Lab and field studies on the establishment of *Cyclocoelum mutabile* (Zeder, 1800) (Digenea) infections in snail communities

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A thesis
in
The Department
of
Biology

Presented in Partial Fulfillment of the Requirements
for the Degree of Master of Science at
Concordia University
Montréal, Québec, Canada

September 1993

ISBN 0-315-87322-1

Abstract

Lab and field studies on the establishment of *Cyclocoelum mutabile* (Zeder, 1800) (Digenea) infections in snail communities

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Lab studies on the transmission of *Cyclocoelum mutabile* to snails revealed that eggs required an incubation temperature of at least 14 °C to hatch. Miracidia hatching from eggs stored for up to 7 weeks at 7 °C before hatching were equally infective to snails as those hatching from fresh eggs. Miracidial longevity and transmission efficiency was maximal at 14 °C. Studies on the susceptibility of 9 species of snails to miracidial infection showed 6 species to be susceptible (>25% infection success), 2 to be refractive (<25% infection success) and 1 to be insusceptible. Smaller snails were more susceptible than larger snails. Temperature had a significant but inconsistent effect on the infectivity success of miracidia. Miracidia did not discriminate between susceptible and refractive species. The number of the different snail species infected in the field could be predicted by the lab susceptibility studies and the abundance of each snail species recovered. The occurrence of infections in a natural

snail community could be related to the period when water temperatures was above 14 °C. Infections did not overwinter in snails in the field. Metacercarial infectivity was unrelated to the snail species in which they developed. Adult coots were less susceptible to infection than juveniles but prior infection by the fluke conveyed no resistance to infection in adult coots. These results are combined to suggest a mechanism behind the seasonal occurrence of *C. mutabile* in coots (*Fulica americana*).

Acknowledgements

I would like to thank all the people who helped me out in any way as assistants while working at the Delta Wetlands and Waterfowl Research Station. In no particular order, these people include A. Aldous, C. de Sobrino, J. Rataczak, B. Paulsen, K. Howard and P. Lee in 1991; P. Klimak, D. Vice, M. Butler, L. Boone, D. Romaine, M. Hartley, A. Kalogeros and J. Hochbaum in 1992. I would like to extend particular thanks to P. Lee and J. Hochbaum, both of whom put in very long days to help see this project through.

Other people at Delta to whom I owe thanks for help in my work include the entire Ward Family, B. Hales, and Sandra Kennedy who made Delta more of a home than a place of work. Special thanks go to both P. Ward and F. Rohwer, who not only allowed this research to be carried out by ensuring funding but also provided me a much greater insight into how the Marsh, waterfowl and nature work. The brown predator allowed me to collect the coots needed in the second season.

Thanks go to Dr. E. Pip, University of Winnipeg, for advising me about snails in the Delta Marsh and for suggestions for overwintering experiments. Thanks also go to Dr. G. G. C. Robinson, University of Manitoba, for allowing me access to the properties of the University of Manitoba Research Station in the Delta Marsh. I would also like to thank G. Selig, Concordia University, for putting up with seemingly endless questions about

statistics and computers. Dr. P. J. Albert kindly helped with the figures.

The Canadian Wildlife Service generously supplied the necessary permits required for the collection of both coots and coot eggs.

Financial support for this research was provided by the North American Wildlife Foundation through a Graduate Research Grant and through a demonstratorship funded by both Dr. J. D. McLaughlin and the Department of Biology, Concordia University.

My two committee members, Drs. Daphne Fairbairn and Jim Grant were both helpful throughout this study and particularly in the analysis of the data. Their comments on this thesis have made it better than it could have been otherwise.

Special thanks go to my supervisor, Dr. J. D. McLaughlin, who has taught me a good deal about parasites, research and how to present it properly. For all my complaining, I really do appreciate the time and effort he has put into my education and particularly for the patience he has shown in the writing of this thesis. I am also grateful for his giving me the chance to carry out this research in the first place.

Finally, I would like to thank Susan Woodfine for providing the understanding, support and encouragement that have made this work all the more enjoyable and rewarding.

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List of population parameters

Parameter	Description
t	time or age.
a	the instantaneous death rate of miracidia at hatching (t = 0).
b	the magnitude of the increase in mortality as the miracidia age.
P_t	the proportion of miracidia alive at age t.
μ_t	the age-dependent instantaneous death rate of the miracidia.
N_t	the number of miracidia alive at time t.
N_{t+1}	the number of miracidia alive at time $t+1$.
L	the mean expected lifespan of a miracidium.
T_{max}	the maximum observed lifespan of a miracidium.
β_t	the age-dependent instantaneous rate of infection of miracidia.
R	the mean number of redia recovered per snail.
M_0	the number of miracidia in the challenge arena at t = 0.
E	exposure time.
$\overline{\beta}$	the mean instantaneous rate of infection of miracidia over the infective lifespan of the miracidium.

$\overline{\mu}$ the mean instantaneous death rate of miracidia over the infective lifespan of the miracidium.

For Susan

Chapter 1.

General Introduction and Literature Review

1.1. Introduction

As in other animals, survival of parasites requires the production of new generations in an ongoing process. Unlike other animals, this proccess usually involves the transmission of different life stages from one host to another. Depending on the parasite, transmission may occur passively, where the infective stages are ingested, or actively, where free-living stages penetrate the host directly. Transmission may also occur through the use of a vector (primarily blood feeding invertebrates) that acquires the infection through a blood meal and transmits it to another host when feeding at a later date. Life cycles involving vectors will not be considered further.

In the context of helminth (worm) parasites, the two major types of hosts involved in the transmission of a particular parasite species are the definitive (final) host and the intermediate host. The definitive host is typically a vertebrate and is the host in which the parasite attains sexual maturity and produces eggs. A variety of organisms may serve as intermediate hosts; some are vertebrates but most are invertebrates. Intermediate hosts are organisms that become infected with eggs or other infective stages that are shed from the definitive host or a preceding intermediate host. The parasite then develops to

an infective stage which is transmissible to the next host in the life cycle. In many cases the next host would be the definitive host but numerous life cycles exist where two or more intermediate hosts are required in succession before the parasite may be transmitted back to the definitive host.

Life cycles may be direct or indirect, depending on the parasite involved. In direct life cycles, transmission is accomplished when eggs or other infective stages passed by the definitive host into the environment enter and establish in another definitive host. Indirect life cycles require an intermediate host, and stages passed by the definitive host must either be eaten or actively seek out and penetrate an intermediate host to continue development. This must be repeated if other intermediate hosts are involved. The definitive host normally becomes infected when infective stages are ingested.

Regardless of life cycle type, virtually all helminths have stages in their life cycles that must pass through the external environment at some point in the transmission process. In all cases, a temporal and spatial overlap of the different parasite stages and subsequent hosts must exist for the transmission of the parasite from one host to another. This "transmission window", incorporating both time and space, may be relatively wide, as in the case of animals living in a stable environment, or comparatively narrow in the case of animals living in seasonal environments. In the case of parasites infecting migratory definitive hosts such as waterfowl, this window may be quite

narrow indeed.

The Digenea, or flukes, on which this study is based, exhibit a variety of life cycles. There may be from one to four or more obligate hosts and up to eight developmental stages. Digenean life cycles involving a single host are restricted to molluscs and are not considered here. Where more than one host is involved in the life cycle, the definitive host is a vertebrate and with few exceptions, the first intermediate host is a snail. A three host life cycle is by far the most common life cycle pattern in digeneans (Pearson, 1972; Gibson, 1987; Shoop, 1988).

