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RELATIVE EFFECTIVENESS OF FOOD, OXYGEN, AND OTHER SUBSTANCES IN CAUSING OR PREVENTING MALE-PRODUCTION IN HYDATINA[1]

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INTRODUCTION

A paper on the effect of oxygen on the life cycle of the rotifer Hydatina senta by Shull and Ladoff ('16) left certain matters in doubt. Whereas water saturated with a mixture of air and oxygen which contained 40 per cent of oxygen caused an increase in the ratio of male-producers to female-producers. saturation with a mixture containing 60 per cent of oxygen was attended by practically no results. Two explanations seemed possible. Either different concentrations of oxygen had different effects or oxygen was incapable of increasing male-production when male-production was already high from other causes. The

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experiment referred to above, in which 60 per cent oxygen produced no effect, was performed at a time when the rotifers were going through one of their well-known 'epidemics' of male-production (Shull, '15 b), whereas the experiment with 40 per cent oxygen came at a period when male-production in the control line was low. No opportunity was found to test these two possible explanations, owing to the loss of the stock cultures of rotifers, and the experiments were published while this question was still unanswered.

The important discovery by Whitney ('14) that rotifers fed upon a green organism, Chlamydomonas, produced many more male-producing offspring than those fed upon other material, raised new questions. Whitney took no account of certain initial chemical differences between the contrasted lines nor of the fact that green organisms yield oxygen as a by-product of photosynthesis. Shull and Ladoff pointed out that although nutrition might on a priori grounds be expected to have the effect which Whitney ascribed to it, the other obvious factors should be eliminated before attributing the residual effect to nutrition. It remained a question, therefore, how much of the increase in male-production which Whitney observed upon feeding with green organisms was due to food, how much to oxygen or other factors.

In the experiments described in this paper an answer to these questions is found. The initial chemical differences referred to above have been eliminated. The effects of oxygen and green organisms have been measured and compared. In addition, the effects of these two agents, which increase male-production, are compared with two of the principal agents known to decrease male-production.

EXPERIMENTS

Effect of sixty per cent oxygen

To show whether saturation with a mixture of air and oxygen of which 60 per cent was oxygen caused any change in the amount of male-production, the following experiments were performed:

Experiment 1. On each of the days named in table 1 three female rotifers were placed in each of two dishes. In one was poured some spring water and scum from a manure solution was added for food. The water put into the other dish was first saturated, by continued agitation, with an air-oxygen mixture produced by removing half the volume of air and replacing it with oxygen. Such a mixture must have been composed of about 60 per cent oxygen and 40 per cent nitrogen. Manure scum was added for food, as in the control dish. The dish was then placed under a sealed bell jar, from which half the air was withdrawn by a filter pump and replaced with oxygen. The air-oxygen mixture under the bell jar must have consisted of about 60 per cent oxygen and about 40 per cent nitrogen.[2]

After twenty-four hours all the parents were removed from these dishes. The young hatching from the eggs laid in that period, experiment and control alike, were reared to maturity in untreated water with manure scum as food. Whatever differences they exhibit, therefore, are due to differences in the conditions which affected the mother or early developmental stages. Table 1 shows the results. The excess of male-production in the presence of oxygen is fairly marked.

Experiment 2. This experiment was designed to contrast the effects of 60 per cent and 40 per cent oxygen mixtures by two simultaneous tests. In the absence of a second suitable bell jar, one test was made with 60 per cent oxygen, the next test with 40 per cent oxygen. It was hoped that by this alternation an epidemic of male-production, if one occurred, would occur in the control of both series of tests. Since, however, it was not feasible to make one test each day, and several days usually elapsed between tests, that object was only partially attained. It happened that on the whole the line used for control produced more male-producers on the days when 60 per cent oxygen was employed than on the days on which the 40 per cent mixture was used. How much this circumstance vitiates the conclusion to be drawn from the experiment is not known.

[2]The method of procuring air-oxygen mixtures of a given composition is here stated again specifically, since Whitney seems to have misunderstood the expressions '60 per cent oxygen' and '40 per cent oxygen.'

TABLE 1

Showing the effect of saturation of the water with an air-oxygen mixture, of which 60 per cent was oxygen, upon the ratio of maleproducers (♂ ♀) to female-producers (♀ ♀) in the rotifer Hydatina senta

DATE	AIR		OXYGEN	
	Number of ♂ ♀	Number of ♀ ♀	Number of ♂ ♀	Number of ♀ ♀
February 26	2	6	2	16
February 26	1	16	2	12
February 27	6	30	1	21
February 27	9	27	15	14
February 29	4	28	17	19
March 1	0	20	2	24
Total	22	127	39	106
Percentage of ♂ ♀	14.8		26.9	

The method of carrying out the tests was precisely the same as described for experiment 1, except that in the 40 per cent oxygen tests the mixture used in saturating the water and the mixture under the bell jar were made up of three parts air and one part oxygen, instead of equal parts of the two as in the 60 per cent oxygen mixture.

