The physiology of growth and sporulation of the yeast Nadsonia fulvescens

THE PHYSIOLOGY OF GROWTH AND SPORULATION OF THE YEAST NADSONIA FULVESCENS

by

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ABSTRACT

The physiology of sporulation of Nadsonia fulvescens was examined. A number of different sporulation and presporulation conditions were studied. Cells grown in dextrose required a carbon source for sporulation, and cells grown in ethanol were able to sporulate without an available carbon source. The carbon sources used by the cells for sporulation were glycerol, dextrose, and ethanol. The cells did not use acetate as a carbon source for sporulation. No direct correlation was found between respiratory quotients and sporulation patterns.

INTRODUCTION

While the ability of yeasts to form spores depends upon the intrinsic nature of the organisms, there are many different conditions by which sporulation can be brought about in ascosporogenous strains. The use of gypsum block or vegetable juices are among the earliest methods developed and have aided in taxonomic studies of yeasts. For physiological studies of sporulation, more defined conditions are required. Conditions widely used for such purposes include a suitable carbon source and buffer in either solid or liquid sporulation media. An example of a medium of this kind described by Fowell (1952) contains sodium acetate and agar buffered at pH 7.

In studying sporulation of Saccharomyces cerevisiae, Miller, Calvin, and Tremaine (1955) observed sporulation on a number of different sugars. Fructose and mannose were more stimulating than glucose or galactose. Dihydroxyacetone, which did not support active growth, did stimulate sporulation (Miller 1957). Sporulation on acetate, glucose, ethanol, pyruvate, or lactate was inhibited by many different amino acids or nitrogen compounds (Miller 1963). Sporulation on dihydroxyacetone or buffer was less sensitive to inhibition by nitrogen compounds. Ammonium sulfate inhibited sporulation only when an exogenous carbon source was present in the sporulation medium.

Results have shown that the kinetics of sporulation are dependent on both sporulation and presporulation conditions. With cells grown in glucose sporulation in medium containing acetate is maximum when the cells are harvested from the growth medium during the early stationary phase (Croes 1967b, Esposito et al. 1969). This appears to be related to the finding that cells grown in the presence of glucose are able to rapidly oxidize acetate only if they are harvested during the late logarithmic or stationary phases of growth (Eaton and Klein 1954). During the early log phase the glucose in the medium represses the enzymes of the citric acid-cycle--a major pathway for acetate oxidation (Polakis and Bartly 1965).

In media containing acetate, sporulation is more rapid and abundant with cells grown on acetate than with cells grown on glucose (Roth and Halvorson 1969).

Acetate metabolism has been shown to play a major role in sporogenesis (Esposito et al. 1969). Acetate metabolism during sporulation results in an increase in dry weight associated with an increase in protein, DNA, and RNA. Acetate-2-^{14}C is incorporated into cells during the early part of sporulation. The label can then be found in the macromolecules of the cells.

The purpose of this research was to study the physiology of sporulation of the species Nadsonia fulvescens. Nadsonia is described by Winge and Roberts (1958) as forming large lemon shaped cells. Spores are formed in a peculiar way. Heterogamous conjugation takes place between a mother cell and its smaller daughter cell;

the daughter cell nucleus migrates into the larger cell, where nuclear fusion occurs. The resulting zygote moves into a bud which has formed on the mother cell opposite the point of attachment of the daughter cell. The bud becomes the ascus.

Experiments were designed to determine the patterns of sporulation with various carbon sources available in sporulation media. A number of different presporulation conditions were used.

MATERIALS AND METHODS

The basal growth medium contained the bulk elements and trace elements described by Wickerham (1951). Pyridoxine hydrochloride was added at a concentration of 400 ug per liter. Unless otherwise stated, all growth media and cell suspensions were buffered at pH 6 with 0.05 M potasium phthalate. Stock cultures were maintained on yeast extract-agar-dextrose (YAD) which contained yeast extract, 1%; dextrose, 2%; KH_2PO_4, 0.1%; $MgSO_4 \cdot 7H_2O$, 0.05%; and agar, 1.5%. Cultures were transferred every two weeks and maintained at 5C. Prior to inoculation of growth media the yeasts were subcultured for 24 hours on YAD medium.