Dispersal of the digenean in space usually involves dissemination of the eggs by a vagile vertebrate acting as a definitive host and by two free living stages in the life cycle (Kennedy, 1976). Dispersal of the digenean in time usually involves a prolonged production of one life stage by another life stage as well as a prolonged survival of the eggs and a long-lived encysted pre-adult stage (the metacercariae). Also, some larval stages appear to have evolved solely to increase the production of a subsequent life stage (e.g. Shoop, 1988) and thereby increasing transmission success. Any reduction in the number of developmental stages also reduces the potential overall transmission success when compared to a more complex life cycle.

A secondary reduction, or 'telescoping' (*sensu* Gibson and Valtonen, 1984) of digenean life cycles is not uncommon and has been adopted by a number of species in response to environmental

pressures (Gibson, 1987). The digenean *Cyclocoelum mutabile* Zeder, 1800, that infects coots (*Fulica americana*), has such an abbreviated life cycle. The consequences of these reductions and the mechanisms by which the parasite counters the associated life history problems are the subject of this study.

In the lab, I examined factors that may influence the transmission of *C. mutabile* to snails in the field. Using these results, I then examined whether the predictions suggested by lab studies could account for what occurs in the field. The findings are then related to the occurence of *C. mutabile* in coots.

This chapter (Chapter 1) starts with a brief introduction followed by a review of digenean life cycles and how they relate to seasonal patterns of prevalence and intensity (percentage of hosts infected and the mean number of parasites per infected host, respe·tively) in waterfowl and intermediate host populations. The life cycle of *C. mutabile* is then discussed with regards to its observed patterns of prevalence in coot populations. As snail-trematode interactions are of great importance to the overall idea behind the thesis, a section is devoted to this as well.

Chapter 2 provides details on basic materials and methods used throughout a number of experiments. These will not be elaborated upon in subsequent chapters.

In Chapter 3, I outline a series of experiments that examines the temperature-dependent hatching dynamics of *C. mutabile* eggs and their survivorship following a prolonged

storage under constant cold conditions. I then examine the temperature-dependent survivorship of miracidia as well as the age-specific infectivity of the miracidia under these same temperature regimes and the infectivity of miracidia from eggs stored for different lengths of time before hatching.

Chapter 4 describes the results of experiments designed to determine what snail species are susceptible to infection by *C. mutabile* and what effect temperature has on the establishment of infections in the snails. The experiment entails a three-way infection schedule involving snail species, snail size class, and temperature. Both infection success and metacercarial production within the snail are used as criteria for the suitability of the snail as potential host. A further experiment is then described that attempts to determine if miracidia preferentially select one species over another when presented with two species of snails.

A series of field experiments are outlined in Chapter 5 that were designed to elucidate the transmission dynamics of *C. mutabile* under natural field conditions and to evaluate the ability of infections to overwinter in the snail hosts. These involved placing lab-infected roots in large cages on small artificial potholes and sampling the snail populations in those potholes to determine how the infective pool of *C. mutabile* develops within that snail community. An attempt was also made to determine if the infections may overwinter in the snails. This was carried out in two ways: (1) by determining the presence of infected snails in late fall one year, followed by an

intensive sampling of the same snail community the following spring; and (2) by placing lab-infected snails in cages in those same potholes and recovering the cages the following spring.

Chapter 6 describes experiments designed to evaluate the relative infectivity of metacercariae from different snail species and to determine what effect age and infection history has on the susceptibility of coots to infection by *C. mutabile*.

In the final chapter of this thesis, Chapter 7, I present an overview of the experimental and field results that attempts to reconcile them with what is known of the seasonal dynamics of the adult flukes from coots.

1.2. Literature Review

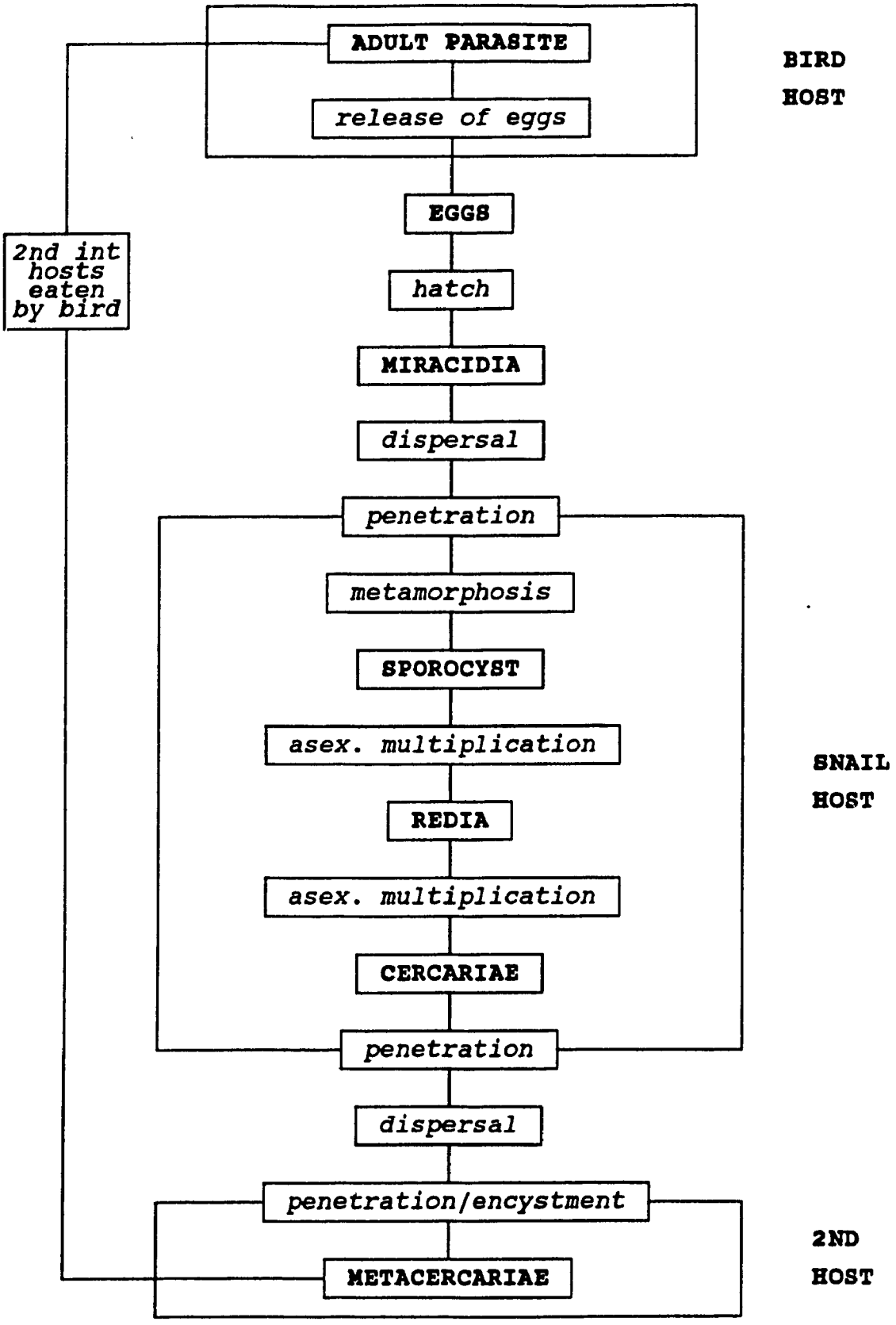
1.2a. Digenean Life Cycles and Seasonal Dynamics

Life Cycles

To understand what makes the *C. mutabile* life cycle unusual, a general understanding of the basic life cycle of digeneans is required. The Digenea, almost exclusively, have indirect life cycles with a mollusc, usually a snail, functioning as the first intermediate host and a vertebrate as the definitive host (Pearson, 1972). As the definitive host most commonly becomes infected through ingestion of an infective stage, the adult fluke typically inhabits the gastro-intestinal tract or its embryological derivatives (salivary gland and naso-lacrimal ducts, eustachian tubes, lungs, liver, pancreatic ducts, and the bile tree) (Shoop, 1988) where they produce many eggs in an ongoing process.