Table 2 shows the results of the experiment. In both of the tests the oxygen mixture produced more male-producers than the control. If the absolute difference between experiment and control be taken as the measure of effectiveness of oxygen, the 60 per cent mixture appears to be a little more potent than the 40 per cent. If the ratio of male-production in the experiment to male-production in the control is the proper measure of effectiveness, then 40 per cent oxygen produced greater results than 60 per cent oxygen. In other experiments the impression has been gained that the relative difference between experiment and control is a better measure of the effect produced than is the absolute difference. In this experiment the ratio of the percentage of male-production in oxygen to the percentage of male-production in the control is 1.51 in the case of the 40 per cent oxygen, 1.41 in the case of the 60 per cent oxygen. The fourteen tests made are too few to use statistically to determine whether

TABLE 2

A comparison of the effectiveness of a 60 per cent oxygen mixture and a 40 per cent oxygen mixture in increasing male-production in the rotifer Hydatina senta. The tests covered the same period of three months, but were made on alternating days

DATE	AIR		60 PER CENT OXYGEN		DATE	AIR		40 PER CENT OXYGEN	
	Number of ♂♀	Number of ♀♀	Number of ♂♀	Number of ♀♀		Number of ♂♀	Number of ♀♀	Number of ♂♀	Number of ♀♀
March 6	7	24	10	20	March 11	1	25	1	27
March 13	0	22	4	38	March 15	6	22	2	38
March 18	0	29	0	31	March 20	0	25	3	21
March 23	0	1	2	6	March 27	12	11	35	2
April 4	3	17	8	16	April 7	1	4	0	6
April 10	0	16	2	20	April 11	0	14	17	9
April 13	14	18	18	7	April 14	1	30	2	29
April 17	21	22	38	23	April 20	3	38	3	45
April 25	3	20	10	25	April 28	1	44	5	37
May 2	4	15	7	19	May 5	17	23	12	17
May 9	1	11	3	15	May 12	1	1	0	17
May 19	8	28	1	13	May 30	0	10	0	23
June 4	0	9	0	24	June 5	3	34	3	22
June 8	1	25	0	16	June 12	0	6	0	19
Total	62	257	103	273		46	287	83	312
Percentage of ♂♀	19.4		27.4			13.8		21.0	

the difference between these two ratios is probably significant or not.

Amount of oxygen dissolved from atmosphere and from Euglena

After repeated tests had shown that the increase of male-production in rotifers reared in oxygenated water was no mere accident, it became a pertinent question whether, and to what degree, the increase of male-production among rotifers fed upon green organisms is dependent upon the liberation of oxygen in photosynthesis. As a preliminary problem, it was necessary to discover how nearly the amount of oxygen dissolved from the air-oxygen mixtures described in this paper and in that of Shull and Ladoff equaled that dissolved in water in which green organisms were kept as food. A number of tests to determine this relation were made, some of them closely resembling the experiments, others differing more or less, but bearing directly on the oxygen content of treated water. The oxygen in solution was measured by the Winkler method.

In this paper all measurements of oxygen are given in number of cubic centimeters per liter of water. This quantity is computed from the tests by means of the formula

$$o = \frac{55.825 \text{ b n}}{\text{t v}}$$

in which 'b' is the number of cubic centimeters of potassium bichromate solution used in standardizing the sodium thiosulphate; 't' is the number of cubic centimeters of sodium thiosulphate required in titration against 'b' cc. of potassium bichromate; 'v' is the volume in cubic centimeters of the water sample tested; and 'n' is the number of cubic centimeters of the thiosulphate required for the water sample. In the tests here described 'b' was always 25, the other factors being variable.

Direct solution of oxygen.—In table 3 are described the tests to determine the amounts of oxygen dissolved in water when the water is agitated in contact with an atmosphere containing an excess of oxygen. Test No. 1, consisting of seven parts, was most nearly like one of the oxygen experiments, and was furthermore most complete. This test indicates that the water used in one of the rotifer experiments contained 6.81 cc. of oxygen per liter at the outset (a). After it was shaken with an atmosphere of which 40 per cent was oxygen, it contained 11.33 cc. of oxygen per liter (b). When the food (manure scum) was added, the water contained only 7.58 cc. of oxygen (c). After standing a day under a bell jar, in an atmosphere of which 40 per cent was oxygen, the oxygen content of the water had risen to 8.35 cc. per liter (d). Had the dish been kept in air instead of under a bell jar, the oxygen content would have fallen to 5.43 cc. per liter (e).

TABLE 3

Showing the effect of agitation of water in contact with an atmosphere containing an excess of oxygen in increasing the oxygen content of the water. The columns of figures were obtained by Winkler's method of determination of dissolved oxygen