Growth media were inoculated using an initial cell concentration of 5×10^4 cells per ml. Cells were grown in 100 ml batches in 500 ml Erlenmyer flasks at an ambient room temperature of 23C. Cultures were aerated by agitation on a 2.5 centimeter stroke New Brunswick rotary shaker at 280 revolutions per minute.

Growth was measured by optical density at 540 nm with a Bausch and Lomb Spectronic 20. Prior to optical density readings, all cell suspensions were sonicated for 10 seconds with a Bronwill Biosonic (power 25, tune 3) to eliminate clumping effects.

Respiration using conventional manometric techniques (Umbreit, Burris, and Stauffer 1957) was measured in a Gilson Differential Respirometer at 23C with air as the gas phase. Respirometer flasks

contained 1.9 ml cell suspension, pH 6; 0.1 ml 10% KOH; and 0.1 ml substrate.

Dry weights of washed cells were measured in tared aluminum foil cups at 80C for 18 hours. Washed cells at an optical density of 1.0 were equivalent to 0.85 mg/ml dry weight.

Ethanol determinations were by the procedure of Feldstein and Klendshoj as described by Conway (1962).

Dextrose was analyzed by the anthrone method (Umbreit et al. 1957).

Three serial transfers into presporulation media for a total of at least 20 generations were made before cells were harvested for sporulation studies. Cells were harvested by centrifugation using a Sorvall RC2-B refrigerated centrifuge at 1500 X g for 10 minutes, washed twice in phthalate buffer at pH 6, and resuspended in sporulation media at a concentration of 1.5×10^7 cells/ml. Ten ml of this suspension was placed in 50 ml Erlenmyer flasks and aerated as above. Sporulation media consisted of either distilled water or buffer and, in some cases, a carbon source.

Sporulation was observed under phase contrast optics. The percent asci was determined by the convention of Croes (1967a) relative to the total count of cells, buds, and asci with a total of at least 500 counted in each determination. Sporangia were not counted and were distinguished from cells on the basis of reduced refractility.

EXPERIMENTAL RESULTS

The growth curve of Nadsonia fulvescens in minimal glucose medium is shown in Figure 1. The mass doubling time was 90 minutes. The stationary phase was reached within 30 hours. The cell concentration in the stationary phase was 60 million cells per ml. The doubling times in glycerol or ethanol were 14 hours.

Table 1 shows the results of ethanol determinations in minimal media at dextrose concentrations of 1% and 0.5%, respectively. After 24 hours incubation in 1% dextrose, a total of 70 mg ethanol were formed per 100 ml growth medium. Ethanol was not detected when dextrose was used at a concentration of 0.5%. The amount of growth was significantly greater with the higher concentration of dextrose. Anaerobic fermentation appears to be greater with higher cell concentrations. Oxygen demand is probably greater than supply at higher cell concentrations.

Table 1. Ethanol Production and Growth.

Carbon Source	Ethanol (mg/100 ml) at 24 hr	Growth, Optical Density at 30 hr
1% dextrose	70	3.0
0.5% dextrose	0	2.4