The life cycle of a typical digenean is outlined in Fig. 1.1. The life cycle begins when the eggs are passed from the definitive host. These eggs are usually unembryonated (fertilized, but with no further development) when shed and require varying lengths of time before development of the first larval stage, the miracidium, is complete. When embryonation is complete, the egg hatches and releases the miracidium. The miracidium is a free-swimming stage that infects the snail host. Relying solely on endogenous energy reserves, the miracidium is short lived (usually under one day) and must locate and penetrate

Figure 1.1. Diagrammatic flow chart of the life cycle of a typical digenean.


ADULT PARASITE
release of eggs
BIRD HOST
EGGS
2nd int hosts eaten by bird
hatch
MIRACIDIA
dispersal
penetration
metamorphosis
SPOROCYST
asex. multiplication
REDIA
asex. multiplication
CERCARIAE
penetration
dispersal
penetration/encystment
METACERCARIAE
SNAIL HOST
2ND HOST

a snail host or it dies. Miracidia locate snail hosts through a complex process involving responses to cues from the physical environment (light, gravity, etc.) that position them in areas where snails are likely to occur and chemical clues from the snail hosts (Wright, 1959; Christensen, 1980; Wright & Southgate, 1981; Sukhdeo & Mettrick, 1987). Although miracidia are often capable of penetrating most snail species they encounter, they can usually only become established and develop further in a narrow range of closely related snail species, and often only certain size classes of those species (see section 1.2c for a discussion on this point).

Once within a suitable snail host, the miracidium undergoes metamorphosis and becomes a mother sporocyst. Over time, the mother sporocyst will produce either further sporocyst generations (daughter sporocysts) or rediae. Both of these are sac-like asexual multiplicative stages; the redia differs in that it has a muscular pharynx and a simple gut whereas the sporocyst does not. The mother sporocyst usually remains close to the miracidium's point of entry in the snail; the secondary stages usually migrate to the digestive gland while some may also occupy the gonad or spill over into adjacent tissues (Wright & Southgate, 1981). The majority of digeneans have a number of sporocyst/redial generations within a snail before the production of the cercariae.

The cercariae of most digeneans are dispersal stages. They are actively swimming larvae that leave the initial snail host

and, like the miracidia, have a brief free-swimming existence in the environment. The cercariae of most species have also evolved an analogous pattern of host - location mechanisms which greatly increase their chances of encountering the appropriate subsequent host (see Haas (1992) for a comprehensive review). In most digenean life cycles, the cercariae penetrate a second intermediate host where they excyst as metacercariae. The second intermediate host for digeneans of water birds is commonly another snail on an insect larva. The definitive host becomes infected upon ingestion of the second intermediate host containing metacercariae. Once begun, cercarial shedding usually continues over the lifespan of the snail and incredible numbers may be produced over the lifespan of the infection. For example, the production and shedding of as many as 10,000 cercariae a day has been reported for *Trichobilharzia ocellata* infecting *Lymnaea stagnalis* (Sluiters, Brussaard-Wüst, & Meuleman, 1980). Thus the great production of cercariae through the development of multiple asexual stages seen in most digenean life cycles may have evolved as a mechanism to increase transmission success through sheer weight of numbers alone (e.g. Pearson, 1972).

Host specificity of cercariae is typically much lower than that displayed by the miracidia and as a result, a greater number of potential hosts are available to the cercariae, as compared to the miracidia. Thus, snails harbouring sporocyst or redial generations are often rarer in the environment than snails harbouring metacercariae in the environment.

In summary, a typical digenean life cycle relies on three mechanisms of distributing itself in space: the vertebrate host disseminating the eggs in the environment and the two free-living larval stages, the miracidium and the cercariae. However, the high specificity and comparably short lifespan of the miracidium may lead to an aggregated distribution of the parasite within a snail community and thus it is the cercarial and subsequent metacercarial stages that are of greatest importance in the transmission of the fluke back to the definitive host. The low host specificity of the cercaria ensures a wide distribution of the parasite in space, the long viability of the metacercaria ensures a wide distribution of the parasite in time.

This process, from the shedding of the eggs to the formation of the metacercariae, usually takes at least 2-3 months to complete. At room temperature, unembryonated eggs take 2-3 weeks to develop before they hatch. Intramolluscan development (mother sporocyst through cercarial shedding) may take up to two months or longer. As temperature plays an important role in the development rates of eggs (Weina, 1986) and of the different larval stages (Nice & Wilson, 1974), this process may be prolonged if environmental temperatures are low. Given that water temperatures in the northern latitudes are on the most part cooler than room temperature (the temperature at which most life cycles have been documented), the life cycle of most flukes from waterfowl in these regions typically rely on one year's adult population seeding the environment with eggs and the subsequent

development of new metacercarial generations over the space of a year.

Seasonal Dynamics

Typically, waterfowl return to the breeding grounds with relatively light digenean infections (Buscher, 1965; Cornwell & Cowan, 1963; Gower, 1938) because of a lack of recruitment of flukes while on the wintering grounds. Recruitment of new infections on the breeding grounds depends upon a number of factors that will ultimately influence the rate at which various species are acquired.

Metacercariae may overwinter or snails may begin to shed cercariae in the spring from overwintered redial infections, thus providing a new metacercarial cohort. If either of these events occur, infection levels will increase as soon as the birds arrive in the spring (Birova, Macko & Spakulova, 1989). Recruitment may continue into the fall or peak earlier and decline (McLaughlin & Burt, 1979). However, intramolluscan stages may reduce host survival (Brown, 1978; Bayne & Loker, 1987) and overwinter mortality of infected snails is common (Reader, 1971; Ménard & Scott, 1987), as is death of the parasite itself (Goater *et al.*, 1989; Fernandez & Esch, 1991). If sporocysts or rediae do not overwinter and only metacercariae are present, infection levels will remain low due to the limited opportunity for the birds to become infected. New infections in snails, derived from overwintered eggs or from eggs passed from hosts infected by the

residual metacercarial population, must become established in the snail population. However, the extended developmental period and the effects of temperature on development rates would delay the production of metacercariae, and hence the increase in infection levels in birds, until later in the year.

In situations where the infections do not survive the winter in the intermediate hosts, no recruitment is possible in the spring and whatever infections establish in the snail population are derived from parasites brought back by returning birds.

