STUDIES ON THE OPALINIDAE OF AUSTRALIAN FROGS

"THE LIFE CYCLE OF ZELLERIELLA BINUCLEATA (RAFF) IN THE FROG LIMNODYNASTES TASMANIENSIS GUNTER." 

by

N. N. TAIT

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CHAPTER 1.  INTRODUCTION

1.1.  TAXONOMY

The Opalinidae are a group of protozoa inhabiting the rectum of Anura. The systematics of the group has been the subject of much controversy. Metcalf's view (Metcalf, 1918), that the Opalinidae represent a primitive stage in the evolution of the Ciliata, has been discredited by more recent workers. A series of papers, culminating with that of Grassé (1952), has pointed out the affinities of the Opalinidae with the Flagellates. Grassé (op. cit.) created a new super-order Opalinina at the same level as the Protomonadina and Metamonadina, clearly indicating his view of their specialized evolution from existing flagellates. Corliss (1955) summarized the ciliate and the flagellate characters of the Opalinidae and agreed with Grasse as regards their specialized flagellate affinities. For these reasons the Order Protociliata of Metcalf (op. cit.) has been discarded.

As regards the systematics within the group, Metcalf (1920) divided the single family Opalinidae thus:
Fam. Opalinidae.

Sub-fam. Protoopalininae. (2 nuclei)
Genus Protoopalina (cylindrical)
Genus Zelleriella (flattened)

Sub-fam. Opalininae. (4 to many nuclei)
Genus Cepedea (cylindrical)
Genus Opalina (flattened)

'Opalinae angustae' (narrow)
'Opalinae latae' (broad)

In describing new species of Opalinidae, it is important to base the identification of the species on the trophont or "adult" stage, rather than the larval stages in which the number of nuclei and the body shapes may vary at different phases. Metcalf (1926) described the immature stages of a species of Opalina in Rana clamitans as follows: "The very young opalinids are long, cylindrical and have two nuclei. They are thus little 'Protoopalinae'. After several weeks they increase the number of nuclei becoming little 'Cepedae'. Later they flatten to form very broad, circular 'Opalinae latae'. Finally, after several weeks more these change into the true 'Opalinae angustae'". Metcalf (op. cit.) also described the immature stages of Opalina in R. catesbeiana tadpoles.
The young Opalina are like small 'Protoopalinae'. They flatten to become 'Zellereiellae' and finally increase their number of nuclei to become at first broad and then narrow Opalina. It is clear that descriptions of new species based on observations of an immature stage could easily give rise to errors. It is important to realise that this applies, not only to the immature stages in the tadpole, but also, as evidence presented here shows, for the various stages in the frog. Since Metcalf (1923,1940) has based many of his species of Opalinidae on preserved material, a major task would be necessary to revise his work.

Very little has been written on the Opalinidae of Australian frogs. Raff (1911, 1912) described Opalinidae from several species of frogs. These are listed in Table 1, in which the generic names have been changed to conform with Metcalf's classification. Raff (1911) described *Z. binucleata* as follows: "This is found in great numbers in *L. dorsalis* and on one occasion I met with it in *L. tasmaniensis.*" This is the only record that Raff makes of Opalinidae in *L. tasmaniensis*. Raff also described *P. intestinalis* (Raff 1911) and *P. acuta* (Raff 1912) from *L. dorsalis*. Forms similar to these have also
now been found in \textit{L. tasmaniensis}. Experimental infections (see Chapter 4) of defaunated specimens of \textit{L. tasmaniensis} with pure cultures of these forms from \textit{L. tasmaniensis} suggest that these species described by Raff are in fact different stages in the life cycle of one species. Since the name \textit{Z. binucleata} was given to the trophont stage, it is proposed that the species of Opalinidae infecting \textit{L. tasmaniensis} should keep the name \textit{Z. binucleata} and that the other names be rejected. It is not known at this stage whether this species is the same as that found in \textit{L. dorsalis}; but on morphological grounds they appear to be identical.

1.2. \textbf{LIFE HISTORY OF OPALINIDAE.}

As emphasized above, it is important that taxonomic studies on the Opalinidae should be accompanied by studies on the life history of the species concerned. There are very few complete descriptions of the life history of the Opalinidae recorded in the literature. The life history of \textit{Opalina} has been described by several workers. Mofty (1959) has given an account of the life history of \textit{Opalina ranarum in Rana temporaria}. He also summarized descriptions of the life history of this species given by several other workers. Perhaps the most thorough account of the life
history and morphology of Opalina is that given by Wessenberg (1961) who based his description mainly on Opalina virguloides from Hyla regella and O. obtrigonoidea maxima from Bufo boreas halophilus. The life histories of these species appear to be basically similar and may be summarized as follows.

During most of the year, large multinucleate trophonts are found in the rectum of the host. With the onset of the breeding period of the frog, trophonts undergo repeated divisions, without compensatory growth, to form tomonts. These encyst and pass out with the faeces. The cysts are ingested by tadpoles. In the gut they excyst and divide to form uninucleate micro- and macrogametes which fuse, giving rise to an uninucleate zygote. The zygote encysts and is voided with the faeces to infect other tadpoles in which they excyst and grow eventually to form trophonts in the newly metamorphosed frog. An extra encystment subsequent to that which gives rise to the zygocyst has also been described in tadpoles.

Sukhanova (1960) described the life history of P. canevi n. sp. in the common spadefoot toad Pelobates fuscus. The elongate trophonts occur in toads during
the non-breeding period of the year. During the spring these divide rapidly to form small uninucleate individuals which encyst and pass out in the faeces to infect tadpoles. Sukhanova (op. cit.) does not include a description of the stages between the trophonts and the small uninucleate precyst forms, nor does he give a description of the stages in the tadpole.

It is believed that no complete study of the life history of a *Zelleriella* has been made. Metcalf (1923) points out the difficulty in classifying the genus due to the superficial similarity of the different species. He states, "It is evident that to review successfully the taxonomy of the *Zelleriellae*, one should have data from the whole life cycle of each species". The only record of a *Zelleriella* in a tadpole was given by Sandon (1938). He described *Zelleriella* (africana B) from a single tadpole of *Rana fuscigula*; no indication was given of the age of the tadpole. In the present study, forms recognizable as *Zelleriella* were found only in metamorphosing tadpoles.

