

## CHAPTER 18. The Physiology of Reproduction: General Role of Nutrition

In previous chapters we have set down, with some discrimination, the contributions to the record of structural aspects of morphogenesis in the water molds. Only in passing have we touched upon the factors that trigger or modify the genetically dictated form these fungi may take. This and the following three chapters review the literature dealing with external and internal factors that influence reproductive morphogenetic expression in the Saprolegniaceae.

Experimental exploration of the physiology of reproduction in the family began with the efforts of Klebs (1896, 1899, 1900). One principle -- or dictum as Kauffman (1926) would have it -- to emerge from Klebs' (1896) thinking is a concept now taken for granted: living organisms are influenced by their structure, by the interaction among their parts, and by their external environment. Put another way (in light of later developing biological concepts) the potential form that an organism can express is controlled genetically but manipulated by internal and external forces. Underlying Klebs' concept of reproduction is the idea that every species has certain potentialities leading toward its own unique nature. The particular chemical or physical conditions prevailing at any given time will dictate the form that species will assume (Pieters, 1915a; Kauffman, 1926). If this Klebsian concept is correct, a given species should express itself morphologically in precisely the same way each time it encounters a specific set of external and internal conditions, barring, of course, any genetic change. A second species subjected to these very same conditions would react in some fashion contrary to the first, and the differences would be discernible in the physical form of the two organisms. It follows that no two species of water mold should react in the same way to uniform conditions. Klebs (1899) pursued this view by experimenting with the influence of nutrition on the nature of reproduction in *Saprolegnia mixta* (= *ferax*). Thus, he established a foundation for the study of reproductive morphogenesis, but left a legacy of little if any analysis.

In the study on *Saprolegnia mixta*, Klebs' aim was to determine what chemical factors in the nutrient environment (and to a lesser extent what physical factors of incubation) incited or altered the various modes of reproduction that this species could exhibit. He presumably worked with bacteria-free cultures, propagating the fungus in a wide variety of chemical and natural compounds: peptone, gelatin, hemoglobin, asparagine, glutamine, oxalic and acetic acids, alkaloids, and various sugars and inorganic salts, among others. Pea broth was the basal medium, and even though Klebs added known quantities of test substances to it, the culture fluid was still chemically undefined. The discrepancies later coming to light when investigators repeated his experiments certainly can be traced in part to his use of pea broth and other undefined additives such as peptone and meat extract.

Klebs discovered that particular organic and inorganic compounds favored sexual or asexual reproduction while others did not. Phosphates (among other minerals) elicited a strong sexual response in *Saprolegnia mixta*, as did hemoglobin and

leucine. Some inorganic salts such as NaCl and KNO<sub>3</sub> in certain concentrations, favored gemma production. Colonies of the fungus grown in water with fibrin added produced sporangia and oogonia simultaneously. Spore formation, Klebs found, was suppressed by ammonium carbonate in the medium, but if a weak acid was added, the inhibitory effect was cancelled. A 5% concentration of sucrose also prevented spore formation by *S. mixta*. While light had no noticeable effect on reproduction by this species, temperature was an influential factor. For sporangium production in *S. mixta* the optimum temperature range was 24-28 °C, and the maximum 32-33 °C. Oogonia were formed at 26-27 °C but the optimum temperature for sexual reproduction (judged by oospore production) was 21-23 °C, a lower range than that for sporangium development (Klebs, 1900). Later work by others was to show that for some species at least, temperatures lower than Klebs reported are more favorable to sporangium formation.

Eventually, studies by Pieters, Kauffman, and others extended Klebs' work substantially, and to some extent refined it. For the most part, however, his general views were substantiated. Klebs concluded that the production of sporangia by *Saprolegnia mixta* made different "demands" on the protoplasm than did the formation of oogonia. By varying the kind and concentration of constituents in the medium, it was possible to induce hyphae to grow vegetatively, to form oogonia alone, or to produce both oogonia and antheridia. Moreover, if the fungus was propagated in a situation where motile spore production was impossible (as, for instance, on a semisolid medium) the sexual apparatus developed.