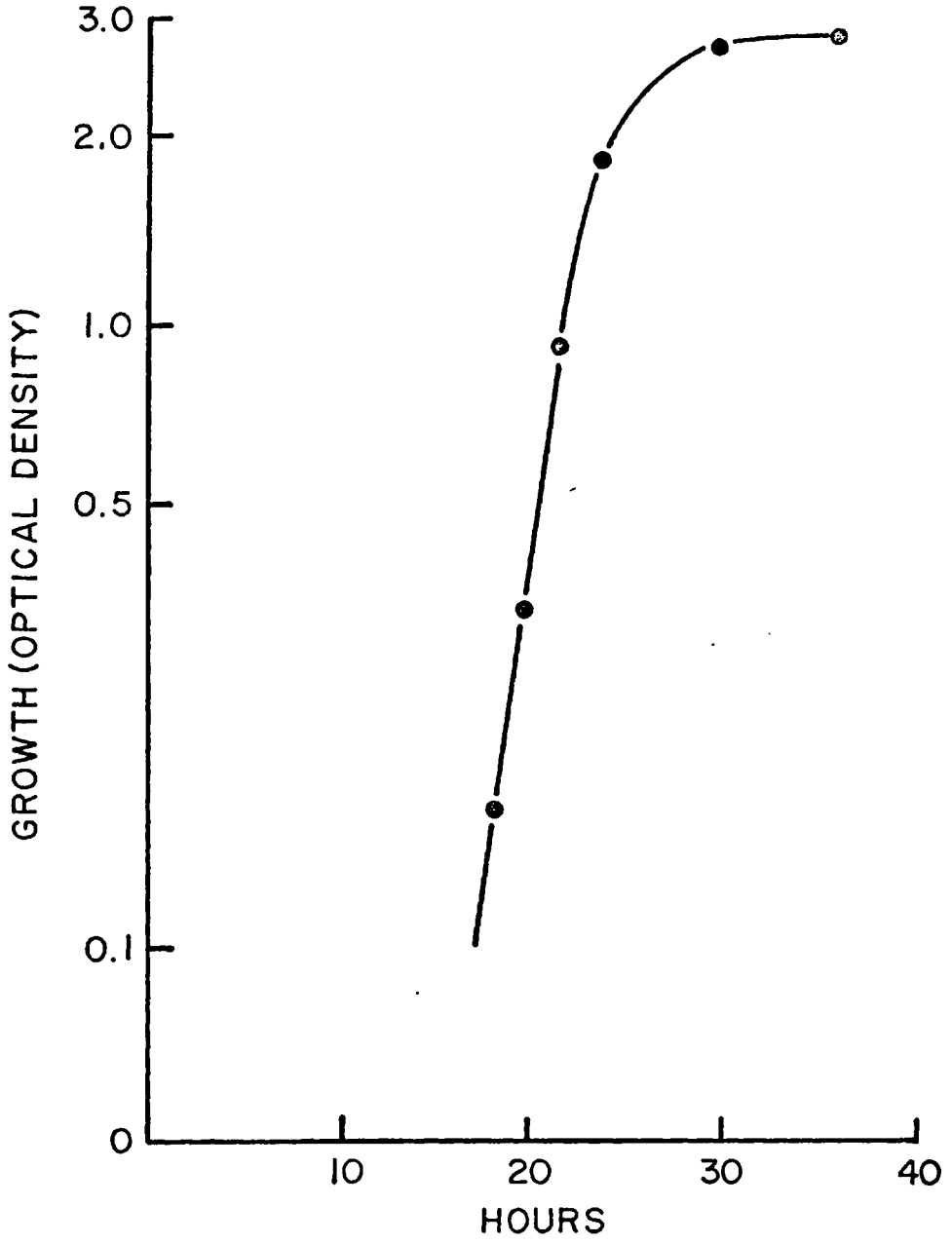

Figure 1. Growth Curve of Nadsonia Fulvescens in Minimal Medium with 0.5% Dextrose.

Growth was measured by optical density at 540 nm.

Dextrose was used at a concentration of 0.5% in respiration and sporulation experiments to prevent the accumulation of ethanol. Croes (1967b) showed that yeasts utilize accumulated ethanol during the stationary phase. By preventing the accumulation of ethanol, cells could be characterized as oxidizing only dextrose. Thus the complicating effect of secondary metabolism of ethanol on respiration and sporulation was reduced. Anaerobic fermentation was not studied.

Cells were harvested for respiration studies by centrifugation, washing twice in buffer and resuspending in buffer at pH 6. Logarithmic phase cells were harvested at an optical density of 1.0, and stationary phase cells were harvested after 40 hours of growth at an optical density of 2.4.

The respiratory capacity of the cells was determined manometrically by measuring oxygen uptake. The Q_{02} values given in Table 2 are microliters of oxygen taken up per milligram dry weight of cells per hour. The Q_{02} values given are the maximum rates reached within one hour of exposure to the carbon source in the manometer flasks. In most cases there was no significant lag period before the maximum rates were reached. However, with logarithmic phase cells grown in dextrose there was a 50 minute lag period before ethanol oxidation reached a maximum rate.