1.3. **OBJECT OF THE PRESENT STUDY**

The main object of the present study is the description of the life history of *Zelleriella binucleata*
(Raff) in the frog *Limnodynastes tasmaniensis* Gunter. Since virtually no work has been done on the life cycle of this genus, the present study could provide a basis for future taxonomic work on the group.
**TABLE 1.**

**OPALINIDAE IN AUSTRALIAN FROGS**

**AS DESCRIBED BY RAFF (1911, 1912).**

<table>
<thead>
<tr>
<th>FROG SPECIES</th>
<th>OPALINIDAE PRESENT</th>
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<tbody>
<tr>
<td><em>Hyla aurea</em></td>
<td><em>P. intestinalis</em> (Stern)</td>
</tr>
<tr>
<td></td>
<td><em>P. hylarum</em> (Raff)</td>
</tr>
<tr>
<td><em>H. ewingi</em></td>
<td><em>P. intestinalis</em></td>
</tr>
<tr>
<td><em>Limnodynastes dorsalis</em></td>
<td><em>P. intestinalis</em></td>
</tr>
<tr>
<td></td>
<td><em>Z. binucleata</em> (Raff)</td>
</tr>
<tr>
<td></td>
<td><em>P. dorsalis</em> (Raff)</td>
</tr>
<tr>
<td></td>
<td><em>P. acuta</em> (Raff)</td>
</tr>
<tr>
<td><em>L. tasmaniensis</em></td>
<td><em>Z. binucleata</em></td>
</tr>
<tr>
<td><em>Crinea signifera</em></td>
<td><em>P. tenuis</em> (Raff)</td>
</tr>
</tbody>
</table>
CHAPTER 2. MATERIALS AND METHODS

2.1. COLLECTION OF FROGS

*L. tasmaniensis* is one of the most common species of frogs to be found around Canberra, Australian Capital Territory. It can be readily collected under logs in the vicinity of water. The frogs become active at night, especially during rain, and often can be collected while they are crossing roads. Specimens for this study were mainly collected on a property 7 miles from Yass, New South Wales, on the shores of Lake George, 20 miles from Canberra, and at Wologorang and Rose Lagoons, 9 and 6 miles from Collector, New South Wales, respectively. The other species of frogs were also collected in these localities.

2.2. METHOD OF OBTAINING QUANTITATIVE COUNTS OF *Z. binucleata*.

The life history studies were made by collecting 20 frogs each month during the year extending from May 1962 to May 1963. The frogs were anaesthetized in ether and the rectum was dissected. The rectal contents were placed in 1 ml. of frog Ringer. The protozoa were killed by adding a drop of Schaudinn's fluid. Total numbers of *Z. binucleata* and the percentages of the different forms
present were determined by using a Fuchs Rosenthal haemocytometer.

2.3. **REPRODUCTIVE STATE OF FROGS**

The reproductive state of the female frogs was determined by noting the size of the ovaries and the development of the oviducts. Testes were preserved in Bouin's fluid, sectioned at 10μ and stained with Mayer's haemalum.

2.4 **DEFAUNATION OF FROGS**

In order to obtain hosts free of Opalinidae, frogs were treated with achromycin (tetracycline hydrochloride) in the following manner. 0.5 ml. of a 10mg/ml. solution of achromycin in frog Ringer was injected into the rectum of each frog by means of a fine cannula tube. This was repeated daily for three days. The frogs were then examined by drawing off a sample of rectal contents by means of a cannula tube attached to a mouth suction tube. The frogs were then left for one week to allow the effects of the achromycin to wear off. They were again examined before the experiment was begun. At all times frogs were kept in individual containers free from contamination with opalinids.
2.5. **METHOD OF OBTAINING OPALINID-FREE TADPOLES**

Opalinid-free tadpoles could easily be obtained by collecting eggs before they hatched, washing them thoroughly and placing them in a culture dish containing clean water.

2.6. **SEROLOGICAL METHODS.**

Immune rabbit serum was obtained by injecting 1 ml. of a suspension of *Z. binucleata* in mammalian Ringer into the ear vein. This was repeated twice weekly for four weeks. The suspension of protozoa was made by filtering the contents of several recta through cheesecloth. The number of *Z. binucleata* in each ml. was counted using the Fuchs Rosenthal haemocytometer. In this way a total injection of approximately 60,000 individuals was given. The rabbit was bled one week after the last injection. The serum was collected and frozen.

Blood from frogs was obtained by making a cut across the back of the neck with a scalpel and collecting the blood in a Pasteur pipette.
CHAPTER 3. LIFE CYCLE OF Z. BINUCLEATA

The life cycle of Z. binucleata has been determined by examination of frogs and tadpoles throughout the year and by experimental infections of defaunated frogs as described in Chapter 4. Fig. 1. is a semi-diagrammatic representation of the life cycle. Wessenberg (1961), in describing the life cycle of Opalina virguloidea and O. obtrigonoidea maxima, used the terms, as first coined by Chatton and Lwoff (1935), trophont for the adult stage and tomont for the divided forms. These terms have been used in the present study. Several morphologically distinct forms of Z. binucleata have been found in the rectum of L. tasmaniensis and it was only after a prolonged series of observations that the relationship between them became clear. The position appears to be as follows.

The adult trophont is a rounded form, flat in cross section, from which the species gets its generic name (fig. 2,A). It occurs in metamorphosing frogs. Wessenberg (op. cit.) states: "Trophonts are found in the rectum of infected hosts while the host is undergoing metamorphosis and typically some are present there during the remainder of the host's existence." In Z. binucleata
this stage is only transitory. Of all the frogs examined, only 16% were infected with this stage. The adult trophont is the largest stage in the life cycle. Large specimens measure on the average 160μ in a straight line through the two nuclei. The cilia occur in longitudinal rows over the whole surface of the body. When removed from the rectum into frog Ringer, they move in a straight line in a horizontal position, occasionally rolling over onto the opposite side. The adult trophonts divide longitudinally giving rise to smaller forms, which are oval but still flattened in cross section (fig. 7,B.C.). These are transitional between the adult trophont and the elongate trophont, which is rounded in cross section (fig. 2,B.). These last forms are typical 'Protoopalinid' types, but from infection experiments (see Chapter 4) have been shown to be a stage in the life cycle of *Z. binucleata*. They represent a more permanent stage in the life cycle occurring in approximately 50% of all frogs examined. Specimens average approximately 550μ in length and 50μ in width. The body is gently spiral in form and is widest at the anterior end, gradually tapering posteriorly. They move with the widest end forwards in a straight spiral motion.
Cilia cover the entire body surface (fig. 2,D.). The two nuclei are situated in the anterior half of the body. Both longitudinal and horizontal divisions have been observed. Prior to longitudinal division the anterior end becomes expanded (fig. 6,A.). A furrow then begins to form from the anterior end and at the same time the nuclei begin to divide synchronously. From several observations it was noted that the products of division of one nucleus pass into separate daughters since, in many cases, observations of specimens with a single dividing nucleus were made (fig. 6,B.). Several specimens with only one nucleus have also been observed. These presumably have been the result of division which has preceded nuclear division. Thus, in many cases, division in *Z. binucleata* results in the formation of individuals with identical nuclei.