1.2b. *Cyclocoelum mutabile* Life Cycle and Seasonal Dynamics

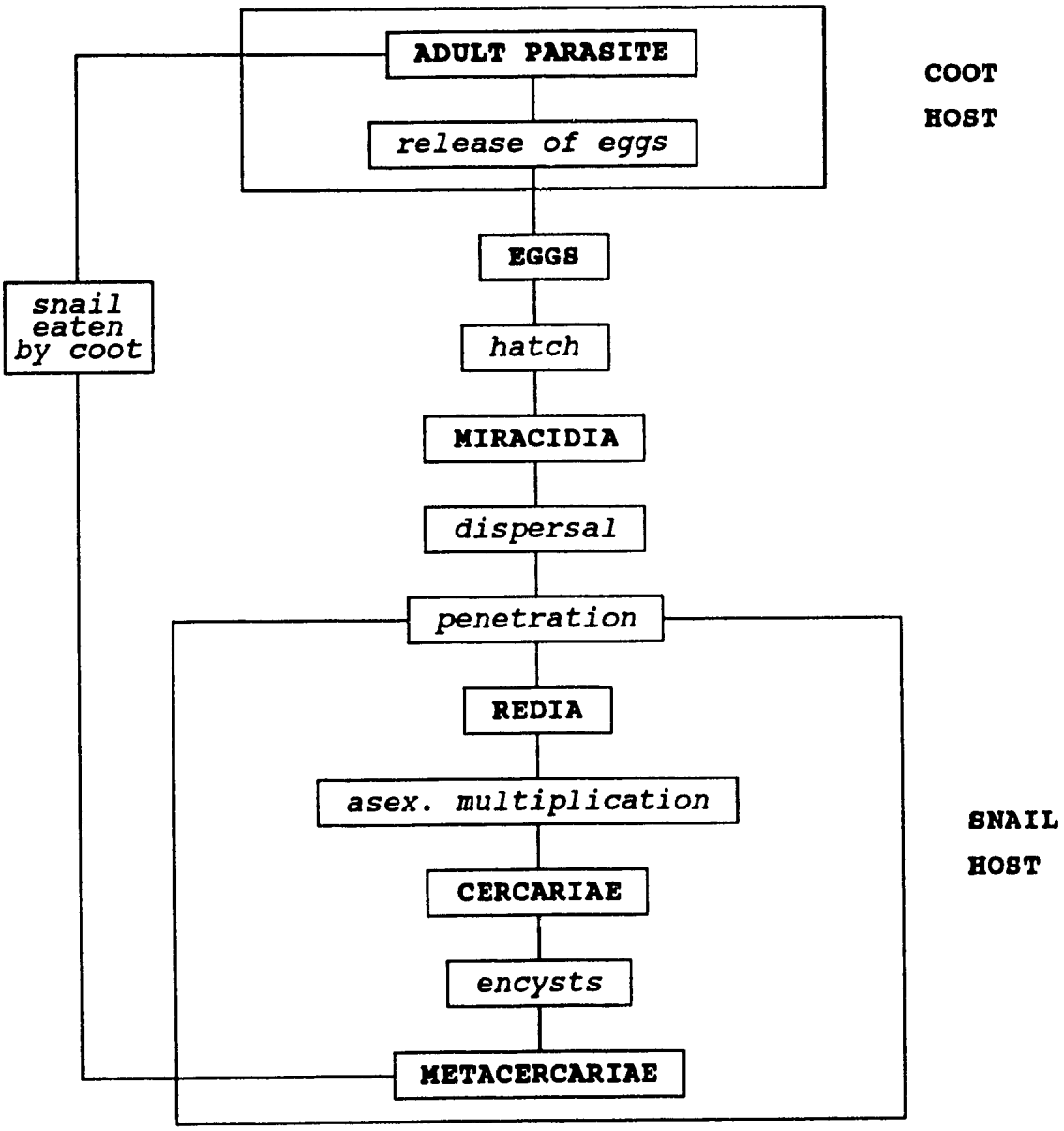
Cyclocoelum mutabile is a large digenean that infects the air sacs of coots (*Fulica americana*). Infections are acquired when snails containing metacercariae are eaten. The metacercariae excyst in the intestine and migrate through the intestinal wall into the body cavity and penetrate the liver 12-24 hours post-infection (pi) (McLaughlin, 1977). The developing worms remain and feed in the liver for about two weeks, then leave. By three weeks pi all worms have migrated into the air sacs, where they mature (McLaughlin, 1977). The lifespan of the fluke is some 10-12 weeks (McLaughlin, 1986). Eggs are liberated from the worm at about 4 weeks pi (McLaughlin, 1983), carried up the trachea by ciliary action, swallowed, and deposited with the host faeces (Feizullaev, 1971).

Coots returning to their breeding grounds in the spring harbour mature infections (Colbo, 1965; McLaughlin, 1986). The infections disappear by mid June and no new infections are acquired until mid July, when recruitment of new infections starts for both young of the year and adult coots. However, other digenean species that encyst as metacercariae within the same snail hosts are acquired by coots from the time they arrive on the nesting grounds, indicating that coots eat snails and become infected by whatever species of metacercariae are present in the snail population. This evidence suggests that metacercariae of *C. mutabile* do not survive the winter in the snail host (McLaughlin, 1986).

C. mutabile displays a secondarily reduced life cycle that seems well adapted for a rapid colonization of snail communities and the rapid production of metacercariae (Fig. 1.2). The eggs of this fluke embryonate and hatch within hours of being placed in water. This eliminates embryonation time required outside the host and any temperature-mediated delays that might occur. The miracidium contains a juvenile redia that is injected into the snail host. Though unusual, this is not unique to the cyclocoelids. The redia is the only one that occurs in the life cycle. Thus, mother sporocyst and the subsequent asexual generations do not occur and cercariae are produced directly. These encyst in the same snail as metacercariae. Metacercariae may be produced within three weeks of the initial infection in the laboratory (McLaughlin, 1976).

While the abbreviated life cycle of *C. mutabile* results in the rapid production of metacercariae, the absence of a free-swimming cercaria limits the dispersal of the parasite in space. Without the presence of the free-swimming cercariae in the life cycle, a highly aggregated distribution of metacercariae would be expected. However, over 80% of the coot population becomes infected over the course of the summer (McLaughlin, 1986), indicating that metacercariae are indeed well distributed in space. Either a large proportion of the population of a particular snail species becomes infected or the miracidium is capable of infecting a wide range of species. The former option would be less efficient as different potholes (ponds) on which

Figure 2. Diagrammatic flow chart of the life cycle of *Cyclocoelum mutabile*.


ADULT PARASITE
release of eggs
COOT
HOST
EGGS
snail eaten by coot
hatch
MIRACIDIA
dispersal
penetration
REDIA
asex. multiplication
SNAIL
HOST
CERCARIAE
encysts
METACERCARIAE

coots live may have very different snail community structures (Pip, 1978; personal observations). The latter option would have the same net effect as cercarial shedding; namely dispersal of metacercariae within a snail community, regardless of its composition. Also, given that the life span of the fluke is 10-12 weeks (McLaughlin, 1986), waterfowl arriving with patent infections must have acquired these on the wintering grounds. It is highly unlikely that transmission is due to the same snail species in both the winter and summer habitats.