TEST NUMBER	TREATMENT OF WATER	VARIABLES IN FORMULA FOR COMPUTATION (SEE TEXT)			NUMBER OF CUBIC CENTIMETERS OF OXYGEN PER LITER
		(n)	(t)	(v)	(o)
1–a	Untreated water, without food or anything else, tested at once for oxygen...	8.9	20.7	88	6.81
b	Water shaken with 40 per cent oxygen atmosphere, tested immediately for oxygen...	14.8	20.7	88	11.33
c	Shaken with 40 per cent oxygen atmosphere, manure scum added, immediately filtered and tested for oxygen....	9.9	20.7	88	7.58
d	Shaken with 40 per cent oxygen, manure scum added, put under bell jar in 40 per cent oxygen atmosphere for a day, then filtered and tested....	10.9	20.7	88	8.35
e	Shaken with 40 per cent oxygen, manure scum added, kept in air a day, filtered and tested....	7.1	20.7	88	5.43
f	Untreated water, manure scum added, then immediately filtered and tested...	7.6	20.7	88	5.82
g	Untreated water, manure scum added, kept in air a day, then filtered and tested....	5.4	20.7	88	4.13
2–a	Shaken with 60 per cent oxygen atmosphere and tested immediately....	14.50	20.8	55	17.67
b	Shaken with 60 per cent oxygen, poured into six watch-glasses, kept under bell jar in 60 per cent oxygen atmosphere one day, then tested....	9.95	20.8	55	12.13
c	Poured into six watch-glasses, set beside bell jar one day, then tested......	6.05	20.8	55	7.88
3–a	Untreated water in twelve watch-glasses, manure scum added, left twenty-four hours, then tested....	5.97	20.7	88	4.57
b	Shaken with 80 per cent oxygen atmosphere, then tested at once for oxygen..	29.25	20.7	88	22.40
c	Shaken with 80 per cent oxygen, put into twelve watch-glasses, manure scum added, kept under bell jar in 40 per cent oxygen twenty-four hours, then tested....	10.23	20.7	88	7.73
4–a	Untreated water tested for oxygen........	13.3	20.7	120	7.47
b	Shaken with pure oxygen and tested immediately for oxygen....	40.5	20.7	120	22.75
c	Shaken with pure oxygen, kept forty-eight hours in atmosphere of 60 per cent oxygen....	32.3	20.7	120	18.14

The control started with 6.81 cc. of oxygen per liter in the untreated water (a). Upon the addition of food (manure scum) the oxygen content fell to 5.82 cc. per liter (f); while after standing a day it further fell to 4.13 cc. per liter (g).

Assuming that the oxygen content of the water in both halves of the experiment changed uniformly during the twenty-four hours in which eggs were being laid, the mean oxygen content of the oxygenated water was

$$\frac{7.58 + 8.35}{2} = 7.965 \text{ cc. per liter;}$$

while the mean oxygen content of the control dishes was

$$\frac{5.82 + 4.13}{2} = 4.975 \text{ cc. per liter.}$$

Absolutely, the difference between experiment and control was about 3 cc. per liter. Relatively, the oxygenated water contained 60 per cent more oxygen than did the untreated water.

Test No. 2 (table 3) is less complete than the foregoing, but indicates approximately the amount of oxygen involved in the 60 per cent oxygen experiments. No test of untreated water at the beginning of the twenty-four hour period described was made and no food was added. It is likely that under these circumstances the untreated water exposed to air would not lose oxygen, but might gain it, so that the average content of such water during the twenty-four hours would be not greater than 7.88 cc. per liter (c). The water shaken with 60 per cent oxygen must have averaged about 15 cc. per liter (a and b), or nearly double the content of the untreated water. This test was not very relevant, but since the comparison was to be made between the 40 per cent oxygen experiments and those in which green food was used, a more accurate test of the 60 per cent oxygen experiments was not made.

Tests 3 and 4 have little bearing on the experiments, and are recorded for whatever interest may attach to the solubility of oxygen at higher pressures.

Oxygen derived from photosynthesis. Euglena was used in these tests. Cultures of a species that formed a sheet of green animals in a somewhat inactive, though not encysted, state along the sides of the aquarium were maintained. A considerable quantity of Euglena was usually available, and it was possible to make nearly as thorough a test as was made with manure scum. Table 4 states in tabular form the nature of the tests and their results.

Test No. 5, of six parts, gives the best idea of the Euglena experiments described in this paper. If the results of this test be applied to one of the Euglena-rotifer experiments, presently to be described, they indicate that the water used in the experiment contained 6.05 cc. of oxygen per liter at the outset (a). When Euglena was added as food, the water contained 6.58 cc. of oxygen per liter (b). After four hours in direct sunlight, it contained 9.27 cc. per liter (c), but on standing overnight the oxygen content fell to 7.35 cc. per liter (d).

In the control the water contained 6.05 cc. per liter at the outset (a); 5.51 cc. after manure scum was added (e), and 4.63 cc. per liter after the dish had stood twenty hours (f).

It should be remarked that probably neither the Euglena nor the manure scum used in test No. 5 was as abundant as in one of the rotifer experiments, though the difference could not have been great. Accepting these results as typical, it appears that the mean oxygen content in one of the Euglena cultures was

$$\frac{\left(\frac{6.58 + 9.27}{2} \times 4\right) + \left(\frac{9.27 + 7.35}{2} \times 16\right)}{20} = \frac{31.70 + 132.96}{20} =$$

8.233 cc. per liter while the mean oxygen content of the control was

$$\frac{5.51 + 4.63}{2} = 5.07 \text{ cc. per liter.}$$

Thus the absolute excess of oxygen in the Euglena dishes over the control was about 3.16 cc. per liter. Relatively the Euglena dishes contained about 62 per cent more oxygen than the manure-scum cultures. As was pointed out above, the quantity

TABLE 4

Showing the effect of Euglena in increasing the oxygen content of water, particularly as contrasted with water containing manure scum. The columns of figures are the results obtained by Winkler's method of determination of dissolved oxygen