Towards the onset of sexual maturity the elongate trophonts divide to form tomonts. Several stages in the division of the tomonts are recognisable. Large tomonts, the result of division of the elongate trophonts, measure on the average 150u in length and 27u in width. They resemble the elongate trophonts except for their smaller size and non-ciliated tail (fig. 2.C.). As these forms
become smaller the body becomes bent in a crescent shape. Finally, forms with the anterior end bent at right angles to the rest of the body are produced. These last forms measure approximately 90μ in length including a very elongate non-ciliated tail (fig. 2,F.). Typically, the tomonts have two nuclei, although uninucleate forms are occasionally found. These presumably have arisen by division of the body without accompanying nuclear division. At the peak of sexual maturity of the host the small tomonts divide to form small uninucleate precystic forms (fig. 2,G.) which are elongate and possess a short posterior pointed tail. The sequence of events leading up to cyst formation appears to be as follows (fig. 3). The elongate, precystic form begins to contract from both the anterior and posterior ends to form a swelling in the region of the nucleus. This process finally results in the production of a drop-shaped form which encysts and passes out with the faeces. The cysts are ingested by tadpoles. Excystment occurs in the tadpole gut. Some information was gained on the process of excystment by gently squeezing cysts between a glass slide and a cover slip. In many cases a specific region of the cyst wall broke away releasing part of the encysted opalinid (fig. 4,A.). Examination of young tadpoles at various
intervals after infection with cysts showed active forms in the tadpole after about 12 hours. These were uninucleate elongate forms (fig. 4,B.). In the tadpole gut the encysted opalinids revolve rapidly within the cyst. Thus the process of excystment may be due to elongation and active movement of the opalinids with the result that a weak region of the cyst wall is broken down. Since gametes have not been observed in the adult frog it would seem that further changes have to occur in the newly excysted gamont to produce the micro- and macro-gametes found in the tadpole 24 hours after infection. Unfortunately the course of these changes has not been observed in *Z. binucleata*. In *Opalina* the newly excysted gamont contains a small varying number of nuclei. The gamont divides to produce binucleate and finally uninucleate gamonts. The latter forms divide to form either micro- or macrogametes. Wessenberg (1961) suggests that meiosis occurs at this point so that each uninucleate gamont would produce 4 gametes. Since the newly excysted gamonts of *Z. binucleata* are uninucleate, it is probable that one meiotic division results in the production of gametes. The process of syngamy has been observed on several occasions and is shown in fig. 5. The macro- and microgametes
are of approximately equal length. However the microgametes are more narrow and are characterised by their sparse ciliation. The microgamete becomes attached by its posterior end to the anterior end of the macrogamete. This attachment is at first very loose, but as fusion proceeds the attachment becomes more firm. Finally complete fusion occurs, resulting in the binucleate zygote (fig. 4.C.). Wessenberg (1961) states that after syngamy the nuclei fuse resulting in a uninucleate zygote. Uninucleate forms of *Z. binucleata* were found together with binucleate forms 24 hours after infection. However, it is not certain whether these are zygotes or newly excysted gamonts, since both these forms have only one nucleus. For *Z. binucleata* to return to the diploid state, (Chen, 1936, 1948, has shown that the trophonts of *Zelleriella* are diploid) it would seem that fusion of the two nuclei of the gametes would have to take place, providing of course that meiosis had occurred previously. Encystment of the zygote has been described in *Opalina*. Here again the single nucleus of the zygocyst and infection cyst would tend to make these indistinguishable in *Z. binucleata*.

In older tadpoles the typical form present is an elongate protrophi (fig. 4,D.). This increases in size
and finally at metamorphosis becomes flattened to form the flattened protrophont (fig. 4,E.). Cyst formation also occurs in large tadpoles by rapid division of the protrophont, to produce uninucleate, elongate and later ovoid precystic forms. The latter encyst and are passed out with the faeces to infect other tadpoles. This results in the presence of all stages of cyst formation as well as gametes and zygotes in the same tadpole.

After cyst formation in the frog the remainder of the tomonts reverse their life cycle so that elongate trophonts are rapidly formed. Later these elongate trophonts become flattened and divide longitudinally and horizontally (fig. 6,C.) to form small flattened trophonts which grow to produce the typical flattened trophont present in metamorphosing frogs.
FIG. 1. SEMIDIAGRAMMATIC REPRESENTATION OF THE LIFE CYCLE OF *Z. BINUCLEATA* IN THE FROG *L. TASMANIENSIS*

1. Adult trophont.
2. Elongate trophont.
3. Large tomonts.
4. Small tomonts.
5. Uninucleate precystic form.
6. Uninucleate precystic form.
7. Infection cyst.
8. Infection cyst.
10. Syngamy.
12. Elongate protrophonts.
13. Flattened protrophonts.
15. Large tomont.
17. Small adult trophonts.
18. Adult trophont.
FIG. 2. STAGES IN THE LIFE CYCLE OF Z. BINUCLEATA IN THE ADULT L. TASMANIENSIS

A. Adult trophont.  
(Scale = 50u)

B. Elongate trophont.  
(Scale = 100u)

C. Large tomont.  
(Scale = 50u)
FIG. 2. STAGES IN THE LIFE CYCLE OF Z. BINUCLEATA IN THE ADULT L. TASMANIENSIS (CONTINUED)

D. Posterior end of elongate trophont to show rounded tip and ciliation.
   (Scale = 25u)

E. Posterior end of large tomont to show pointed tip and ciliation.
   (Scale = 10u)
FIG. 2. STAGES IN THE LIFE CYCLE OF *Z. BINUCLEATA* IN THE ADULT *L. TASMANIENSIS* (CONTINUED)

F. Small tomont.
   (Scale = 25u)

G. Uninucleate elongate precystic form.
   (Scale = 25u)

H. Infection cysts
   (Scale = 50u)
FIG. 3. STAGES IN CYST FORMATION OF Z. BINUCLEATA

A. Small tomont.
B. Elongate, binucleate precystic form.
C. Uninucleate, elongate precystic form.
D. Contraction of precystic form.
E. Oval precystic form.
F. Infection cyst.
FIG. 4. LIFE CYCLE OF Z. BINUCLEATA IN THE TADPOLE OF L. TASMANIENSIS

A. Squash preparation of an infection cyst to show emerging gamont.
   (Scale = 10u)

B. Newly excysted gamont
   (Scale = 10u)

C. Binucleate Zygote.
   (Scale = 10u)
FIG. 4. LIFE CYCLE OF Z. BINUCLEATA IN THE TADPOLE OF L. TASMANIENSIS (CONTINUED)

D. Elongate protrophont from large tadpole. (Scale = 50u)

E. Flattened protrophont from metamorphosing frog. (Scale = 50u)
FIG. 5. STAGES IN SYNGAMY

A. Attachment of microgamete to Macrogamete.

B. Fusion of microgamete to macrogamete.

C. Zygote.
FIG. 6.  ASEXUAL DIVISION IN THE ELONGATE TROPHONT

A. Dividing trophont. Note wide anterior edge, division groove and synchronously dividing nuclei.
   (Scale = 100u)

B. Daughter trophont. Note nucleus in telophase.
   (Scale = 100u)

C. Horizontal division of trophont.
   (Scale = 100u)
CHAPTER 4. INFECTION OF DEFAUNATED FROGS WITH VARIOUS STAGES IN THE LIFE CYCLE OF Z. BINUCLEATA.

These experiments were carried out in order to determine whether the various forms of Opalinidae found in _L. tasmaniensis_ were stages in the life history of one species or whether they represented individuals of different species, as described by Raff (1911, 1912).

