bold's (1967) medium successfully lowered its nitrogen content and altered its food

quality. Closer analysis of Chlorella B may reveal the nature of these changes. Reduced quantities of protein (Ketchum and Redfield, 1949; van Oorschot, 1955) and photosynthetic pigments (Bongers, 1956) and perhaps more lipid (van Oorschot, 1955) are expected.

In this study nitrogen-deficient algae were prepared by transferfering healthy algae to a medium devoid of nitrogen (nitrogen starvation method). The nitrogen exhaustion method has been used by others. Syrett (1962) commented that these two methods do not necessarily produce algae with identical properties. Fogg (1959) speculated on possible differences. In view of these opinions, future research might be wise to clarify the particular method used. Since fluctuations of inorganic nitrogen in most lakes tend to be gradual rather than abrupt, the nitrogen exhaustion method may be preferred.

Influence of Food Quality on Induction of Males

The influence of food quality on male offspring was investigated in both experiments. In Experiment 1, numbers of male offspring were generally higher in cultures fed algae rather than yeast (Tables 5A and 5B). Cultures fed Chlorella A, on the whole, also produced more males than those fed Chlorella B (Tables 5A and 5B).

Percentages of male offspring were highest in cultures fed Chlorella A and lowest in cultures fed yeast at both population densities (Table 6). At the lower density, mean percentages for Chlorella A, Chlorella B, and yeast were 2.42%, 1.71%, and 1.26%, respectively. At the higher density, mean percentages for Chlorella A, Chlorella B, and yeast were 3.50%, 1.24%, and 0.67%, respectively. Superiority of Chlor-

ella A in induction of males seemed likely.

This superiority was eventually confirmed through a series of analyses of variance. Although differences between food qualities were insignificant at the lower density, at the higher density significant differences were found between Chlorella A and Chlorella B and between Chlorella A and yeast (Table 11). On the average, cultures given Chlorella A produced almost three times as many males as cultures given Chlorella B and more than five times as many males as cultures given yeast (Table 6). Food quality can initiate sexual reproduction in daphnia.

The difference in male-inducing potential between Chlorella A and Chlorella B was again borne out in Experiment 2. Male offspring percentages exceeded one per cent in over half the cultures fed Chlorella A, but in only one culture fed Chlorella B (Table 15). Percentages in some Chlorella A cultures ranged as high as three or four per cent (Table 15). Mean percentages for Chlorella A and Chlorella B were 1.40% and 0.43%, respectively. This difference was significant (Table 17).

The difference between Chlorella A and yeast is questionable. The mortality rate of original females was nearly three times higher in yeast cultures as in cultures fed either algal quality (Tables 5A and 5B). This undoubtedly contributed to fewer offspring in the yeast-fed cultures and may have influenced male production also.

Most research on male induction has concentrated on physical or biological factors in the environment and overlooked ingested chemical factors. Action of these factors in the female, however, was always

assumed to have a chemical basis. By taking a slightly different approach, it is hoped this study may further elucidate the chemical mechanism involved in sex determination in cladocerans.

The male-inducing substance in Chlorella probably exists in related algae also, but sweeping generalizations to more distantly related species should be guarded against for the present. Speculations as to its identity are still premature; it may or may not be α-tocopherol, the active substance found in algae and littoral grasses in Gilbert and Thompson's (1968) work. The male-inducing substance in von Dehn's (1955) yeast extract resided in the sterol-fat fraction.

The present study indicates that nitrogen is required in the synthesis and/or maintenance of the active substance since nitrogen deficiency promotes its catabolism in the algal cell. Isolation and identification of the substance are awaited.

Questions for further research should include: does the active substance serve as a precursor or remain unchanged after ingestion? is the material taken up by the male embryos or does it remain in the mother?

Percentages of male offspring were smaller in Experiment 2 than in Experiment 1 at the same population density (Figures 5 and 9). Mean percentages for Chlorella A in Experiments 1 and 2 were 3.50% and 1.40%, respectively. Mean percentages for Chlorella B in Experiments 1 and 2 were 1.24% and 0.43%, respectively. These represent large differences. Fluctuations in the experimental culture temperature may be responsible for the differences. Deviations from the ideal temperature can cause male production to decline (Stimpfl, 1971). Tempera-

ture variance in Experiment 2 ($15^{o} \pm 3.5^{o}C$) was greater than in Experiment 1 ($15^{o} \pm 0.5^{o}C$) due to problems with the environmental chamber generated by unusual weather conditions.