The main principle to emerge from Klebs' research (1899), however, dealt with the morphogenetic shift of a fungus *in vitro* from vegetative growth to reproduction. Colonies propagated in a rich nutrient medium would remain vegetative essentially indefinitely if the nutrients were renewed. If these same well-nourished colonies were transferred from the nutrient medium to water they would sporulate in a few days. Furthermore, Klebs discovered, if the sporulating mycelium then was left in water that was not changed, it would develop the sexual apparatus. Hyphae provided with ample nutrients also would produce oogonia if transferred to an agar medium. It is in part from Klebs' report of 1899 that the practice arose of starving watermold mycelium (by transferring it to water from a nutrient medium) to induce the reproductive phase. However, as Hawker (1956) so cogently pointed out, "fruiting" is not always induced by total starvation, whereas it is often favored by transferring well-nourished mycelium to a dilute medium. In any event, the idea of inducing sporulation by transferring hyphae from a nutrient solution to water has gained favor, and certainly is to some extent effective with, for instance, species of *Aphanomyces*. One notion to emerge from the work of Klebs was that the conditions required to advance vegetative growth were less stringent than those needed to promote reproduction. Vegetative vigor, then, could be considered "antagonistic", he thought, to sexuality.

A spate of papers reporting research along the lines initiated by Klebs shortly thereafter made their appearance. The common denominator in all the subsequent studies was the utilization by various watermolds of a wide variety of natural extracts,

compounds, or substrates for their possible effects on reproduction. Coincident with Klebs' papers (but in one instance predating them) were reports published by Maurizio (1896a, 1899) on the effect of various nutrients on the growth and reproduction of water molds from fish and fish eggs. Maurizio succeeded in inducing oogonium formation in some cases by growing species on such substrates as mealworm decoctions, meat extract, and egg albumin. The production of sporangia, he noted, was bound to the nutrient level of the culture: as long as the nutrients lasted the fungus "needed nothing", and continued to develop. As the food supply decreased, however, oogonia appeared. Maurizio reckoned that most of the saprolegniaceous fungi were adapted to heterogeneous nutrients, and therefore to a saprophytic (saprotrophic) existence. Like Klebs, Maurizio (1899) also experimented briefly with aspects of culture other than nutrition. While the water mold found on fish survived immersion in ice, he reported, low temperatures clearly prevented sporulation. A steady temperature of 38.5-39.5 °C applied to the mycelium for 90 seconds hindered reproduction, and at 40 °C the colonies were killed in 2-3 minutes. Maurizio thought it not impossible that such temperature "sterilization" could be applied to fish culture practices. Reduced light, he found, had no influence on reproduction by the species he examined.

Among the first and most extensive analyses of reproduction in water molds, patterned after Klebs' experiments, was that by Horn (1904). He used cultures of *Achlya polyandra* de Bary (= *debaryana*) primarily, and sought to uncover structural variations in the species. Horn, too, experimented with natural-product substrates such as pea broth and plum extract, and also tested various concentrations of mineral elements and weak acid and alkaline solutions for their influence on morphology of the *Achlya*. Among the modifications induced in sporangia of *A. polyandra* on mycelium grown in weak acids or in solutions of metal salts were the production of lateral exit pores, reversion to vegetative growth, and endogenous clumping of the protoplasts. Aqueous mixtures of peptone with KNO<sub>3</sub> or NaCl within certain concentration limits supported sporulation, but sucrose or glucose broths in concentrations above 0.9% inhibited this event. Using lecithin, for example, in combination with various mineral salts and weak ionic solutions, Horn found that the morphology of the sexual apparatus in *A. polyandra* was modified chiefly in three ways. Oogonia of the fungus were unpitted if the mycelium was grown in water, but abundantly pitted when the medium contained peptone with glucose or sucrose. Leucine in the culture broth promoted the elongation of oogonial stalks, and leucine or peptone plus MgSO<sub>4</sub> favored androgynous antheridial branch production. In a peptone-leucine solution with this same magnesium salt, oogonium formation was stimulated. Coker (1909) also demonstrated that calcium nitrate (in a hemoglobin solution) was necessary for oogonium initiation and development by *Leptolegnia caudata*.

Coker (1923) experimented at great length with the effects on water mold reproduction of natural substrates (egg yolk or peptone, for instance) fortified with salts of calcium, potassium, and sodium in particular. The rather extensive data he assembled on several species in the family appear to confirm the chief conclusions to emerge from Klebs' work. Reproduction by various isolates, Coker found, was sparse

or absent in solutions of mineral salts plus 5% maltose and 0.1% peptone, the richness of these concoctions favoring vegetative growth. Mineral salts alone in 0.1% concentrations generally supported gemma-forming mycelium of *S. mixta*. Solutions of mineral salts in distilled water with egg yolk favored oogonium-producing mycelium in some water molds, suggesting that the Klebsian premise -- low nutrient concentrations favor reproduction -- was correct.