TEST NUMBER	TREATMENT OF WATER	VARIABLES IN FORMULA FOR COMPUTATION (SEE TEXT)			NUMBER OF CUBIC CENTIMETERS OF OXYGEN PER LITER
		(n)	(t)	(v)	(o)
5–a	Spring water, without treatment, tested at once for oxygen	7.9	20.7	88	6.05
b	Spring water, Euglena added, then immediately filtered and tested for oxygen	8.6	20.7	88	6.58
c	Spring water, Euglena added, kept in direct sunlight four hours, then filtered and tested	12.1	20.7	88	9.27
d	Spring water, Euglena added, kept four hours in direct sunlight, sixteen hours in diffuse light and darkness, filtered and tested	9.6	20.7	88	7.35
e	Spring water, manure scum added, then immediately filtered and tested	7.2	20.7	88	5.51
f	Spring water, manure scum added, left twenty hours, then filtered and tested	5.4	20.7	88	4.63
6–a	Spring water in flat dish, Euglena added, kept cool in direct sunlight four and one half hours, then filtered and tested for oxygen	8.8	21.5	88	6.49
b	Spring water in flat dish, manure scum added, kept in diffuse light four and one half hours, then filtered and tested	3.0	21.5	88	2.21
7–a	Untreated water in ten watch-glasses, Euglena and a little manure solution without scum added, kept cool in direct sunlight five hours, then in diffuse light and darkness fourteen hours, filtered and tested	5.73	20.75	67	5.75
b	Untreated water in ten watch-glasses, manure scum and a little water from Euglena culture (but without Euglena) added, kept in diffuse light and darkness nineteen hours, then filtered and tested	3.83	20.75	67	3.84

TABLE 4—*Continued*

TEST NUMBER	TREATMENT OF WATER	VARIABLES IN FORMULA FOR COMPUTATION (SEE TEXT)			NUMBER OF CUBIC CENTIMETERS OF OXYGEN PER LITER
		(n)	(t)	(v)	(o)
8–a	Spring water in bottle, Euglena and a little manure solution without scum added, bottle sealed without air bubble, agitated on clinostat for two hours in direct sunlight and twenty-two hours in diffuse light and darkness, filtered and tested	16.91	20.75	115	9.89
b	Spring water in bottle, manure scum and a little Euglena water (but without Euglena) added, bottle sealed without air bubble, agitated on clinostat two hours in direct sunlight and twenty-two hours in diffuse light and darkness, filtered and tested	4.1	20.75	115	2.39
9–a	Spring water in bottle, Euglena added, bottle sealed without air bubble, agitated on clinostat thirty-two hours in diffuse light and darkness, filtered and tested	20.2	20.7	115	11.84
b	Spring water in bottle, manure scum added, bottle sealed without air bubble, agitated on clinostat thirty-two hours in diffuse light and darkness, filtered and tested	3.55	20.7	115	2.08

of Euglena used in the rotifer experiments was probably greater than in the oxygen tests just described, so that the excess of oxygen in the Euglena dishes in the experiments was probably somewhat more than 62 per cent.

The decrease of the oxygen content of the water over night, in the Euglena culture, may be in part due to other organisms, since my Euglena culture was not quite pure. In this respect the tests described here apply more correctly to the rotifer experiments of this paper than they do to Whitney's experiments. Probably, however, a greater part of this overnight decrease is due to escape of oxygen into the air. Quiet water in contact with ordinary atmosphere will not, as a rule at least, retain as much as 9.27 cc. of oxygen per liter.

The remaining tests in table 4 (Nos. 6 to 9) are so unlike any of the rotifer experiments that it is unnecessary to explain them further than they are explained in the table. All of them contribute, however, to the proof that Euglena under various circumstances markedly increases the oxygen content of the water in which it lives.

Euglena versus oxygen and other substances as male-producing or male-repressing agents

Since the quantity of oxygen produced by Euglena and dissolved in the water is about the same as that dissolved directly from the atmosphere in the rotifer experiments, the effects of Euglena and of oxygen in increasing male-production may be directly compared. Any excess of male-production in the Euglena experiments over that in the oxygen experiments may therefore be attributed to another factor, probably the food.

The following experiments were designed to compare the effectiveness of Euglena with that of oxygen among the male-increasing agents, and with manure solution and creatin among the male-repressing agents.

Experiment 3. Euglena versus oxygen. In each of three dishes was placed the same number of rotifers, the number ranging from four to eight on different days. Two of the dishes were filled with spring water; to one of these Euglena was added as food, to the other manure scum. In the third dish was placed water that had first been saturated with an air-oxygen mixture composed of about 40 per cent of oxygen and 60 per cent of nitrogen. Manure scum was added to the water in the third dish, which was then set under a bell jar in an atmosphere of which 40 per cent was oxygen. The chemical composition of the medium in the three dishes at the beginning of the experiment was equalized by adding to the Euglena-fed culture as much manure solution (without scum) as was added to the other two dishes with the scum, and by adding to the manure-scum cultures as much water (without Euglena) from the Euglena stock as was introduced into the first dish with Euglena. It was easy to obtain this water practically free of Euglena, since the Euglena was a quiescent form that produced an incrustation along the sides of the jar, while very few individuals were actively swimming.