Failure to remove offspring from the cultures at regular intervals prevented discrimination between broods in time; nevertheless, some data suggests that male production increased in later broods. The length of all male offspring was taken in Experiment 2. Body length is a rough estimator of age in daphnia. Approximately one-half of the males measured less than one millimeter; another one-third were only slightly longer (Table 13); thus, most males were relatively newborn. Higher percentages of males in later broods were decreased by inclusion with earlier broods.

Although increasing the sample size in Experiment 2 helped reduce the large sample variance in Experiment 1, some parameters still varied greatly between individual cultures: fecundity, male production, and mortality of original females (Tables 12A and 12B). Sampling error was avoided by examining all daphnia. Since several past studies have also encountered unusually large variances with daphnia, variability in this study was presumed real and inherent in the animal.

Influence of Food Quality on Induction of Sex Intergrades

Three varieties of sex intergrades were discovered. They differed with respect to the shape of their rostrum (Figures 6, 7, and 8). All other sex characters appeared female. The rostrum is one of the most frequently modified sex characters (Banta, et al., 1939).

The rostrum varied in appearance from roughly male (P-intergrades)

to more or less female (Q-intergrades). Sex characters in intergrades exhibit various degrees of modification (Banta, et al., 1939).

R-intergrades were most plentiful (Table 14). Q-intergrades resembled R-intergrades but possessed a fuller rostrum. Q-intergrades were not as common as the P-intergrades (Table 14).

Cultures fed Chlorella A generally contained more sex intergrade offspring than cultures fed Chlorella B (Tables 12A and 12B). Cultures fed Chlorella A also contained more of each intergrade variety than cultures fed Chlorella B (Table 14). Cultures without any intergrades occurred in the Chlorella B group.

Percentages of sex intergrade offspring were higher in the Chlorella A cultures (Table 15). Intergrades percentages here ranged from 0.17% to 3.15%; they ranged from zero to 1.14% in the Chlorella B group.

The mean percentage of intergrade offspring for the Chlorella A cultures was more than twice the mean for the Chlorella B cultures: 1.06% and 0.38%. This difference was significant in the analysis of variance (Table 18). Thus, algal food quality can also induce intergrade offspring. The superiority of Chlorella A in induction of intergrades is especially interesting, since Chlorella A was also superior in induction of males.

The decision to tabulate sex intergrades was initially made because of a suspected link between intergrade production and male production. External factors are probably responsible for the appearance of intergrades also (Banta, et al., 1939). If androgen is involved in intergrade development as it is in male development, the mechanisms by which intergrades and males are produced may be related.

Examining this relationship more closely, a correlation analysis was performed on the transformed percentages of male and intergrade offspring. Correlation coefficients for the Chlorella A and Chlorella B offspring were -0.24 and 0.66, respectively. The latter correlation was significant (Table 19). The absence of a correlation between the Chlorella A male and intergrade offspring appears to negate a common induction mechanism dependent on the active male-inducing substance. The correlation, however, in the Chlorella B data suggest another common mechanism, related perhaps to some other substance in both algal qualities. This mechanism could also be related to some environmental factor such as temperature or photoperiod. A mechanism dependent on temperature has some support. Low temperatures not only induce male offspring, but are effective in inducing intergrades (Ginsburger-Vogel, 1975). This whole area requires much more study.

SUMMARY

1. Growth of Chlorella sp. in the modified Bold's medium significantly lowered its nitrogen content and altered its food quality.

2. Mean percentages of male offspring were significantly higher in Daphnia pulex cultures fed Chlorella A rather than Chlorella B. Cultures given Chlorella A produced almost three times as many male offspring as cultures given Chlorella B.

3. The mean percentage of sex intergrade offspring was also significantly higher in cultures fed Chlorella A rather than Chlorella B. Three varieties of intergrades were discovered, which differed with respect to the shape of the rostrum. All other sex characters appeared female.

4. A significant correlation between Chlorella B male and intergrade offspring, but not between Chlorella A male and intergrade offspring, suggests a common method of induction, but one unrelated to the male-inducing substance in Chlorella A.

5. Crowding was ineffective in inducing males at population densities of 2 females/20 ml and 5 females/20 ml. Overcrowding in the cultures probably erased density distinctions.

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