Frogs were defaunated as described on page 10. Frogs in which only one stage was present were used as donors. 6 frogs were infected with adult trophonts on 18th June, 1963. Examination on the 20th June, 1963 showed three frogs infected with adult trophonts and forms mid-way between adult trophonts and elongate trophonts. By the 23rd June, 1963 the forms present had become more elongate. On the 30th June, 1963, all the forms present were of the typical elongate trophont stage. Photographs of these stages are shown in fig. 7. This experiment shows that _Z. binucleata_ passes through a 'Protoopalina' stage in the frog as well as in the tadpole.

Six frogs were infected with small trophonts on the 3rd August, 1963. These were examined on the 5th August, 1963. Three of the frogs contained large tomonts. By the 12th August, 1963, many elongate trophonts were present.
Photographs of these stages are shown in fig. 8. This experiment shows that the life cycle of *Z. binucleata* is reversible. This occurs in natural infections after cyst formation.

These results confirm observations on natural infections in which intermediates between the several well-defined morphological stages in the life cycle of *Z. binucleata* have been observed.

More work along the lines of Cleveland (1955) working on the flagellates of *Cryptocerous* could be carried out to determine whether these various stages can survive in frogs at various states of reproductive maturity. The low infection rate found would tend to suggest that this is not the case.
FIG. 7. INFECTION OF DEFAUNATED FROGS WITH ADULT TROPHONTS

A. Adult trophont used to infect frogs on 18vi 63.
   (Scale = 100u)

B. Divided forms found on examination on 20vi 63.
   (Scale = 50u)
C. Elongate form found on examination on 23vi 63.
   (Scale = 50u)

D. Typical elongate trophont found on 30vi 63.
   (Scale = 100u)
A. Small tomont used to infect frogs on 3viii 63.
   (Scale = 50u)

B. Large tomonts found on 5viii 63.
   (Scale = 25u)

C. Elongate trophonts found on 12viii 63.
   (Scale = 100u)
CHAPTER 5. DISTRIBUTION OF Z. BINUCLEATA IN THE RECTUM OF THE HOST.

Many workers have noted that the Opalinidae are found in greater numbers in the anterior part of the rectum. Wessenberg (1961) states that trophonts of Opalina are found between the faecal mass and the mucosa. Tomonts enter the faecal mass where encystation occurs.

Observations on the distribution of Z. binucleata in the rectum were made by dissecting the rectum from freshly killed frogs. The rectum was then bissected and the two halves placed in 0.5 ml. of frog Ringer. A sample of each was then taken and the percentage and number of each form counted using the Fuchs Rosenthal haemocytometer.

In immature frogs very few opalinids were found in the posterior half of the rectum. Of these most were small tomonts. However, in mature frogs large numbers of small tomonts and cysts were found in the posterior half of the rectum.

Examination of freshly passed stools of immature frogs showed, in most cases, very few opalinids. Where they did occur they were at the small tomont stage. No trophonts were found in the faeces. Examination of the faeces of mature frogs revealed in many cases large numbers
of small tomonts and cysts. From these results it can be concluded that in immature frogs very few opalinids are present in the posterior half of the rectum. However, at the onset of sexual maturity the small tomonts enter the faecal mass. Here they form the precystic stage and encystment takes place. The cysts, together with large numbers of small tomonts and precystic forms, are then voided with the faeces.

Sections of recta were made to determine the intimacy of the protozoan to its host. Material was fixed in liquid air and the freeze drying technique, using an Edward's Tissue Dryer, was used. Sections revealed trophonts closely applied to the mucosa (fig. 15, A.). Active secretion of mucus occurs in the cells of the rectal mucosa which may provide nourishment for the protozoa. Sections of the recta of sexually mature frogs revealed large numbers of small tomonts in the faecal mass.
Many workers have correlated the life cycle of the Opalinidae with the sexual cycle of the host, (e.g. Bieniarz, 1950, Mofty and Smyth, 1960, McConnachie, 1960, and Wessenberg, 1961. The host species examined by these workers had restricted breeding periods so that a strong correlation could be made between cyst formation in the opalinids and the breeding period of the host. *L.tasmaniensis*, on the other hand, has an extended breeding period. In the vicinity of Canberra, the breeding period extends from August to May. Thus a correlation between the life cycle of *Z. binucleata* and the sexual cycle of *L.tasmaniensis* has to be made largely on individual frogs, since frogs of all ages and states of sexual maturity are found throughout the year.

The reproductive state of female frogs could easily be determined by examination of the size of the ovaries and the development of the oviducts. Female frogs could thus be classified on this basis into the following categories:

- **Ovaries very immature ............... stage 1.**
- **Ovaries beginning to develop, oviducts undeveloped ...................... stage 2**
Ovaries and oviducts well developed ... stage 3.
Ovaries undeveloped, oviducts well developed ...
........... stage 4.

Table 2 represents the average percentage of the different stages of the life cycle of *Z. binucleata* present at corresponding stages in the sexual development of the host. Thus, maximum cyst formation occurred in female frogs in which the ovaries and oviducts were well developed. However, only 38% of all frogs examined in this reproductive state contained cysts. This was probably due to two factors. Firstly, the reproductive category was too large for precise typing so that some of the frogs included in the category had not reached that state necessary for the production of cysts. Secondly, cyst formation appears to be dependent on gonad development and is independent of whether the frog has actually bred or not. Presumably, breeding depends on environmental conditions. Thus, in frogs in which breeding has been delayed, cyst formation has ceased and the life cycle of *Z. binucleata* has proceeded in the reverse direction towards the eventual production of adult trophonts. This is indicated by an increase in the percentage of elongate trophonts and adult trophonts in frogs at stage 3. Examination of post breeding
frogs showed a decrease in the number of frogs containing cysts. This is due partly to the uncertainty of the time lapse from breeding to examination. However, examination of frogs in the act of oviposition also showed that very few of them contained cysts. Most of these frogs contained the adult and the elongate trophont stage as well as tomonts. This increase in adult and elongate trophonts in post-breeding frogs is shown in Table 2. Thus, in summary, it seems that cyst formation occurs in female frogs when the ovaries have reached an advanced state of development. It is apparently independent of oviposition, although given the right environmental conditions, these three phenomena are coincidental.

In male frogs, a similar situation was observed. Sections of testes of frogs showed that cyst formation occurred in frogs in which the development of spermatozoa had reached a maximum. Fig. 10,C,D. shows sections of testes of frogs that contained cysts. The seminiferous tubules are large and contain numerous bundles of mature spermatozoa. Fig. 10,A, shows a section of a testis of an immature frog (0.8 gms. in weight). All stages of spermatogenesis are present, completely filling the small seminiferous tubules. A few bundles of spermatozoa are present. The frog contained 20% elongate trophonts,
55% large tomonts and 24% small tomonts. Comparisons can be made between this and fig. 10,B. The latter is a section of the testis of a mature frog containing 20% divided trophonts, 69% large tomonts and 11% small tomonts. The small number of spermatozoa present indicates that breeding has taken place, cyst formation had ceased and the life cycle reversed towards the production of trophonts. Fig. 10,E, is a section of the testis of a post-breeding frog, whose rectum contained large numbers of cysts. The seminiferous tubules are large and contain few spermatozoa. Thus, the same picture as that obtained for female frogs is seen in the male, i.e., cyst formation occurs at the maximum stage of development of spermatozoa, after which the cycle is reversed towards production of trophonts. Cyst formation was independent of breeding.