The parent rotifers were removed from their dishes from twenty-four to forty hours later. All young rotifers hatching from eggs laid in that time were reared to maturity, in all three lots alike, in spring water with manure scum as food. The male-producers and female-producers are recorded in table 5.

TABLE 5

Comparison of the effects of Euglena and manure scum as food, and of oxygenated and untreated water, upon the proportion of male-producers in the rotifer Hydatina senta

DATE	EUGLENA AS FOOD IN UNTREATED WATER		MANURE SCUM IN OXYGENATED WATER		MANURE SCUM IN UNTREATED WATER	
	Number of ♂♀	Number of ♀♀	Number of ♂♀	Number of ♀♀	Number of ♂♀	Number of ♀♀
March 20	13	43	1	22	2	37
March 22	21	33	15	33	1	21
March 23	14	18	0	22	0	21
March 26	29	28	1	29	0	18
March 28	3	27	5	9	0	1
March 29	25	55	2	77	3	74
April 2	4	62	4	41	10	44
April 4	14	61	4	64	0	59
April 16	1	46	0	28	0	35
April 18	1	16	23	45	13	58
April 19	3	68	0	53	3	57
April 23	6	27	0	20	0	5
April 25	0	47	5	51	4	30
April 26	0	34	3	47	0	54
April 30	0	57	0	31	0	40
May 2	0	32	1	86	0	69
May 3	1	25	9	26	3	46
May 7	2	43	0	47	1	18
May 9	4	67	0	41	0	50
May 14	0	29	1	21	0	20
Totals	141	818	74	793	40	757
Percentage of ♂♀	14.7		8.5		5.0	

Comparison of the second and third divisions of the table indicates that oxygenation of the water increases the proportion of the male-producers by 3.5 per cent. The first and third divisions show that Euglena increases male-production by 9.7 per cent, of which 3.5 per cent is presumably attributable to the oxygen liberated in photosynthesis, leaving 6.2 per cent to be caused by some other factor, probably the food itself. The food, if this assumption is correct, is a little less than twice as effective as the oxygen.

Experiment 4. Euglena versus manure scum, spring water versus manure solution. The effects of the four agents named, in conjunction with one another and in opposition to one another, are here tested. On each of the dates named in table 6 four rotifers were placed in each of four dishes. In two of the dishes was placed manure solution, the food in one of these being Euglena, in the other manure scum. Into the other two dishes was put spring water, the food in one of them being Euglena, in the

TABLE 6

A comparison of the effects of Euglena and manure scum, and of spring water and manure solution, upon the proportion of male-producers in the rotifer Hydatina senta

DATE	EUGLENA AND MANURE SOLUTION		EUGLENA AND SPRING WATER		MANURE SCUM AND MANURE SOLUTION		MANURE SCUM AND SPRING WATER	
	Number of ♂♀	Number of ♀♀	Number of ♂♀	Number of ♀♀	Number of ♂♀	Number of ♀♀	Number of ♂♀	Number of ♀♀
February 10	4	14	5	14	0	10	0	23
February 15	11	10	13	15	0	24	3	24
February 21	16	20	16	16	0	11	9	8
February 26	7	10	7	12	1	15	5	21
February 28	5	41	10	41	2	27	3	30
March 1	1	33	5	29	4	23	2	31
March 7	0	9	10	4	0	0	0	13
March 12	5	47	29	23	0	16	12	26
March 14	5	33	1	32	0	38	8	37
March 16	0	34	2	14	8	16	0	25
Totals	54	251	98	200	15	180	42	238
Percentage of ♂♀	17.70		32.88		7.69		15.00	

other manure scum. The two dishes receiving manure scum as food received also a small amount of water from the Euglena stock (without Euglena), and the dish receiving Euglena as food in spring water received also a small amount of manure solution (without scum). This was done in order to equalize the initial chemical composition of the media in the four dishes, except as it was intentionally made different by manure solution and spring water. After from twenty-four to forty hours the rotifers were removed from their dishes. All offspring hatching from eggs laid in that time were reared to maturity, all four lots alike, in spring water with manure scum as food.

Comparison of the third and fourth divisions of table 6 shows that manure solution reduces the proportion of male-producers by 7.31 per cent, but this reduction is more than offset by feeding with Euglena, as in the first division of the table. That is, Euglena increases male-production more than manure solution reduces it. If, however, as indicated by experiment 4, over one-third of the total effect of Euglena is due to the oxygen liberated in photosynthesis, then Euglena as food is less powerful in increasing male-production than manure solution is in decreasing it. The maximum male-production was obtained, as was to be expected, by using both Euglena and spring water (second division of table 6). The minimum is obtained from manure scum and manure solution together. This minimum is approximately doubled by substituting either spring water for manure solution or Euglena for manure scum. Making both substitutions practically doubles the results of a single substitution.

Experiment 5. Oxygen versus creatin, Euglena versus creatin. On each of the dates in table 7, three to five rotifers were placed in each of four dishes, the number in each dish being the same on any one day. The four dishes were treated as follows: 1) One lot was immersed in a dilute solution of crude creatin, of the concentration indicated in table 7, and fed Euglena. 2) One dish was filled with a measured quantity of water which had first been saturated with an atmosphere of which 40 per cent was oxygen. Enough of a more concentrated creatin solution was added to produce the same final concentration as in the preceding dish.