1.2c. Determinants of Snail-Digenean Compatibility

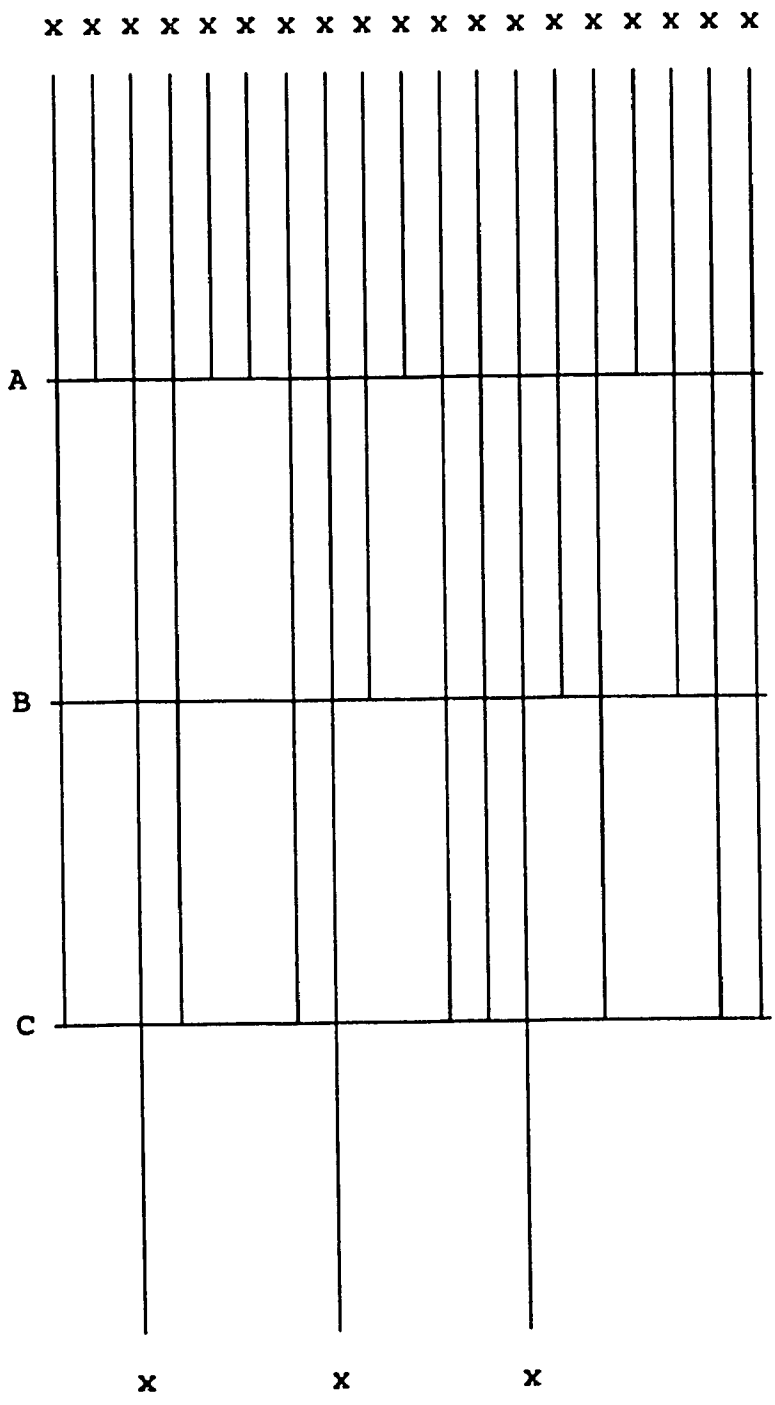
Most authors contemplating the origins of present-day digeneans have assigned the present first intermediate host, the snail, the status of the original host; the other present-day host being acquired later in the evolution of the parasite's life cycle (Gibson, 1987; Pearson, 1972; Llewellyn, 1987; Shoop, 1988). One of the principal features of this proposition is the high specificity of the parasite to its first intermediate snail host (=long association) as opposed to the lower host specificity (=relatively short association) the parasite displays for its definitive host (Pearson, 1972; Wright, 1960; Heyneman, 1960). In any event, it is the specificity for the snail host by the digenean that is of interest here.

With some notable exceptions, each species of digenean is capable of infecting only a limited number of the snail species. In some cases, only certain geographic strains of a snail species or a particular size class are susceptible (van der Knaap & Loker, 1990; Dikkeboom, van der Knaap, Meuleman & Sminia, 1985). This specificity has ethological, ecological, physiological and immunological components (see Fig. 1.3).

Ethological components involve the responses of the miracidium to the environment that act to concentrate the miracidia in the snail's general habitat and to stimuli arising from the snail host (Saladin, 1979; Combes, 1991). These have been briefly discussed already and will not be reiterated here. Ecological restraints that limit the specificity of potential

Figure 1.3. Factors limiting the compatibility of digeneans and snails. Factor "A" represents the ecological and ethological factors keeping the host from being exposed to the parasite in the first place (ie. parasite rarely encounters snail host). Factor "B" represents the suitability of the snail host for that digenean species (ie., does the host supply all the parasite's basic life needs?). Factor "C" represents the outcome of the anti-parasite internal defence response mounted by the snail against the parasite and the steps used to counter or limit the effect by the snail. (Modified from Holmes, 1987.)

POOL OF POTENTIAL
SNAIL HOSTS



REALIZED UTILIZED
SNAIL HOSTS

snail-digenean combinations arise from the ethological component or from aspects of the biology of either the parasite or hosts. Quite simply, if the miracidium and host do not occupy the same space (temporal and spatial), the snail will be neither challenged nor, obviously, infected; an 'ecological screen' (Holmes, 1987) or 'encounter filter' (Combes, 1991) is in place. These factors may be of great importance in the limiting of potential snail-digenean combinations in nature.

While ethological and ecological factors may limit contact with the spectrum of potential hosts in nature, this is not the case in the lab where any digenean-snail combination may be examined. Here, unless miracidia fail to recognize a snail as such, ecological and ethological factors may be considered negligible.

Snails are generally classified as being either *susceptible* or *insusceptible* to infection by a specific digenean. However, insusceptibility may be due to one or both of the following: (1) host physiological *suitability* - the ability of a snail to provide the larval parasite's biological needs; or (2) host *resistance* - the host snail's immune response to invasion by the parasite (Lie, Jeong & Heyneman, 1987).

Although most of the accumulated knowledge of the immune system in snails comes from studies of three economically or medically important snail species (*Helix pomatin*, *Biomphalaria glabrata*, and *Lymnaea stagnalis*), they display remarkably similar mechanisms of defence and other pulmonate species probably

utilize the same or similar lines of defence (van der Knaap & Meuleman, 1986).

As the immune system of snails utilizes neither immunoglobins nor lymphocytes, and anamnestic responses to specific antigens are lacking, Bayne (1983) suggested this system should more properly be known as an internal defence system. Nonetheless, this system is well capable of distinguishing between self, effete self and non-self and significantly reduces the number of digenean species capable of establishing within a snail. Thus, "resistance to foreign agents (digenean larvae) is the rule; compatibility of host and parasite is the exception" (Bayne & Yoshino, 1989: 399). However, the distinction between compatibility and incompatibility is not absolute. Intra- and interspecific differences occur in both the parasite and snail and may only be detected by comparison of many criteria such as infectivity of the miracidium, rate of development of the intramolluscan larval stages, and production and infectivity of the resulting cercariae/metacercariae (Cheng, 1968). The system comprises both cellular and humoral components that act alone or in conjunction with each other to detect and destroy, or otherwise hamper the development of the parasite.

Sminia & van der Knaap (1986) outlined four types of cells involved in the internal defence of snails. These are the antigen-trapping endothelial cells, pore cells, reticulum cells, and the haemocytes. The first three cell types are anchored in the snail tissue and their role in defence against digeneans is

questionable. It is the haemocytes, amoeboid cells found both in connective tissue and floating free in the circulating haemolymph, that are most involved with cellular defence reactions.

Within the haemocytes, two distinct subpopulations exist, the granulocytes and the hyalinocytes, the former comprising about 90% of the total of all haemocytes (Locker & Bayne, 1986). These two are distinguished by their relative sizes and their ability to spread on glass plates *in vitro*. Depending on the author, they are variably considered to be precursors of the other or separate cell types altogether (van der Knaap & Loker, 1990). As the granulocytes are considered most important in the snail's defence against digeneans, only they will be considered further and will be referred to, alternatively, as amoebocytes or haemocytes from here on.

In resistant hosts, amoebocytes typically first gather in the general area of the invading parasite, possibly mediated through chemotaxis (van der Knaap & Loker, 1990). The larva is then surrounded and encapsulated by several layers of haemocytes (van der Knaap & Meuleman, 1986). Killing of the parasite proceeds through the release of a number of toxic elements by the haemocytes and mechanical action of the innermost cells. Upon breaching of the parasite's surface membrane, the haemocytes quickly devour the animal and then disperse (Lie *et al.*, 1987).

The enzymes and enzyme inhibitors that have been identified in snail haemolymph and haemocytes, and presumably those used in

the degradation of the parasite body wall, include lysozyme, acid and alkaline phosphotases, aminopeptidase, amylase and lipase (Lie *et al.*, 1987). In a comparative study of haemocytes from six different snail species, Adema, Harris & van Deutekom-Mulder (1992) demonstrated peroxidase activity in four of the six species studied as well as the generation of super oxide by all species, indicating the potential role of these elements in the destruction of the parasites as well.