Correlation of the number of frogs containing various stages in the life cycle of _Z. binucleata_ and the time of year is shown in fig. 9. Only mature frogs (above 3 gms.) were used in this calculation, since frogs metamorphosed at all times of the year; this fact tended to obscure the picture of the cycle of _Z. binucleata_ in relation to the sexual cycle of the host. Maximum cyst formation occurred in the spring, i.e., during August, September and
October, after which cysts occurred at a lower level for the rest of the year except for June and July, during which months no cysts were observed. From observations made during the year on the quantity of spawn in farm dams in the study areas it was noted that spawn production was at its maximum during the spring after which spawn was found at all times of the year except for June and July. Peak production of elongate trophonts occurred in October-November, somewhat later than that of the cysts. Adult trophonts increased steadily, reaching a maximum in February-March. This indicates that, after cyst formation, the life cycle of *Z. binucleata* is reversed with a rapid production of divided trophonts in the summer; eventually adult trophonts are produced in Autumn. During the winter, a build up of small tomonts, which will eventually form cysts in Spring, takes place.
<table>
<thead>
<tr>
<th>Stage of reproductive maturity of the frog</th>
<th>No. of Frogs</th>
<th>% of stages of <em>Z. binucleata</em> present</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Adult Trophont</td>
</tr>
<tr>
<td>Stage 1</td>
<td>30</td>
<td>1</td>
</tr>
<tr>
<td>Stage 2</td>
<td>11</td>
<td>-</td>
</tr>
<tr>
<td>Stage 3</td>
<td>30</td>
<td>1</td>
</tr>
<tr>
<td>Stage 4</td>
<td>28</td>
<td>3</td>
</tr>
</tbody>
</table>
FIG. 9. Graph of the number of mature frogs containing various stages in the life cycle of *Z. binucleata* during various times of the year.

--- Cysts.

----- Elongate trophonts.

---- Adult trophonts.
NUMBER OF FROGS WITH VARIOUS STAGES OF Z. BINUCLEATA

MONTH


16 14 12 10 8 6 4 2
FIG. 10. T.S. OF L. TASMANIENSIS TESTES

A. Immature frog.
(500x)

B. Post-breeding frog.
(200x)
FIG. 10.  T.S. OF L. TASMANIENSIS TESTES  (CONTINUED)

C. Mature frog.
(200x)

D. Mature frog.
(100x)
FIG. 10. T.S. OF L. TASMANIENSIS TESTES

E. Post-breeding frog.
(200x)
CHAPTER 7.  HOST SPECIFICITY.

In the study area near Yass, New South Wales, several species of frogs were found breeding in the same general area. Particular attention was paid to a farm dam that was not fed by a stream and so contained water only after rain. During this time the dam filled to a depth of about twelve inches. The sides were gently sloping and tussocks of grass caused small pools to be formed around the banks. After rain the dam rapidly dried up with the result that large populations of tadpoles were left stranded. Eggs of at least five species of frogs have been collected in this dam. Since most, if not all these species of frogs appear to be infected with a different species of Opalinidae, the problem arises as to whether the host specificity is due to an ecological separation, due to a micro-differentiation in breeding habitat or to a different breeding period; or whether it is due to a physiological barrier. The five species studies are:

L. tasmaniensis Gunther
Crinea signifera Girard
C. parainsignifera Main
Hyla verreauxi Dumeril
Pseudophyrene bibroni Gunther
Broadly speaking these species can be separated on the basis of their breeding habitat preference. *L. tasmaniensis* constructs a floating foam egg mass usually in moderately deep water. However, spawn has been found around the edges of the dam in shallow water and in the small pools formed by the tussock grass. *H. verreauxi* also tends to breed in deeper water, but lays submerged eggs that are attached to the stems of water plants and debris. *C. signifera* and *C. parainsignifera* spawn around the shallow edge of the dam. The eggs are laid singly and are attached to the submerged grass and debris. *P. bibroni* constructs depressions or burrows in the mud around the edge of the dam. The eggs are either washed into the dam during rain or else released as the general water level increases.

The breeding period of most of these frogs is extensive and a great deal of overlapping occurs. Full data on the breeding habits of the frogs in the Australian Capital Territory is not available but from the author's observations and records made by Mr. R. Pengilly (Zoology Department, Australian National University), a general picture of the breeding period of all these frogs could be formed. *H. verreauxi* has the most extensive breeding
period and has been recorded breeding at all times of the year. *L. tasmaniensis* breeds during the greater part of the year. The only two months when spawn was not found were June and July. *P. bibroni* breeds from March to mid-April, (Jacobson, 1963). *C. signifera* and *parainsignifera* have extensive breeding periods. The former probably does not breed during midsummer and the latter does not breed during midwinter in the Australian Capital Territory.

Thus, while these species have different breeding micro-habitats and breeding periods, overlapping between species did occur and it is fairly certain that the young tadpoles would come in contact with the opalinid cysts of another species of frog. An experiment with *L. tasmaniensis* was carried out to determine whether older tadpoles were capable of infection. Two month old *L. tasmaniensis* tadpoles which had been reared free of Opalinidae, as described on page 11 were placed in a culture dish containing infection cysts from a *L. tasmaniensis* frog. The tadpoles were examined after one week. All the tadpoles had become infected. Another experiment was carried out in which two month old uninfected and infected tadpoles were placed together in a culture dish. After one week the uninfected
tadpoles had become infected, presumably by cysts from the infected tadpoles. The importance of these experiments is that they show that the various species of frogs are very likely to come in contact with cysts of another species of frog at some stage in their larval life either before or after they have become infected with their own species of Opalinidae. No information was obtained on the possibility of multiple infection of different species of Opalinidae. The opalinid stages in the tadpole are very similar in appearance and, due to active asexual division and cyst formation, there is a great range of sizes of forms present at any one time.

A thorough study of the life cycles of the Opalinidae found in the various species of frogs has not been made and so it is impossible to describe them as new species at this stage. Numerous specimens of all these frogs have been examined both from the study area and other areas around Canberra. A brief description of the Opalinidae found in these species of frogs is given below.

*C. signifera*. This species of Opalinidae has been described by Raff (1911) as *Opalina tenuis* and renamed *Protoopalina tenuis* by Metcalf (1923). It is a very elongate species and specimens measuring 1,300u have been recorded (fig. 11,B.).
Numerous smaller forms are also usually present, presumably the result of asexual division. Tadpoles and metamorphosing frogs have been examined and in no case was the flattened 'Zelleriella-type' found. Thus on Metcalf's classification this species is probably a protopaladinid.

*C. parainsignifera* is also infected with *P. tenuis*. However, the infection level is very low (Hoy, 1963). Some 30% of *C. parainsignifera* are infected with *P. tenuis* while the infection level of *C. signifera* is close to 100%.