Respiration by logarithmic phase cells differed both quantitatively and qualitatively from stationary phase cells. Rates of respiration of dextrose or ethanol were higher for logarithmic phase cells. The rate of glycerol respiration was greater with

Table 2. Respiration by Nadsonia Fulvescens

Growth		Carbon in Respirometer	Q_{O2}
Carbon Source	Age		
dextrose	log phase	dextrose	79
		glycerol	7
		acetate	0
		ethanol	63
	stationary phase	dextrose	61
		glycerol	12
		acetate	17
		ethanol	44
ethanol	log phase	acetate	31
		ethanol	71
glycerol	log phase	glycerol	20

stationary phase cells. Acetate was oxidized at a low rate by stationary phase cells but not at all by logarithmic phase cells. It is likely that the acetate oxidizing enzymes are repressed by dextrose during logarithmic growth. At an optical density of 1.0 there remained 258 mg dextrose per 100 ml medium. Only 23 mg dextrose per 100 ml remained after the early stationary phase was reached (30 hours). Cells grown in ethanol were able to oxidize either ethanol or acetate. In general, glycerol oxidation occurred at a low rate compared to oxidation of dextrose or ethanol.

The sporulation characteristics of the cells in sporulation media were determined by counting the percentage of asci at 12 hour intervals. The results show the lag period before asci were detected, the rate at which sporogenesis occurs and the maximum percentage reached with each sporulation condition used. It was found that the kinetics of sporulation is a function of both sporulation and pre-sporulation conditions.

The results of sporulation by logarithmic dextrose grown cells are shown in Table 3. Cells grown in dextrose and harvested during the logarithmic phase require a carbon source for sporulation. Of the carbon sources used in the sporulation media, glycerol supported best sporulation. In the presence of glycerol, sporulation was detected after 24 hours incubation both at pH 7 and pH 6. Sporulation on ethanol or dextrose began considerably later. Rate of sporulation in the presence of glycerol appears to be greater at pH 7 than at pH 6.

Table 3. Sporulation of Cells Grown in Dextrose and Harvested During Log Phase.

Sporulation Media	pH	Percentage of Asci					
		Hours in Sporulation Media					
		12	24	36	48	60	72
0.5% dextrose	7	0	0	3	10	19	19
2% glycerol	7	0	23	48	49	-	-
1% sodium acetate	7	0	0	0	0	0	0
1% ethanol	7	0	0	0	4	5	5
1% ethanol and 0.005% dextrose	7	0	0	4	23	38	39
0.005% dextrose	7	0	0	0	0	0	0
Phthalate buffer	7	0	0	0	0	0	0
2% glycerol	6	0	12	37	58	58	-

A trace of dextrose added to ethanol greatly enhanced sporulation when compared to either ethanol or dextrose alone. The trace of dextrose was added since it was shown by Croes (1967b) that in Saccharomyces cerevisiae exposure to ethanol as the only carbon source did not allow optimum sporulation. It was pointed out, however, that a low concentration of glucose might stimulate activation of the respiratory enzymes. A possible explanation of my results is that the trace of dextrose stimulates activity of the acetate oxidizing enzymes and that they are involved in sporulation. It must be noted that even though logarithmic phase cells of Nadsonia were able to oxidize ethanol (Table 2) they were unable to oxidize acetate. Moreover, if this explanation is true then the rapid sporulation on glycerol must not be dependent upon acetate oxidizing enzymes.

With stationary phase cells grown in dextrose (Table 4) the extent of sporulation was generally better than with logarithmic phase cells. In contrast to results with logarithmic phase cells, a trace amount of dextrose had very little stimulatory effect on sporulation of stationary phase cells in ethanol. In the presence of dextrose sporulation of stationary phase cells was considerably greater than sporulation of logarithmic phase cells. The sporulation pattern with glycerol was not greatly changed. Sporulation did not occur in phthalate buffer in the absence of added carbon or in acetate medium.

Table 4. Sporulation of Cells Grown in Dextrose and Harvested During Stationary Phase.