According to van der Knaap & Loke (1990), numerous studies have indicated that humoral factors alone are incapable of killing trematode larvae. However, Jourdane & Théron (1987) have reported that up to 15% of *Schistosoma mansoni* successfully penetrating the snail host, *Biomphalaria glabrata*, degenerate in the absence of any haemocytic action. Thus, the existence of any humoral factors acting alone has not been proven one way or the other.

Titres of haemocytes are often much higher in infected snails than in uninfected ones. In some cases, such as in the snails *Biomphalaria glabrata* and *Bulinus* spp., this is mediated through the stimulation of a so-called 'amoebocyte producing organ' by infection with digeneans (Lie *et al.*, 1987). Its function appears to be solely the production of haemocytes and its productivity is correlated with the susceptibility of the snail. Similar structures in other snails have yet to be reported but this may reflect the level of research directed towards these two species (intermediate hosts of the medically-

important schistosomes) as compared to that directed towards other snail species.

That humoral factors, namely agglutinins produced by the haemocytes, act in an important role in the regulation of haemocytic cytotoxicity is beyond doubt: injection of plasma from resistant snails into otherwise susceptible snails transfers resistance (Granath & Yoshino, 1984). Incubation of haemocytes from susceptible snails with plasma from resistant snails *in vitro* also confers phagocytosis capability to the otherwise incapable haemocytes (Fryer & Bayne, 1989). These studies indicate that the agglutinins found in the snail plasma also act as opsonins and Bayne & Yoshino (1989) have suggested that compatibility between parasite and snail may occur when the parasite fails to elicit or blocks the opsonization process. However, haemocytes from resistant snails may kill sporocysts in the absence of any plasma and the humoral factors associated with it (van der Knaap & Loker, 1990). Immune mechanisms in snails are far from clear-cut.

Susceptibility to infection changes with snail size more than with snail age (Anderson, Mercer, Wilson & Carter, 1982; Anderson & Crombie, 1984). This has been attributed to two factors: (1) larger snails have thicker body wall and thus are simply more difficult to penetrate, (2) the internal defence system of larger snails is more highly developed. In addition to juvenile snails having lower numbers of circulating haemocytes (van der Knaap & Meuleman, 1986), Dikkeboom *et al.* (1985) have

shown that haemocytes of juvenile *Lymnaea stagnalis* are less efficient at phagocytizing rabbit red blood cells and that agglutination and opsonic activities of the plasma are lower than that in plasma from adult snails.

Activation of the snail's defence system via both plasma- and cell-associated effects may occur in as little as 1.5 hours post exposure, as seen in *Lymnaea stagnalis* exposed to *Trichobilharzia ocellata* (Amen, Baggen, Bezemer & de Jong-Brink, 1992). Taft & Gasque (1991) have reported that activation may occur even during attachment of the miracidium and the injection of the redia of *Cyclocoelum oculeum* into *Gyraulus parvus*. In this system, haemocytes were seen to attach to the redia as it entered the snail body (Taft & Gasque, 1991). Thus, a parasite's defence mechanisms must be up and running as soon as it enters the snail host and must continue to operate throughout the duration of infection as it remains under the constant threat of the host's internal defence system.

How a parasite evades this immediate and constant host vigilance is thought to occur either through passive or active mechanisms (Yoshino & Boswell, 1986; Bayne & Loker, 1987). Passive mechanisms allow the parasite to avoid being recognized as non-self by the host. This may be carried out by molecular mimicry - where the parasite's innate antigenic coat is similar to host antigens, or by antigenic masking - where the parasite acquires host antigens from the host plasma. While many digeneans have antigenic coats that are bound by anti-snail

antisera, and near maximal coating of sporocysts of snail plasma-derived antigens may be accomplished within 3 hours pi (Bayne & Loker, 1987), the potential value of both strategies remains in dispute (van der Knaap & Loker, 1990). Two other possible passive mechanisms involve exploiting a blind-spot in the snail's recognition system (van der Knaap & Loker, 1990). The parasite may either lack the specific antigens necessary for recognition as non-self or it may produce an epitope that is unique to compatible parasites.

Active mechanisms may be either direct - where the parasite destroys the attacking haemocytes, or indirect - where the parasites act to suppress the snails internal defence system (Yoshino & Boswell, 1986). Indirect mechanisms are thought to be mediated through secretion of some substance by the parasite (Loker & Bayne, 1986). Recently, Amen & de Jong-Brink (1992) have shown that, in the *T. ocellata* - *L. stagnalis* combination, the newly established parasite releases a factor that induces the release or synthesis of a factor, probably a neuropeptide, by the snails central nervous system, which in turn suppresses haemocyte activity.

Without doubt, many combinations of these evasive strategies are employed by digeneans infecting snails. For example, in the *Trichobilharzia-Lymnaea* combination, miracidia display evidence of molecular mimicry, the young primary sporocysts and cercariae show evidence of molecular masking and older and primary sporocysts show no such passive abilities (Yoshino & Boswell,

1986). Presumably, the other stages may suppress the internal defence response as previously outlined. That cercariae show the ability for antigenic masking is of interest in that Amen *et al.* (1992) have shown an increased activation of the snails internal defence system (both cellular and humoral components) at the initiation of cercarial emergence. How other snail-digenean combinations prove successful remains to be seen.

Chapter 2.

General Materials and Methods

2.1. Host Animals

2.1a. Coots

All coots used in laboratory and field studies were hatched from eggs collected from coot nests in the Minnedosa, Manitoba, Pothole area. Eggs were incubated in a Robbins Hatch o' Matic incubator. When pipped, eggs were transferred to a Robbins Hatch o' Matic hatcher for hatching. The baby coots were left within the hatcher for 1 day following hatching prior to being transferred to pens. The pens for baby coots were plywood and masonite rings approximately 2m in diameter and 30cm high. A brooder lamp suspended about 40cm off the floor provided the youngest coots with warmth and a mesh screen covered with crepe paper kept them off the floor. When old enough, they were transferred to larger pens. One side of each pen allowed the coots free access to water in a flow-through swimming trough. Baby coots were initially hand-fed a mash of duck feed (Duck Starter, Feed Rite Mills, Winnipeg, Manitoba) and water until 5-10 days of age. At this time they fed on their own and were supplied duck feed, *ad lib*.

A total of 276 and 295 eggs were collected in 1991 and 1992, yielding 213 and 144 baby coots, respectively. Technical problems with the incubators resulted in low hatching success in 1992.

2.1b. Snails

Snails used in this study originated from the Delta Marsh (Delta), from a pond in nearby Portage la Prairie, Manitoba (PlaP), or from a creek just south of Portage la Prairie (creek). Snails came from three sources: (1) populations maintained in the lab since 1990 and originally collected at Delta (lab), (2) egg packets collected in the field and reared in the lab (eggs), or (3) wild-caught (field). Table 2.1 summarizes the origin of all the snails used in all studies. Snails from lab populations and those raised from eggs were known to be free of digenean infections. Wild-caught snails were checked individually for the presence of sporocysts or redia and/or the shedding of cercariae prior to being exposed to *C. mutabile* miracidia. Snails that were raised from egg packets collected from both Delta and PlaP were pooled and no attempt was made to differentiate between the two populations of snails used. Snail identifications were made following Clarke (1973; 1981), Burch (1989), and Harman & Berg (1971); nomenclature follows Clarke (1981).

Each species of snail was maintained separately in the lab. Plastic tubs (38x25x14cm or 31x16x8cm) (Sterlite Inc., Montréal, Québec) or plastic pails (22cm in diameter by 21cm in height) (Reliance Products Ltd., Winnipeg, Manitoba) were used as aquaria. All of the "aquaria" used were aerated and kept under ambient temperature and light conditions. Some aquaria were fitted with plastic lids to prevent the more amphibious species from escaping.