*P. bibroni*. The Opalindae from this species of frog has not been described before. What is probably the trophont stage is a large sphere-shaped form with a posterior, pointed, non-ciliated tail (fig. 11,A.). These measure approximately 120μ in length and 60μ in width.

*H. verreauxi* is infected with a large species of Opalindae resembling the elongate trophont of *Z. binucleata*. It differs in that the posterior end is more rounded. Although numerous frogs and metamorphosing tadpoles have been examined, no trace of a "Zelleriella" stage was present. Thus this species is probably a 'protoopaladinid'.

Since it is unlikely that ecological separation plays a direct role in determining host specificity, cross infection experiments were carried out between the Opalindae
of *C. signifera* and *L. tasmaniensis*. Frogs were defaunated as described in page 10. Cross infections were made by inserting a cannula tube into the rectum of the donor frog and withdrawing some of the contents which was examined under the microscope to make sure opalinids were present. The opalinids were then injected into the rectum of the recipient defaunated frog. Table 3 summarises the results of infection of *L. tasmaniensis* with *P. tenuis* from *C. signifera*. No infection survived for more than one day. The fact that many infections survived for one day indicates that the Opalinidae did not die in transit. A control was also set up in which the defaunated *L. tasmaniensis* donors were infected with their own species of Opalinidae. In this instance the Opalinidae survived in the recipient host.

Experiments involving the infection of tadpoles with foreign species of Opalinidae were also carried out. Tadpoles of *L. tasmaniensis* reared free of Opalinidae were subjected to the cysts of *P. tenuis* from *C. signifera*. Examination of these tadpoles at weekly intervals showed that opalinids were present. At the time of writing, 3 months after infection they were still infected with large numbers of Opalinidae.
From these results it seems that there is some physiological basis controlling host-specificity. The nature of this is not known although experiments suggest that some immunological process may be involved. In preparing Opalinidae for examination, by removing the rectum, it was noted, in many cases, that they soon became immobilized. From experiments described below this effect is believed to be due to the presence of serum in the blood released during dissection of the rectum. Serum was obtained from \textit{L. tasmaniensis} frogs as described on page 11. \textit{Z. binucleata} were collected by withdrawing rectal contents through a fine cannula tube. Protozoa collected in this way remained active for some hours although in a few cases immobilization occurred. This was probably due to damage of the rectal wall by the cannula. Only protozoa that remained active for half an hour were used for subsequent experiments. Table 4 shows the results of adding a drop of \textit{Z. binucleata} culture to \textit{L. tasmaniensis} serum at various dilutions. The reaction is very dramatic and is shown in fig. 12. Immobilization of the protozoa appears to be caused by matting and sticking together of the cilia (fig.12,A.) The cytoplasm then contracts from the cell wall and rounds
off into spheres (fig. 12,C.). Finally the cell wall breaks down, releasing these spheres of cytoplasm still surrounded by part of the cell wall and matted cilia (fig. 12,D.). In undiluted serum the whole process takes about one minute and at dilutions of 1:16 the process takes 5 minutes. The same reaction occurs in normal rabbit serum although, as table 3 shows, the titre is not as high. The reaction of *Z. binucleata* to immune rabbit serum prepared as described on page 11 appears different. Table 3 shows immobilization at dilutions of 1:64 of the immune serum, after treatment for 5 minutes. The cilia, instead of becoming matted, clump together at their distal ends (fig. 14). Although the animal becomes quickly immobilized at high concentrations of antiserum, the cilia still continue to beat for some time. No lysis occurred even at high concentrations of antiserum.

Natural and acquired antibodies have been described in mammals against various protozoa. Robertson (1939a) described the reaction of certain ciliates belonging to the Glaucoma–Colpidium group in normal and immunized rabbit serum. In unheated normal rabbit serum agglutination and lysis occurred. The protozoa were unaffected in heated normal serum. In immune serum agglutination occurred in higher dilutions but lysis did not take place. The same
reaction occurred in heated immune serum but the agglutination titre was lower than in the unheated serum. Bernheimer and Harrison (1940, 1949) described immobilization of \textit{Paramecium} in both normal and immune rabbit serum. In immune serum accumulation of a semi-solid material occurs at the distal extremities of the cilia. The cilia then stick together at their distal ends. Using the fluorescent antibody technique, Beale & Kacser (1957) demonstrated the accumulation of antibodies in a thin layer around the entire surface of the organism and in globules at the clumped tips of the cilia. Although immobilization occurs in both immune and normal serum the reactions are not the same. Immobilization in normal serum is dependent on complement, while in immune serum immobilization occurs even though the complement has been destroyed. Lysis of \textit{Paramecium} has not been reported in either normal or immune serum. Sinclair (1958) also demonstrated the immobilization effect of normal guinea pig serum on \textit{P. aurelia}, while immobilization and lysis occurred in \textit{Tetrahymena pyriformis} at high concentrations of normal serum. Experiments involving the addition of complement to immune serum were performed by Robertson (1939a & b). It was found that additions of complement to antiserum produced lysis only at very high dilutions, in which region
the immune serum and the complement by themselves had no effect. Sinclair (op.cit.) was unable to confirm this lytic effect of complement on the immune reaction of *T. pyriformis*.

Experiments were performed to determine the effect of complement (normal guinea pig serum) on *Z. binucleata*. The results were summarised in table 4. After one hour lysis had occurred at dilutions of 1:64. Normal guinea pig serum which had been heated at 56°C to inactivate complement did not cause lysis of *Z. binucleata*. However, immobilization did occur. The immobilization effect of inactivated serum was similar to that of immune serum in that the cilia became clumped at their tips and continued to beat for some time after immobilization. These facts are consistent with the view that lysis by fresh (non-inactivated) guinea pig serum is dependent on complement. Although the problem has not been investigated in this study, nor in the published work of others on other protozoa, it is likely that lysis by normal guinea pig serum involves a primary reaction between natural antibodies and the antigen on the surface of the opalinid, followed by fixation of complement and subsequent lysis. Lysis of red cells from various species and of certain bacteria by
normal serum has been shown to depend on such a mechanism. Additions of complement to immune serum were made to determine whether the immobilizing antibody in immune serum could mask the lytic effect of the complement. Equal proportions of complement and immune rabbit serum were diluted in frog Ringer. The results of addition of \textit{Z. binucleata} are summarized in table 5. The region of lysis formation was comparable to that of similar dilutions of the complement alone. The results of these experiments are inconclusive except that they demonstrate that the immobilization effect of immune serum does not mask or inhibit the lytic effect of the complement. The fact that the titre of immobilization tended to be somewhat higher than the titre of lysis suggests that the reaction is a progressive one and that immobilization precedes lysis.

It was hoped that serological techniques could be employed to determine specific or subspecific differences in the Opalinidae of different species of frogs. It was surprising to find that immune serum against \textit{Z. binucleata} produced the same immobilization reaction in \textit{P. tenuis} (fig. 14,B). This indicates that they possess common antigents, but does not preclude the possibility that there are also antigenic differences. This is in contrast to
the work of Bernheimer and Harrison (1940) demonstrating the specificity of antiserum against three species of *Paramecium* and between strains of a single species of *Paramecium*, Bernheimer and Harrison (1941).