Sporulation Media	pH	Percentage of Asci				
		Hours in Sporulation Media				
		12	24	36	48	60
0.5% dextrose	7	0	0	23	46	47
2% glycerol	7	0	27	62	64	64
1% sodium acetate	7	0	0	0	0	0
1% ethanol	7	0	0	12	36	44
1% ethanol and 0.005% dextrose	7	0	0	14	40	46
0.005% dextrose	7	0	0	0	0	0
Phthalate buffer	7	0	0	0	0	0

The results of sporulation of cells grown in ethanol are shown in Table 5. The most striking characteristic of cells grown in ethanol is their rapid sporulation in buffer alone not previously observed. Sporulation was greatest in distilled water. Sporulation was slightly inhibited by phthalate buffer and by the addition of a trace of dextrose. Addition of carbon sources such as dextrose, 0.5%; glycerol, 2%; or ethanol, 1% delayed sporulation. However, the sporulation pattern with glycerol was quite similar to that observed with both logarithmic phase and stationary phase dextrose grown cells. Sporulation was slightly better at pH 6 than at pH 7 in both buffer and glycerol media. There was no sporulation in acetate medium.

In order to determine the effects of amino acids in the pre-sporulation medium on sporulation, cells were grown in basal medium containing 0.5% dextrose and 0.5% casein hydrolysate. The sporulation patterns for logarithmic phase cells are shown in Table 6, and the results for stationary phase cells are shown in Table 7. In both cases a carbon source was required for sporulation. The unique characteristic of these cells was that sporulation was better in 0.5% dextrose than in 2% glycerol. This difference is most marked with stationary phase cells. As was previously observed (Tables 3 and 4), a trace of dextrose stimulated sporulation on ethanol by logarithmic phase cells to a greater extent than with stationary phase cells. In general sporulation appeared earlier and to a greater extent with stationary phase cells compared to logarithmic phase cells following growth in a complex medium.

Table 5. Sporulation of Cells Grown in Ethanol and Harvested During Log Phase.

Sporulation Media	pH	Percentage of Asci				
		Hours in Sporulation Media				
		12	24	36	48	60
0.5% dextrose	7	0	0	1	32	40
2% glycerol	7	0	19	46	56	57
1% sodium acetate	7	0	0	0	0	0
1% ethanol	7	0	0	20	34	38
1% ethanol and 0.005% dextrose	7	0	0	18	33	38
0.005% dextrose	7	11	30	36	37	-
Distilled water	-	35	71	71	-	-
Phthalate buffer	7	16	43	43	-	-
Phthalate buffer	6	23	48	48	-	-
2% glycerol	6	0	38	53	56	-

Table 6. Sporulation of Cells Grown in Complex Medium and Harvested During Log Phase.*

Sporulation Media	pH	Percentage of Asci				
		Hours in Sporulation Media				
		12	24	36	48	60
0.5% dextrose	7	0	0	28	36	38
2% glycerol	7	0	0	18	30	30
1% sodium acetate	7	0	0	0	0	0
1% ethanol	7	0	0	8	23	34
1% ethanol and 0.005% dextrose	7	0	0	27	35	35
0.005% dextrose	7	0	0	0	0	0
Phthalate buffer	7	0	0	0	0	0

*Complex medium contained 0.5% dextrose and 0.5% casein hydrolysate in addition to the basal growth medium.

Table 7. Sporulation of Cells Grown in Complex Medium and Harvested During Stationary Phase.*

Sporulation Media	pH	Percentage of Asci				
		Hours in Sporulation Media				
		12	24	36	48	60
0.5% dextrose	7	0	19	59	60	60
2% glycerol	7	0	6	29	36	36
1% sodium acetate	7	0	0	0	0	0
1% ethanol	7	0	2	23	32	32
1% ethanol and 0.005% dextrose	7	0	5	27	35	35
0.005% dextrose	7	0	0	0	0	0
Phthalate buffer	7	0	4	4	4	-

*Complex medium contained 0.5% dextrose and 0.5% casein hydrolysate in addition to the basal growth medium.

The data in Table 8 summarizes data presented in Tables 3, 4, 5, 6, and 7. Maximum percentages of asci observed within 60 hours exposure of cells to sporulation conditions are shown. Sporulation of logarithmic phase dextrose grown cells in 0.5% dextrose was significantly less than sporulation of dextrose grown stationary phase cells in the same sporulation medium.

Logarithmic phase dextrose grown cells sporulate best in glycerol and ethanol plus a trace of dextrose. They sporulate to a lesser degree in dextrose or ethanol. It is interesting to note that the presence of 0.005% dextrose markedly stimulates sporulation of these cells in ethanol. With the exception of logarithmic phase dextrose grown cells, ethanol supports good sporulation of cells grown under other conditions.