TABLE 7

Showing the effects of oxygen, Euglena, and creatin upon the proportion of male-producers in a Nebraska line of the rotifer Hydatina senta

DATE	STRENGTH OF CREATIN SOLUTION IN PER CENT	EUGLENA AND CREATIN SOLUTION		MANURE SCUM AND OXYGENATED CREATIN SOLUTION		MANURE SCUM AND UNTREATED CREATIN SOLUTION		MANURE SCUM AND UNTREATED SPRING WATER	
		Number of ♂♀	Number of ♀♀	Number of ♂♀	Number of ♀♀	Number of ♂♀	Number of ♀♀	Number of ♂♀	Number of ♀♀
May 16	0.01	0	18	0	23	0	26	0	26
May 17	0.02	0	21	0	2	0	17	0	9
May 21	0.01	4	14	0	29	0	25	0	21
May 23	0.01	0	19	6	24	1	32	0	35
May 24	0.02	18	14	0	13	1	21	0	19
May 28	0.02	17	24	0	19	0	41	2	42
May 30	0.03	0	10	3	43	4	27	9	43
May 31	0.025	9	30	0	40	4	55	3	63
June 4	0.02	4	17	3	21	3	47	10	61
Totals		52	167	12	214	13	291	24	319
Percentage of ♂♀		23.7		5.3		4.2		6.9	

Manure scum was added as food, and the dish was set under a bell jar in an atmosphere of which 40 per cent was oxygen. 3) A third dish was filled with the dilute creatin solution, manure scum was added as food, and the dish set in air. 4) The fourth dish, as general control, was filled with untreated spring water, manure scum was added as food, and the dish was set in air.

After from twenty-four to forty hours the rotifers were removed from the dishes. The offspring hatching from eggs laid in that period were reared to maturity in spring water, being fed with manure scum.

The rotifers used in this experiment were received from Prof. D. D. Whitney, who collected them at Lincoln, Nebraska.

Experiment 6. This is a duplication of experiment 5 in every respect, except that the rotifers used were part of a line received from Prof. A. L. Treadwell, who in turn obtained them from Prof. D. D. Whitney, then at Wesleyan University. They are presumably from the same New Jersey stock as was used in Dr. Whitney's former work. All the preceding experiments in this paper, with the exception of experiment 5, were performed with these New Jersey rotifers.

Table 8 shows the results of experiment 6.

While the last three divisions of table 7 show differences of the kind expected, these differences are small. Comparisons based on the first three divisions of that table would indicate that Euglena is about 18 times as potent in increasing male-production as is oxygen. Or, deducting from the total effect of Euglena the 1.1 per cent which a comparison of the second and third divisions of the table would indicate was due to oxygen, the effect of the Euglena as food would be nearly 17 times as great as that of oxygen.

A different result, however, is found in table 8. The first three divisions of the table show that oxygenation increases the proportion of male-producers 5.1 per cent, Euglena 20.2 per cent. Deducting from the total effect of Euglena the fraction chargeable to oxygen alone (20.2 − 5.1 = 15.1), Euglena as food is nearly three times as effective as oxygen in increasing male-production.

TABLE 8

Showing the effects of oxygen, Euglena, and creatin upon the proportion of male-producers in a New Jersey line of the rotifer Hydatina senta

DATE	STRENGTH OF CREATIN SOLUTION IN PER CENT	EUGLENA AND CREATIN SOLUTION		MANURE SCUM AND OXYGENATED CREATIN SOLUTION		MANURE SCUM AND UNTREATED CREATIN SOLUTION		MANURE SCUM AND UNTREATED SPRING WATER	
		Number of ♂♀	Number of ♀♀	Number of ♂♀	Number of ♀♀	Number of ♂♀	Number of ♀♀	Number of ♂♀	Number of ♀♀
June 6	0.02	19	11	2	29	2	19	0	28
June 7	0.02	3	2	5	44	5	33	8	27
June 11	0.02	3	19	0	21	0	6	0	11
June 13	0.02	2	22	6	15	1	29	0	12
June 14	0.02	4	28	6	26	1	29	4	23
Totals		31	82	19	135	9	116	12	101
Percentage of ♂♀		27.4		12.3		7.2		10.6	

Response to oxygen in the Nebraska line

The failure of the Nebraska line in experiment 5 (table 7) to show any marked effect of oxygen is the only inharmonious result obtained in this study. It seemed possible that this line was not as sensitive to oxygen as the New Jersey line used in the other experiments. If this were the case, any other response to oxygen might prove less marked in the Nebraska line than in the New Jersey line. Such a response to oxygen in this species was found in earlier experiments. In a former paper (Shull, '15a) it was shown that races of rotifers might differ very markedly in the place of egg-laying. One race laid its eggs very largely on the bottom or sides of the dishes; another race chiefly attached to the surface film of the water. In connection with later oxygen experiments (Shull, '18), it was shown that placing the dishes in an atmosphere containing an excess of oxygen caused the eggs to be laid more largely on the bottom than when the dishes were kept in air. If the Nebraska line lacked responsiveness to oxygen, this lack might be evidenced by failure of oxygen to alter the place of egg-laying. This possibility gave rise to the following experiment.