Table 2.1. Species, original collection site(s), and form of the snails, upon collection and just prior to use, used in this study.

Snail species	Collection location	Form of collection
Lymnaea stagnalis jugularis (Say, 1817)	Delta & creek	Lab & eggs
Stagnicola (stagnicola) elodes (Say, 1821)	Delta	Lab & field[a]
Physa gyrina gyrina Say, 1821	PlaP	Field & eggs[b]
Physa jennessi skinneri Taylor, 1953	Delta	Field
Armiger crista (Linnaeus, 1758)	Delta	Field
Gyraulus circumstriatus (Tryon, 1866)	PlaP	Field
Gyraulus parvus (Say, 1817)	Delta	Field
Helisoma (Pierosoma) trivolvis subcrenatum (Carpenter, 1856)	Delta	Eggs
Promenetus exacuous exacuous (Say, 1821)	Delta	Field

[a]All *S. elodes* used in snail specificity studies were from lab-stock; those used in miracidial choice studies were all collected from the field. [b]*P. gyrina* of size class 1 used in the snail specificity studies were obtained from eggs laid in the lab by field-caught snails, all other size classes examined were from mixtures of field-caught and similarly lab-raised snails.

Except for *Physa gyrina*, the water used was that from Delta Marsh diluted about 50-50 with tap water. *P. gyrina* did not tolerate marsh water and the water from the pond in Portage la Prairie where they were collected was used instead. This was diluted in the same way as the water from Delta Marsh. Aquaria were cleaned periodically by siphoning off about half of the water and most of the settled particulate matter. Fresh clean water was added to keep the aquaria filled to within 3-8cm of the top.

All snails were maintained on romaine lettuce, Nutra Fin Staple Food Tablets (Rohlf C. Hagan Inc., Montréal, Québec) and provided with powdered chalk as a source of calcium carbonate. Lymnaeids were fed raw lettuce; all other species received boiled lettuce.

2.2. Parasite material

New laboratory populations of *Cyclocoelum mutabile* were established annually. Adult flukes were obtained from coots collected by shotgun in the spring of both 1991 and 1992 from the Delta Marsh and from potholes near Minnedosa, Manitoba. A total of 6 birds were taken the first year, 2 of which were infected and provided a total of 4 worms. Similarly, a total of 6 worms were recovered from 3 of the 19 birds collected during the spring of 1992. The birds were necropsied within 12 hours of collection and the parasites retrieved. Necropsy of birds involved a simple examination of the airsacs.

Worms were teased apart in tap (Lake Manitoba) water at room temperature (20-25 °C) and the eggs pooled. Eggs began to hatch about 3-4 hours later. Miracidia were collected and 2-3 were placed in each well of 24-well tissue culture plates containing juvenile *Stagnicola elodes*. This species was used in the initial infections because it is readily cultured in the laboratory, is easily infected and infected individuals may survive for several months. After about 12 hours, exposed snails were transferred to plastic tubs and kept at room temperature. A total of about 400 snails was infected in this manner each spring. Metacercariae developing in these snails were used to infect the laboratory raised coots which in turn provided material for the experiments.

Beginning at about five weeks pi, a fraction of the snails was crushed and the metacercariae pooled and fed to naive coots by oral intubation (25-30 metacercariae/bird). The first group of coots received metacercariae when they were about 15 days old. Although the parasites are ovigerous within 4 weeks pi, care was taken to use only worms that were between 6 and 12 weeks of age in an effort to ensure that the miracidia hatching from the eggs were all approximately equal in infectivity. (Studies on other species have shown that the first eggs produced may be less viable, smaller and produce smaller miracidia which may in turn be shorter lived and less infective to snails.) Thus, successive groups of coots were infected every few weeks and those harbouring worms of the appropriate age were selected for necropsy when needed.

2.3. Harvesting of eggs and miracidia

Eggs used in this study were obtained from the feces of lab-infected coots or dissected directly from the adult worm following necropsy. Although it was desirable to use only eggs from feces (the normal source of eggs), this method is very time consuming and one cannot anticipate collection of sufficient numbers of eggs for many of the experiments. Infected coots may not defecate when isolated and highly variable numbers of eggs may be recovered on successive days). Thus the large number of eggs required in most of the experiments necessitated the use of eggs dissected directly from worms. Eggs from feces were used in all studies examining the hatching dynamics of the eggs (except the egg age-dependent infectivity studies) and in half the survivorship studies whereas eggs from worms were used in all infection and transmission studies.

2.3a. Eggs from feces

Feces from lab-infected coots were collected by isolating the birds in boxes (9 x 12 x 13 inches) fitted with lids and with 1/4 in^2 mesh on the bottom. Feces were collected every 2-3 hours from waxed paper placed below the boxes. At each collection, the feces were pooled and diluted in water. This slurry was then passed through a series of screens, the eggs being trapped on a 37 μm screen. These eggs and remaining debris were transferred to a Petri dish and the eggs collected by pipette under a dissecting microscope. For all experiments involving hatching

time, timing (t = 0) began as soon as the feces were mixed with the water. Collection was halted as soon as the first free-swimming miracidium was seen in the Petri dish.

2.3b. Eggs from worms

Collection of eggs from worms was carried out as in 2.2, with three exceptions: (1) worms were obtained from lab-infected coots, killed by a sharp blow to the head. (2) Only the anterior 1/3 of the worm was used to obtain eggs as this provided only the most mature eggs. This method yields thousands of eggs. (3) Temperatures were controlled as outlined in 2.3c, below. Experimental time was initiated as soon as the worms were dissected (t = 0).

2.3c. Temperature considerations

When eggs or miracidia were to be tested at temperatures other than room temperature, all manipulations (i.e., dilutions of the feces, dissection of the worms and collection of the eggs and miracidia) were carried out with water and equipment kept at the experimental temperatures (± 1 °C) within incubators. A fibre optic light source was used to ensure minimal warming during observations. Two air conditioners helped maintain the room in which the experiments were carried out at temperatures near that required for each experiment.

2.4. Statistical analyses

All statistical analyses were performed following proceedures outlined by Sokal & Rohlf (1981), Zar (1984) and SPSS Inc. (1990). Unless otherwise indicated, all assumptions required for the different parametric analyses utilized were met, otherwise nonparametric analyses were employed. Analyses were performed using 'SYSTAT' (Wilkinson, 1990), 'SPSS' (SPSS Inc., 1990), or 'NCSS' (Hintze, 1992), depending on the analysis. Description and rationales for the particular statistical methods employed are presented where appropriate in each chapter.

Chapter 3.

Transmission of *Cyclocoelum mutabile* (Digenea) to snails: the influence of environmental temperature on the egg and miracidium

3.1. Introduction

In most migratory waterfowl in North America recruitment of digenean infections begins during migration and continues on arrival on the breeding grounds as a result of infective pools established the previous year (Gower, 1938; Buscher, 1965). These infections become patent and eggs are returned to the environment to infect new cohorts of intermediate hosts and, ultimately, definitive hosts.

Cyclocoelum mutabile (Zeder, 1800) is a large digenean that infects the airsacs of coots (*Fulica americana*). Adult coots arriving in the spring bring patent infections with them. However, the parasite population dies out by mid to late June (Colbo, 1965; McLaughlin, 1986). In contrast with other digenean species that are transmitted by the same snail species, no recruitment of new *C. mutabile* infections occurs during the spring. Thus infective pools established the previous year do not appear to survive the winter or, if they do, are for some reason unavailable to coots. It appears therefore that new infective pools must be established in local snail populations each spring as young of the year coots become infected by mid

July.