Ethanol grown cells sporulated under all conditions tested with the exception of acetate. Indeed, sporulation was observed to occur in distilled water and buffer alone.

It was expected that the maximum percentages of sporulation would be greatest with cells grown in complex medium since the availability of preformed small molecules (amino acids) would allow more energy to be stored in polymers such as glycogen or fat and then be utilized during sporulation. However, this was not generally true. Sporulation did, however, appear to progress at a greater rate with cells grown in complex medium than with dextrose grown cells in sporulation media containing dextrose or ethanol.

Table 8. Maximum Sporulation Observed within 60 Hours Using Various Presporulation and Sporulation Conditions.*

Sporulation Conditions	Presporulation Conditions**				
	Dextrose		Ethanol	Complex	
	log	stat	log	log	stat
0.5% dextrose pH 7	19	47	40	38	60
2% glycerol pH 7	49	64	57	30	36
1% sodium acetate pH 7	0	0	0	0	0
1% ethanol pH 7	5	44	38	34	32
1% ethanol and 0.005% dextrose pH 7	38	46	38	35	35
0.005% dextrose pH 7	0	0	37	0	0
Phthalate buffer pH 7	0	0	43	0	4
Phthalate buffer pH 6	-	-	48	-	-
2% glycerol pH 6	58	-	56	-	-
Distilled water	-	-	71	-	-

*Cumulative data from previous tables.

**The abbreviation "log" is used to designate cells harvested during logarithmic phase of growth. The abbreviation "stat" is used to designate cells harvested from growth during the stationary phase.

DISCUSSION

It can readily be seen that sporulation patterns depend in large part on presporulation conditions. In general, cells harvested during the stationary phase sporulate better than cells harvested during the logarithmic phase, and cells grown in ethanol sporulate best of all. This may be due to increasing ability to oxidize acetate. However, a strong correlation between respiratory quotients and sporulation was not found. While the respiratory quotient for glycerol was low, sporulation on glycerol was rapid and abundant.

Stimulation of sporulation by a trace of dextrose with ethanol appears to be related to physiological age. This stimulation was greater with logarithmic phase cells than with stationary phase cells. It is likely that the trace of dextrose stimulated respiratory activity since logarithmic phase cells harvested from a medium containing dextrose were unable to oxidize acetate. Stationary phase cells, which were able to oxidize acetate, were exposed to a low concentration of dextrose as the dextrose was depleted from the medium.

The unavoidable question becomes: What is the role of the acetate oxidizing enzymes in sporulation? Three possibilities are: (1) they play no role at all, (2) they are necessary for utilization of a carbon source for energy and metabolites for sporogenesis, or (3) they are synthesized incidentally as sporogenesis progresses.

From the discussion above it would appear that the first proposal is unlikely even though sporulation did not occur in media containing acetate.

To further evaluate these proposals it seems important to consider the time lag between placing the cells in sporulation media and the appearance of asci. This time lag is considerable when dextrose or ethanol is used as a carbon source in the sporulation media. Perhaps the time is required for the acetate oxidizing system to become active. If so, then the short time lag when glycerol is used would indicate that the utilization of glycerol for sporulation does not depend upon an active acetate oxidizing system. This rationale would not necessarily preclude the second proposal. The necessity of the acetate oxidizing enzymes for sporulation may depend upon the carbon source available.

Rapid sporulation in the absence of a carbon source by cells grown in ethanol would not preclude either the second or third proposals. These cells did carry the acetate oxidizing system. The energy and metabolites for sporogenesis were apparently supplied from within the cells. Sporulation of ethanol grown cells was inhibited by the addition of carbon sources to the sporulation media; however, the resulting sporulation patterns are not greatly different from those of stationary phase cells grown in dextrose.

It thus appears that yeast cells are conditioned for sporulation by increased respiratory activity as dextrose concentrations are reduced and two carbon compounds are metabolized.

Sporulation of Nadsonia fulvescens appears to differ physiologically from sporulation of Saccharomyces cerevisiae in two major ways. The rapid and abundant sporulation in distilled water that has been shown to occur with Nadsonia fulvescens has not been reported for Saccharomyces cerevisiae. Moreover, while acetate sporulation medium is widely used for Saccharomyces cerevisiae, acetate is not used as a carbon source for sporulation by Nadsonia fulvescens.

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