Experiment 7. On each of the days named in table 9 several rotifers of the Nebraska line were placed in each of two dishes of water and fed manure scum. One dish was set under a bell jar in an atmosphere of which 60 per cent was oxygen, the other placed under a bell jar in ordinary air. After a period of from sixteen to eighteen hours, the eggs laid on the bottom and at the surface film in each of the dishes were counted. As a check upon the results, a similar test was simultaneously made upon the New Jersey line. Table 9 gives the results.

While the Nebraska line is here shown to be responsive to oxygen, the controls show that it normally laid more of its eggs at the bottom than did the New Jersey line. In view of this normal difference between the two lines, it is difficult to judge of the relative effect of oxygen upon them, and the discrepant results in experiments 5 and 6 probably remain unexplained.

TABLE 9

Showing the number of eggs laid at the surface film of water and at the bottom of the dish by two lines of rotifers, one from Nebraska, the other from New Jersey, when kept in air and in a 60 per cent oxygen atmosphere. The excess of oxygen causes more eggs to be laid at the bottom, but it is questionable which line is most affected this way, since their behavior is different under the same conditions

DATE	NEBRASKA LINE				NEW JERSEY LINE			
	Air		Oxygen		Air		Oxygen	
	Eggs laid at surface	Eggs laid at bottom	Eggs laid at surface	Eggs laid at bottom	Eggs laid at surface	Eggs laid at bottom	Eggs laid at surface	Eggs laid at bottom
December 8	5	25	0	22	16	12	2	29
December 11	28	23	1	44	28	16	3	22
December 13	4	24	0	28	1	5	1	3
December 20	6	24	0	13	32	26	0	25
Totals	43	96	1	107	77	59	6	79
Percentage at surface	30.9		0.9		56.6		7.0	

DISCUSSION

While this paper was in preparation an article by Whitney ('17) on a similar subject, the relative effectiveness of oxygen and food as sex determiners, was published. These two papers, however, in no sense duplicate, since they approach the problem from different angles and with different criteria of judgment.

A considerable part of Whitney's paper is devoted to experiments to show that green organisms as food increase the production of males in other rotifers than Hydatina. It is to be regretted that many of these experiments were performed with mass cultures without controls. If these rotifers are like Hydatina in producing males in 'epidemics,' the lack of controls is most unfortunate. It is well known to every student of Hydatina that, especially in lines that produce only a moderate proportion of males, these males often appear in well-defined 'waves,' and it has been shown that these epidemics not infrequently have a rather regular periodicity (Shull, '15b). If, in an experiment without control, a line which regularly produces numerous males once a month, the experimenter, ignorant of the interval between periods of many males, should attempt to alter male-production by introducing something into the culture five or six days before the beginning of the period of males was due, he could use any one of a dozen agents and obtain the same result, namely, an increase of male-production a few days later.

Notwithstanding the lack of controls, Whitney's conclusion that the use of green organisms for food in these several species of rotifer increases male-production is probably correct; it is undoubtedly correct in the case of Hydatina, as Whitney ('14) satisfactorily showed in an earlier paper and as I have confirmed in my experiments. The chief question is how much of this effect of green organisms is due to nutritive differences, how much to other agents associated with the green organisms.

One of Whitney's experiments bears upon this point, but the results are not easily understood. That experiment is represented by his table 5 (Whitney, '17). Information on which one could base a judgment of the significance of this experiment is not given. The cultures were apparently of some size, all of them containing green organisms. These cultures had been in existence from five to thirty days, presumably in the sunlight (either direct or diffuse), before the beginning of the experiment. Some of them were then excluded from the sunlight, but for how long a time is not stated. If the duration of the darkness was not prolonged, it is to be expected that the average oxygen content would be appreciably greater than in a culture which had never been kept in the light. The experiments described in table 4 of this paper, test No. 5, indicate that oxygen accumulated in photosynthesis is only very gradually lost. It is not clear, then, that the oxygen content of the cultures in the darkness was as much lower than that of the sunlight cultures as Whitney supposed. It is almost certain that, unless they were excluded from the sunlight for a considerable period, these dark cultures contained appreciably more oxygen than a culture never kept in the sunlight would contain.

It is a pertinent question, therefore, whether the smaller amount of oxygen in the cultures in darkness was as effective as the larger quantity in the sunlight. Some of Whitney's cultures in the darkness were aerated, others not, and he appears to have suspected that if oxygen were a male-producing agent, the more oxygen there was present the more males there should be. In the experiments described in this paper there is little difference, in its effect on male-production, between water saturated with a 40 per cent oxygen atmosphere and water saturated with a 60 per cent oxygen atmosphere, although it was shown that 60 per cent oxygen atmosphere left the oxygen content of the water very plainly greater. What difference there was indicated that the lower concentration of oxygen was the more effective. It cannot be accepted without question, therefore, that aeration of the water, in addition to the use of green food with its incidental oxygen, should further increase the male-production.