The effect of normal frog serum on Opalinidae seems to be also unspecific. Fig. 13 shows the reaction of *P. tenuis* from *C. signifera* in the serum of *L. tasmaniensis*.

When *Z. binucleata* were removed to frog Ringer it was observed, on several occasions, that they became attached by the anterior end to the glass surface. Preparations of opalinids in methyl green acetic were made to detect the presence of trichocysts or similar structures. In many cases a network of thin strands of material was observed around the surface of the organism (fig. 15,C.). Noirot-Timotheé (1958) has described a stratified layer of vesicles inside the pellicle of *Opalina ranarum*. He believes that the innermost layer of large vesicles gives rise by budding to the layers of smaller vesicles just below the pellicle. Pitelka (1956) has also described vesicles in *Opalina obtrigonoidea* mostly concentrated just inside the pellicle. It is possible that these structures are responsible for the production of this material. Specimens of *Z. binucleata*, sectioned as described on page 22 showed a dark red layer
below the pellicle when stained with the periodic acid
Shiff technique after digestion in salivo (fig. 15,D.).
This PAS positive material thus has a corresponding
distribution to that of the vesicles as described in
electron microscope studies. Acetylation, followed by
staining with the PAS technique, showed a negative reaction.
This would tend to indicate the presence of a carbohydrate-
protein material probably a glyco- or mucoprotein (Leblond
et al, 1957). The significance of this material is not
known, but it may provide a means of attachment of the
opalinids to the rectal epithelium. Fig. 15,A, shows an
opalinid closely associated with the rectal epithelium.
The opalinids were found to be closely packed together in
the rectum (fig. 15,B.). Fig. 15,D, shows strands of
material connecting two individuals together. This
intimate connection of the opalinids to the rectal
epithelium and to each other would bring the opalinids into
a position whereby an inflammatory reaction could be
produced by the host in response to the presence of foreign
opalinids. This could perhaps result in the release of
blood, serum components of which would rapidly kill the
opalinids.
## TABLE 3

**INFECTION OF DEFAUNATED *L. TASMANIENSIS* FROGS WITH *P. TENUIS* FROM *C. SIGNIFERA***

<table>
<thead>
<tr>
<th>DONOR FROGS (L. tasmaniensis)</th>
<th>Days after infection with <em>P. tenuis</em></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td>1</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>+</td>
</tr>
<tr>
<td>3</td>
<td>+</td>
</tr>
<tr>
<td>4</td>
<td>+</td>
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<tr>
<td>5</td>
<td>-</td>
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<tr>
<td>6</td>
<td>-</td>
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<td>7</td>
<td>-</td>
</tr>
<tr>
<td>8</td>
<td>+</td>
</tr>
<tr>
<td>9</td>
<td>-</td>
</tr>
<tr>
<td>10</td>
<td>+</td>
</tr>
</tbody>
</table>
**TABLE 4**

**IMMOBILIZATION OF Z. BINUCLEATA IN VARIOUS SERA.**

<table>
<thead>
<tr>
<th>SERUM</th>
<th>DILUTION IN FROG RINGER</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1:1</td>
</tr>
<tr>
<td>Normal R.S.</td>
<td>-(L)</td>
</tr>
<tr>
<td>Immunized R.S.</td>
<td>-</td>
</tr>
<tr>
<td>NORMAL F.S.</td>
<td>-(L)</td>
</tr>
</tbody>
</table>

- = Total immobilization after 5 mins.
+ = Active
(L) = Lysis
### TABLE 5

**THE IMMOBILIZATION AND LYtic REACTIONS OF Z. BINUCLEATA IN COMPLEMENT**

<table>
<thead>
<tr>
<th>Concentration</th>
<th>5</th>
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<th>15</th>
<th>20</th>
<th>25</th>
<th>30</th>
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<th>40</th>
<th>45</th>
<th>50</th>
<th>55</th>
<th>60</th>
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<td>1:256</td>
<td>L</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>1:128</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>1:64</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>L</td>
<td>L</td>
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<td>1:32</td>
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<td>-</td>
<td>-</td>
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<tr>
<td>1:16</td>
<td>+</td>
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<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<td>1:4</td>
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<td>L</td>
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<td>L</td>
<td>L</td>
<td>L</td>
<td>L</td>
<td>L</td>
<td>L</td>
<td>L</td>
</tr>
<tr>
<td>1:2</td>
<td>L</td>
<td>L</td>
<td>L</td>
<td>L</td>
<td>L</td>
<td>L</td>
<td>L</td>
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<td>L</td>
<td>L</td>
<td>L</td>
<td>L</td>
<td>L</td>
<td>L</td>
</tr>
</tbody>
</table>

**TIME IN MINUTES**

- = Total Immobilization
+ = Active
L = Lysis
### TABLE 6

**IMMOBILIZATION AND LYTIC REACTIONS OF Z. BINUCLEATA**

IN A 1:1 MIXTURE OF COMPLEMENT AND IMMUNE SERUM

<table>
<thead>
<tr>
<th>Dilution</th>
<th>Total Immobilization</th>
<th>Active</th>
<th>Lysis</th>
</tr>
</thead>
<tbody>
<tr>
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<td>+ + + + + + + + + + +</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1:128</td>
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<td></td>
</tr>
<tr>
<td>1:64</td>
<td>+ + + + - L L L L L L</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1:32</td>
<td>+ + + - L L L L L L</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1:16</td>
<td>+ + L L L L L L L L</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1:8</td>
<td>+ L L L L L L L L L</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1:4</td>
<td>+ L L L L L L L L L</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1:2</td>
<td>L L L L L L L L L L</td>
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<td></td>
</tr>
<tr>
<td>1:1</td>
<td>L L L L L L L L L L</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

5 10 15 20 25 30 35 40 45 50 55 60

**TIME IN MINUTES**

- = Total Immobilization

+ = Active

L = Lysis
FIG. 11. **OPALINIDAE FROM FROGS FOUND BREEDING IN THE SAME AREA AS L. TASMANIENSIS**

A. Trophont from *P. bibroni*.
   (Scale = 50u)

B. Trophont of *P. tenui* from *C. signifera*.
   (Scale = 500u)
FIG. 12. EFFECT OF *L. TASMANIENSIS* SERUM ON *Z. BINUCLEATA*

A. Immobilization - cilia matted.
(Scale = 25u)

B. Cytoplasm contractin from cell wall.
(Scale = 25u)
FIG. 12.  EFFECT OF L. TASMANIENSIS SERUM ON Z. BINUCLEATA
(CONTINUED)

C. Cytoplasm rounding into spheres.
   (Scale = 100u)

D. Released sphere of cytoplasn surrounded by pellicle and matted cilia.
   (Scale = 100u)
FIG. 13. EFFECT OF L. TASMANIENSIS SERUM ON P. TENUIS FROM C. SIGNIFERA

A. Immobilization.
   (Scale = 200u)

B. Cytoplasm contracting from pellicle.
   (Scale = 200u)

C. Cytoplasm rounding into spheres.
   (Scale = 200u)
A. Effect of immune rabbit serum against *Z. binucleata* on *Z. binucleata*. Note clumped cilia.