Some experimental work that most nearly duplicated that published by Klebs was reported by Kauffman in 1908. Aside from using dead flies, wasps, and bits of dried beef as substrates, Kauffman concocted a number of nutrient solutions in which to grow his water molds. In general, these broths consisted of mineral salts (potassium, calcium, sodium, ammonium, and zinc) in combination with hemoglobin, peptone, or leucine. He characterized the isolates (chiefly *Saprolegnia hypogyna* and *S. mixta*) in the various media as to degree and kind of reproduction. In mixtures of potassium, sodium, and calcium salts with hemoglobin, antheridial branches were induced in *S. hypogyna*, a species thought to be singularly free of such structures. Of the various concoctions Kauffman tried, potassium phosphate or disodium phosphate plus hemoglobin were most effective in this regard. Klebs (1899) had found these same metal salts to stimulate antheridial hyphae in *S. mixta*, but the phosphate salt of calcium did not (Kauffman's later findings were contradictory with respect to calcium). Since both androgynous (monoclinous?) and diclinous antheridial branches were inductively formed in *S. hypogyna*, Kauffman concluded that branch origin was of no diagnostic importance in speciation. Other solutions that Kauffman tried had various effects such as inducing large pits in the oogonia developed by mycelium propagated in hemoglobin and peptone, and the appearance of multiple septations in oogonial stalks on hyphae from colonies grown in a calcium phosphate solution.

The parallels between the experimental work of Pieters (1915a, c) and that of Klebs and Kauffman are many. Pieters (1915a, c), like Klebs, did not concern himself with chemically defined media but only with uncovering the effects of culture adjuvants. Pieters (1915a), however, added a dimension to the study of nutrients and water mold reproduction that had not been applied up to that time, namely, an attempt to quantify results and to standardize the amount of inoculum. Although he referred to his various media as synthetic ones, they in fact contained peptone; by synthetic he simply meant that he did not use flies or grubs in the cultures as was common practice.

Pieters (1915a-c) experimented with four species: *Saprolegnia ferax*, *S. monoica* (=ferax), *Achlya racemosa* and *A. prolifera*. The basic media contained 0.01-2.0% peptone, and various salts and carbohydrates in concentrations of 0.1-1.0%. Three principal conclusions emerge from Pieters' experiments (1915a). First -- except in the case of *A. prolifera* -- fructose (then known as levulose) was the most effective sugar for supporting mycelium leading to oogonium production, and in combination with hemoglobin, for stimulating antheridial hyphae to form. Maltose had a parallel though not as effective an influence; sucrose was not used by the fungi. Second, phosphates in the media favored oogonium development as evidenced by the fact that the increased weight of mycelium production in media with phosphates was proportionately much less than

the increased stimulation of oogonia. Not all the species tested, however, responded in this fashion. Thirdly, Pieters found that there was a definite minimum nutrient concentration necessary for the full development of the water molds he tested. Increasing the concentration above a given level did not necessarily promote reproductive vigor. Mycelium in a nutrient-deficient solution, Pieters concluded, would show poor vegetative vigor, but would produce more and larger oogonia than well-nourished hyphae.

Pieters (1915b) noted that oogonium configuration in *Saprolegnia monoica* was considerably modified by the concentration of hemoglobin in combination with certain hexoses in the medium. Solutions of hemoglobin plus fructose supported mycelium which produced more oogonia with attendant antheridial branches than did hemoglobin alone or in combination with glucose.

In what was an apparent contradiction to earlier observations of others, Pieters (1915c) found that sporangia were formed by some species of water molds grown in pea broth. Such a rich nutrient medium should not support sporangial development if Klebs' hypothesis was correct. Pieters put isolates in pea broth then subjected some cultures to vibration while leaving others stationary. The agitated mycelium produced no sporangia, but they were present on hyphae in the stationary vessels. In explanation, Pieters advanced the notion that the hyphae in stationary culture were effectively starving because fresh medium was not being brought to them; accordingly, the fungus produced sporangia. He of course ignored what some predecessors had called attention to, namely, that sporangia of water molds usually were produced more abundantly by aerial hyphae or by those lying on the surface of water than by the submerged filaments. It was assumed this response was too high versus low levels of oxygen. In any case, Pieters saw no contradiction to the principle Klebs had established, namely, since pea broth was not readily available to the hyphae, it was a starvation medium, and sporangium production was thereby favored.