Disregarding the above objections to the experiments on which table 5 of Whitney's paper is based, we may examine the results of those experiments. Instead of comparing only selected parts of his table 5, the whole table may be included. Since in some of the cultures more mothers were used than in others, merely adding together the offspring of parents that were treated in the same manner would give to those cultures having many mothers undue weight. A more just method of combining like cultures is to average the percentage of male-producers in all cultures; each culture is then as weighty as the others, regardless of the number of parents used. By this method of combination, Whitney's table 5 is converted into the following:

	Per cent male-producets
Sunlight, with aeration	54.5
Darkness, with aeration	19.0
Darkness, without aeration	40.6

It is difficult to see what these results mean, though Whitney concludes from them that oxygen is not a male-producing agent. Just why one is to infer that the quantity of food is the cause of the differences shown, is equally obscure. The argument for oxygen is at least as good as the argument for food.

The remainder of Whitney's paper is devoted mainly to the effect of oxygen upon the protozoan and bacterial food supply of the rotifers in experiments like those of Shull and Ladoff. He finds that the increased oxygen content of the water favors more rapid multiplication of these food organisms, and infers that the rotifers were therefore better fed. That there is a certain low concentration of food at which an increase of available food would mean an increase of nutrition is probable. If food were in greater concentration than this, it is not clear that further increase in the amount of food at hand would cause an increase in the amount eaten. A man who sits down alone at a table with a hundred pounds of steak and fifty pounds of potatoes eats no more than if only fifty pounds of steak and twenty-five pounds of potatoes are set before him.

If the above criticism is not valid, and it is after all merely quantity of available food that determines male-production, that fact should be very simply and easily discovered by controlled experiments, involving large numbers of individuals, and the next desirable step in the investigation is perfectly clear.

The criticisms of Whitney's methods and conclusions in the foregoing paragraphs may seem to indicate a wider divergence between his conclusions and mine than really exists. Certainly the conclusions reached in this paper and in that of Shull and Ladoff differ less from Whitney's conclusions than Whitney's recent article would lead one to suppose. Thus when Whitney ('17, p. 114) writes, "According to Shull and Ladoff, one would expect many male-producing females to be produced in sunlight and Chlamydomonas with the accompanying excess of oxygen, but no male-producing females would be expected to be produced in darkness and Chlamydomonas with no excess of oxygen," he overdraws the indictment somewhat. The quoted statement could be correct only if Shull and Ladoff had denied that food had *any* effect on male-production, and had asserted that *all* of the increase of male-production obtained by Whitney was due to oxygen instead of food. No such idea was even suggested. Indeed, there are frequent passages in the paper of Shull and Ladoff that indicate the reverse. Thus on page 138 it is stated "our results may be interpreted as being largely in support of Whitney's contention." Again, page 156, "part of the increased male-production following the use of Chlamydomonas as food in Whitney's cultures, was due to the oxygen liberated by the green flagellates as a by-product of photosynthesis." Likewise, page 157, "We suspect * * * Whitney's conclusion that the food conditions influence male-production is correct." All that was insisted upon by the authors of these passages was that (page 157) "all the factors obviously associated with Chlamydomonas in the cultures should be separately tested before any residue of influence is assigned to nutrition."

Two factors associated with Chlamydomonas in Whitney's earlier experiments were 1) an initial difference in the chemical content of the water and 2) dissolved oxygen produced during photosynthesis. In this paper the former factor has been eliminated and the second has been measured. After deducting the measured effect of the oxygen from the total effect of the green organism used for food, there is a residual effect which it seems fair to attribute to nutrition. According to the results of experiments described in the foregoing pages, this residual effect due to nutrition is at least several times as great as the effect of the accompanying oxygen.

SUMMARY

Repetition of the experiments with an excess of dissolved oxygen, in which the excess was obtained by saturating the water with an atmosphere of which 60 per cent was oxygen, showed that oxygen is a male-producing agent. The experiments of Shull and Ladoff had demonstrated that a smaller excess of oxygen (obtained from a 40 per cent oxygen atmosphere) increased male-production, but left in doubt the effectiveness of a 60 per cent atmosphere. Rather direct comparison of the two concentrations of oxygen, in this paper, shows that there is little difference between them. The lower concentration may, perhaps, be a little the more effective in inducing male-production.

The amount of oxygen dissolved in the water in these experiments has been measured by the Winkler method. The cultures which were first saturated with an atmosphere of which 40 per cent was oxygen and were then enclosed in a vessel in such an atmosphere, contained on the average about 60 per cent more dissolved oxygen than did the untreated controls.

Cultures in which Euglena was used for food were also compared, relative to their oxygen content, with cultures in which manure scum was used as food. The Euglena cultures under the conditions of the experiments, contained on the average about 62 per cent more oxygen than the manure scum cultures. Presumably, therefore, when Euglena increases male-production in Hydatina, as much of that increase is due to oxygen as is directly produced by saturation with a 40 per cent oxygen atmosphere.

In experiments with Euglena as food, after deducting the increase in male-production presumably due to oxygen liberated, it was found that Euglena was two or three times as effective as the oxygen, and in one case many times as effective. The experiment in which Euglena appeared to be many times as effective as its liberated oxygen was performed upon a different line from that of the other experiments. This line was tested to discover whether it was responsive to oxygen in another way (laying eggs at surface film or bottom of dish). While it was plainly responsive to oxygen, there is some doubt whether it was as responsive as the other line used.

Euglena as a male-producing agent was compared with manure solution as a male-repressing agent. The repressing effect of manure solution was a little more than offset by the Euglena (including the effect of the oxygen liberated by the Euglena).

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