(Scale = 100u)

B. Effect on same serum on *P. tenuis* arrow indicates a group of cilia clumped at their tips.

(Scale = 25u)
FIG. 15. PRODUCTION OF A MUCOID MATERIAL BY Z. BINUCLEATA

A. Intimate contact of *Z. binucleata* (indicated by arrow) with the rectal epithelium.

B. Section of several *Z. binucleata* to show dense packing within the rectum.
FIG. 15. PRODUCTION OF A MUCOID MATERIAL BY Z. BINUCLEATA (CONTINUED)

C. Z. binucleata after treatment with methylene green acetic. Arrow indicates the anastomosing strands of mucoid material.

D. Section of Z. binucleata in situ, stained with the P.A.S. technique after digestion in saliva. Note the region of P.A.S. positive material below the pellicle. Arrow indicates position of mucoid strands apparently joining two individuals together.
CHAPTER 8. DISCUSSION

8.1. LIFE HISTORY OF Z. BINUCLEATA

The present taxonomy of the Opalindae appears to be inadequate. This is especially true of the Opalinidae of Australian frogs. The inadequate state of the taxonomy of the group is due, to a large extent, to the lack of life history studies. No complete life history study has been made on the genus Zelleriella. The life cycle of Z. binucleata shows many more morphologically distinguishable stages than that of the various species of Opalina (Mofty, 1959; Wessenberg, 1961), and Protoopalina, (Sukhanova, 1960). Several of these stages were previously described as a separate genus and species (Raff, 1911, 1912). Evidence for this was obtained from infection of defaunated frogs with these forms. Observations of intermediate stages in natural infections between the various forms show that there is a continuous graduation of change during the course of the life history. This emphasizes the necessity for complete life history studies before a taxonomic work can be undertaken. All stages in the life cycle of Z. binucleata are not present in any one frog. Thus this species differs from that of Opalina as described by Mofty (1959) and Wessenberg (1961) in that the trophont stage of Z. binucleata
is not persistent throughout the life of the frog. The adult trophont is apparently only a transitory phase occurring in metamorphosing and post-breeding frogs.

A correlation of the life history of *Z. binucleata* with the reproductive state of the host was observed in that cyst formation occurred in reproductively mature frogs. However, since *L. tasmaniensis* breeds throughout the greater part of the year, this correlation had to be made largely on individual frogs. From observations of the quantity of spawn found in farm dams throughout the year it was noted that this was at its maximum during Spring. Spawn was then found throughout the year except for June and July. This can be correlated with a maximum cyst formation during the spring, after which cysts were found much less frequently during the rest of the year except for June and July, when no cysts were found.

After cyst formation the life cycle is reversed. This is indicated by an increase in the number of frogs containing elongate trophonts in summer, followed by an increase in the adult trophonts reaching a maximum in autumn.

The cysts of *Z. binucleata* are uninucleate. Excystment results in the release of uninucleate gamonts. The changes that then occur resulting in the production of
micro- and macrogametes are not clear. It seems that the uninucleate gamonts divide meiotically to produce gametes which fuse to produce the diploid zygotes. Whether the zygote encysts, as described in *Opalina*, is not known. The result is that in the older tadpoles elongate, binucleate forms are found. These flatten in the metamorphosing frog to become typical 'Zelleriellas'. Cyst formation occurs in older tadpoles resulting in the formation of uninucleate precystic forms, infection cysts, gamonts, micro- and macrogametes and zygotes. Thus the process of cyst formation in the tadpole resembles that in the frog.

Correlation of cyst formation in the opalinid with the reproductive cycle of the host has resulted in the suggestion that the sex hormones may be involved in this change. Evidence for this is lacking at the moment. Since cyst formation occurs in the tadpole it would seem that a separate mechanism would have to be postulated for this phenomenon.

8.2. **HOST SPECIFICITY**

A great deal more work has to be done to determine, not only the nature of the response of *Z. binucleata* to normal serum and the differences between this reaction and
that towards immune serum, but also the part played by natural and/or acquired immunity in relation to host specificity of Opalinidae. The lumen of the gut is normally considered out of the range of immunological reactions. The experiments performed here do not offer a solution to the problem of the nature of host specificity, since the serum of natural, as well as unnatural hosts was extremely effective in killing the protozoan. Clearly if blood or plasma proteins gain access to the rectum, the opalinids would be eliminated even in the natural host. It is not impossible that opalinids in the unnatural host might excite an inflammatory reaction resulting in the release of the lytic plasma components into the rectum and that adaptation of a parasite to a given host might involve rather specific modification so that this inflammatory response of the rectal epithelium is inhibited.

The distribution of a glyco- or mucoprotein material below the pellicle can be correlated with the presence of numerous vesicles as described in electron microscopic studies of several workers. This material is extruded as fine strands which possibly provide a mechanism for adhesion to the rectal epithelium and to each other. This would prevent the opalinids from being
voided with the faeces and provide a more intimate association with the host. This would seem necessary for a hypothesis of host specificity based on an immunological mechanism.

Transfaunation studies of Opalinidae from two species of frogs found breeding in the same area showed that foreign species of Opalinidae were unable to survive in adult frogs. However, infection of opalinid-free tadpoles with foreign species of Opalinidae showed that these were able to survive in the tadpole. The meaning of these experiments is not clear since at the time of writing (3 months after infection) the tadpoles had not metamorphosed and still contained large numbers of Opalinidae. The fact that foreign Opalinidae can survive in tadpoles and not in frogs is not inconsistent with the view that some immunological reaction may be involved in host specificity.
SUMMARY

The following points summarize the main findings in this thesis.

a. The life cycle of Zelleriella binucleata in the frog Limnodynastes tasmaniensis is described.

b. Z. binucleata passes through a 'Protoopalina' stage in its life cycle both in the tadpole and the frog. Several previously described species of Protoopalina have been found to be stages in the life cycle of Z. binucleata. This emphasises the need for complete life history descriptions in making a taxonomic study of the group.

c. A correlation between cyst formation in Z. binucleata and reproductive maturity of the host is postulated.

d. A method of defaunating frogs of Opalinidae by the use of achromycin is described.

e. Several species of frogs found breeding in the same habitat as L. tasmaniensis were found to be infected with different species of Opalinidae.

f. From transfaunation studies of defaunated frogs there appears to be a physiological basis for host specificity.
g. The mechanism for this host specificity is not known but the presence of a potent antibody in the serum of frogs may provide an explanation. The effect of this serum as well as normal and immune rabbit serum is described. Immobilization occurs in immune serum, whereas normal serum causes immobilization and lysis.

h. Sections of recta show that there is close contact of the opalinids with the rectal epithelium and between individual opalinids. This is probably due to the presence of a glyco- or mucoprotein secretion.
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