Chaze (1925) isolated a nonsexual *Saprolegnia* from fish, and propagated the fungus on a variety of natural substrates, some prepared as broths or extracts, others being used as solid or semisolid media. The *Saprolegnia* did not sporulate on such materials as bread, fish bouillon, wort, carrots, rice, potatoes, or decoctions of *Glycine max* (L.) Merr. Only by starving the fungus could Chaze induce sporulation by the mycelium, thus supporting Klebs' view. Much later, Fowles (1976) was to use a variety of natural materials in attempts to induce reproduction in species of *Aphanomyces*. Substrates such as snakeskin, porcupine quills, cow horn shavings, and dry, sterilized *Drosophila* were ineffective. On the other hand, some natural product extracts in agar-base media supported oogonial development while other infusions did not.

The final most intensive study following slavishly, as it were, the Klebsian notion of the relationship between nutritional level and reproductive vigor in water molds was that reported in 1932 by Kanouse. Although as late as 1948(c) A. W. Ziegler used the classical "stand by" ingredients (leucine, hemoglobin, mineral salts) in culture media in attempts to induce antheridial branch formation in *Achlya crenulata*. Kanouse experimented with ways to induce asexual apparatus to form in *Saprolegnia parasitica*

(=*diclina*), a species notorious for being refractory toward expressing sexuality. Her approach was to control vegetative as opposed to reproductive manifestations by gradually reducing the nutrient concentration in the media in which she grew the water molds. Aside from trying the usual natural substrates used to propagate water molds -- fish eggs, whole flies (or only their legs) for example -- Kanouse also used broth cultures containing hemoglobin, leucine, peptone, various sugars, and salts of potassium, calcium, and magnesium. Peptone together with leucine, glucose, or maltose, and peptone plus glucose in agar supported oogonium-producing mycelium. When leucine was used alone, sporangium development was favored. None of the hemoglobin and salts mixtures which Kauffman (1908) had found to be supportive of sexual reproduction in *S. hypogyna* and *S. mixta* were effective for *S. parasitica*. Similarly, Kanouse found that Pieters' (1915a, b) report of the stimulating effect of hemoglobin, and phosphates on sexual reproduction in *S. monoica* did not hold true for *S. parasitica*. She concluded that the Klebsian premise was operable with this species from fish and fish eggs, and believed that the production of oogonia by *S. parasitica* in four weeks (on hempseed) was a positive response to a decreasing nutrient level.

Inevitably with the passage of time, culture techniques became much more refined than earlier mycologists would (or could) make them, and specific compounds were substituted in chemically defined media (Mullins and Warren, 1975; Yang and Schouties, 1972, among others). Investigators still looked to the Klebsian principle as the functional explanation for the shift from vegetative to reproductive phases of development in the Saprolegniaceae. Unestam (1969a), for example, raised the question of why planonts were not produced by cultures of *Aphanomyces astaci* in nutrient media. The method which Unestam employed was to submerge spores of *A. astaci* in 6% peptone broth for 20 hours, then add a glucose-mineral salts medium, and incubate the spore suspension for an additional 45 hours. The medium was then withdrawn, pond water was substituted, and at various time intervals up to five hours the culture water was replaced. Only when the medium was removed and pond water substituted did sporulation occur. Low concentrations of phosphates and metallic and mineral salts inhibited spore formation in *A. astaci*, and the magnitude of spore motility was greatly reduced in media containing fructose and sucrose. To Unestam, the experimental data he uncovered suggested that sporulation in the crayfish plague fungus could proceed in the presence of growth-inhibiting metabolites provided those inhibitors did not also block differentiation.

In another water mold, *Achlya ambisexualis*, the basic concept developed by Klebs also applied to sex cell induction, Mullins and Warren (1975) discovered. They used a variety of culture media such as water alone, Barksdale's (1970) enriched medium, and various chemically defined media in which the male strain of this dioecious species had grown prior to use. Four phases of morphogenesis were apparent in *A. ambisexualis*: vegetative growth without induction, male induction of antheridial branches, female induction of oogonia, and the physical contact of the male and female elements. In the absence of exogenous nutrients (as, for example, in water) male induction commenced about two hours after compatible cultures were mated. If the medium in which the

mating colonies were tested had a low nutrient level, male induction was consequently delayed. With an increasing level of nutrition in the "mating water," there was a correspondingly extended delay in the initiation of sex cells. Mullins and Warren (1975) interpreted these observations to support one of Klebs' main tenets, namely, that nutritional levels favorable for vegetative growth were unfavorable for reproduction.

Those reports giving more narrowly directed or more precisely refined attention to the influence on reproduction by specific factors are treated in the following chapters. While the experimentation by Klebs, Kauffman, Pieters, Kanouse, and others was crude by current standards, they did serve to direct future productive efforts in the exploration of the physiology of reproduction in the Saprolegniaceae.