The life cycle of *C. mutabile* seems well adapted for rapid establishment and development of metacercariae in snail populations. Eggs are embryonated when passed from the coot and hatch soon after contact with water. The miracidium which is released contains a young redia, the only one in the life cycle. The redia is injected directly into the snail. It produces cercariae which encyst as metacercariae within the same snail (McLaughlin, 1976). At room temperature, metacercariae may be produced within three weeks. Snails infected with eggs passed by coots in May or June could, therefore, produce the metacercariae necessary to infect new cohorts of coots in mid to late July.

By virtue of its effect on both the survivorship and infectivity of transmission stages, temperature is one of the most important factors influencing the transmission dynamics of digeneans (Evans, 1985). Thus, how well the egg and miracidium cope with the range of ambient temperatures that occur in the spring will have a profound effect on the establishment of infections in snails each year and ultimately on the transmission back to coots.

This study examines the effect of temperatures on the survival and hatching dynamics of *C. mutabile* eggs and on the longevity and infectivity of the miracidia.

3.2. Materials and Methods

Eggs used in the following experiments were obtained from either the feces of lab-infected birds (passed eggs), or dissected directly from the uterus of worms obtained at necropsy (dissected eggs) as outlined in Chapter 2. Unless otherwise stated, all experiments were carried out within incubators at *temp* ± 1 °C under constant, incandescent illumination.

The original objective of these experiments was to examine the effect of a range of temperatures likely to be encountered by eggs shed in the spring (20, 16, 12, 8 and 4 °C) on the survival and infectivity of miracidia of *C. mutabile* to the snail host at these temperatures. However, hatching did not occur at or below 12 °C and the experiments were modified to include trials at 14, 16 and 20 °C; 14 °C being considered the effective lower limit for hatching of *C. mutabile* eggs.

3.2a. Temperature-dependent Hatching Dynamics

Passed eggs were used exclusively in this experiment. Two trials were run at each temperature. In each trial, single eggs were placed in separate wells of eight, 24-well tissue culture plates. Timing of the experiment ($t = 0$) began when the feces were diluted to obtain the eggs. Eggs were examined every hour for the first 48 hours and then every 12 hours for the following five days. An egg was considered hatched when the miracidium was free of the egg. The effects considered in this experiment were

the overall hatching success and hatching times of eggs incubated at each temperature.

3.2b. Egg Viability Following Prolonged Cold Storage

Passed and dissected eggs were collected at 12 °C and placed in 16 (passed eggs) or 18 (dissected eggs), 24-well tissue culture plates, as described above. They were stored for up to four weeks at 12 °C. At intervals of 12, 24, 48 and 96 hours and 1, 2, 3, and 4 weeks (dissected eggs only), two plates were selected at random, removed, and warmed to 14 °C. The plates were monitored daily for one week. Eggs not hatching by day 7 were considered dead. Two control plates held at 12 °C were monitored under the same schedule as the experimental plates. Three separate trials were performed using passed eggs; a single trial was also performed with dissected eggs. Hatching success was the only variable considered.

3.2c. Miracidial longevity

This experiment used both passed and dissected eggs. Young (< 30 min old) miracidia hatching from eggs incubated at 14, 16, or 20 °C were pipetted to individual wells of 9-spot depression plates and maintained at the corresponding temperature. Their activity was monitored every hour until they died. A miracidium was considered dead when it was motionless and failed to respond to mechanical stimulation. Two trials of six plates each were performed on miracidia hatching from each egg source. Longevity

of miracidia at different temperatures and from the different egg sources was compared.

3.2d. Miracidial Infectivity: Effect of Miracidial Age

Dissected eggs were incubated at 14, 16 and 20 °C. Three newly hatched miracidia were placed in each well of a series of 24 - well tissue culture plates and returned to the corresponding incubator. At set intervals, (0, 1, 2, 3....12, 16, and 20 hours for each incubation temperature), a single snail (*Gyraulus parvus*, size class = 3.0 - 5.0mm) was added to each well and the plate returned to the incubator. (Snails were allowed to acclimate to the experimental temperatures for at least two days prior to infection.) Snails were exposed for two hours then transferred to plastic cups containing 250ml of culture water at the same temperature used in the experiment. The snails were held at this temperature for an additional 24 hours then kept at 20 ± 2°C for the duration of the experiment. Snails were dissected within 3-4 weeks pi and the number of redia found was recorded. The 1:1 ratio of redia to miracidium permitted a direct estimation of infection success. Three trials consisting of one plate (24 snails) at each interval were done at each temperature.

3.2e. Miracidial Infectivity: Effect of Egg Age

Dissected eggs were placed in five *Drosophila* vials, screened with 37μm mesh at each end (Lee *et al.*, 1992), and

placed in an aerated aquarium at 7 °C. At intervals of 0, 1, 3, 5 and 7 weeks, one randomly-selected container was removed, the eggs rinsed into a Petri dish and left to hatch at room temperature (20 ± 2 °C). Three newly-hatched (< 1/2 hour old) miracidia were added to each well of a 24-well tissue culture plate containing a single *G. parvus* (shell diameter of 3-5mm). The snails were exposed for 2 hours then removed from the wells and placed in plastic trays. They were dissected at 3-4 weeks pi and the number of rediae in each snail recorded.

3.2f. Statistical Analysis

The distribution of hatching times of *C. mutabile* eggs incubated at different temperatures was compared using multiple Kolmogorov-Smirnov 2-sample tests of pooled results of the two trials carried out at each temperature. Hatching success of eggs incubated at different temperatures and stored for different lengths of time at 12 °C prior to being raised to 14 °C were compared using the Wilks *G* statistic. Statistics and models used in the miracidial survivorship and infectivity portions of this experiment will be discussed in those sections of the results. In all cases, results were accepted as significant at $P < 0.05$. A Bonferroni adjusted critical value ($P \leq 0.05/2(3) = 0.008$) was used for the pairwise comparisons of hatching distributions and for infectivity success of miracidia of the same age but incubated at different temperatures if significant differences were found in the 2x3 analyses. A Bonferroni correction was also

employed when comparing the survivorship dynamics of miracidia from different egg sources and at different temperatures. In this case, five comparisons were to be made (between temperatures for each egg source (2) and between egg sources at each temperature (3)) and results were accepted as significant at $P < 0.005$.

3.3. Results

3.3a. Temperature-Dependant Hatching Dynamics

Mean hatching success of eggs incubated under the three temperature regimes did not differ significantly (G = 1.71, P = .426) (Fig. 3.1). The distribution of hatching times of eggs incubated at different temperatures differed significantly (K-S Z = 3.791, 3.711, 2.326 for pairwise tests of 14x16, 14x20, and 16x20 °C; $P < .0005$ for each pairwise test). More eggs hatched sooner at higher temperatures and a second surge in hatching was more pronounced at 20 °C than at lower temperatures (Fig. 3.2). Despite this difference, a general trend was seen at each temperature (Fig. 3.2). No hatching occurred within the first two hours at any temperature. The majority of eggs hatched within the first 12 hours. A second, smaller, peak occurred between about 22 and 30 hours incubation. A few eggs hatched between the two peaks and after the second peak. No eggs hatched beyond 6 days incubation.

3.3b. Egg Viability Following Prolonged Cold Storage

The hatching success of eggs from passed and dissected eggs stored for varying lengths of time at 12 °C before being raised to 14 °C is shown in Fig. 3.3. A heterogeneity G test showed the hatching success of the three trials using passed eggs did not differ significantly (G = 2.59, $0.25 < P < 0.50$) so the results from the three trials were pooled. No significant

Figure 3.1. Hatching success of *Cyclocoelum mutabile* eggs incubated at 14, 16 and 20 °C.

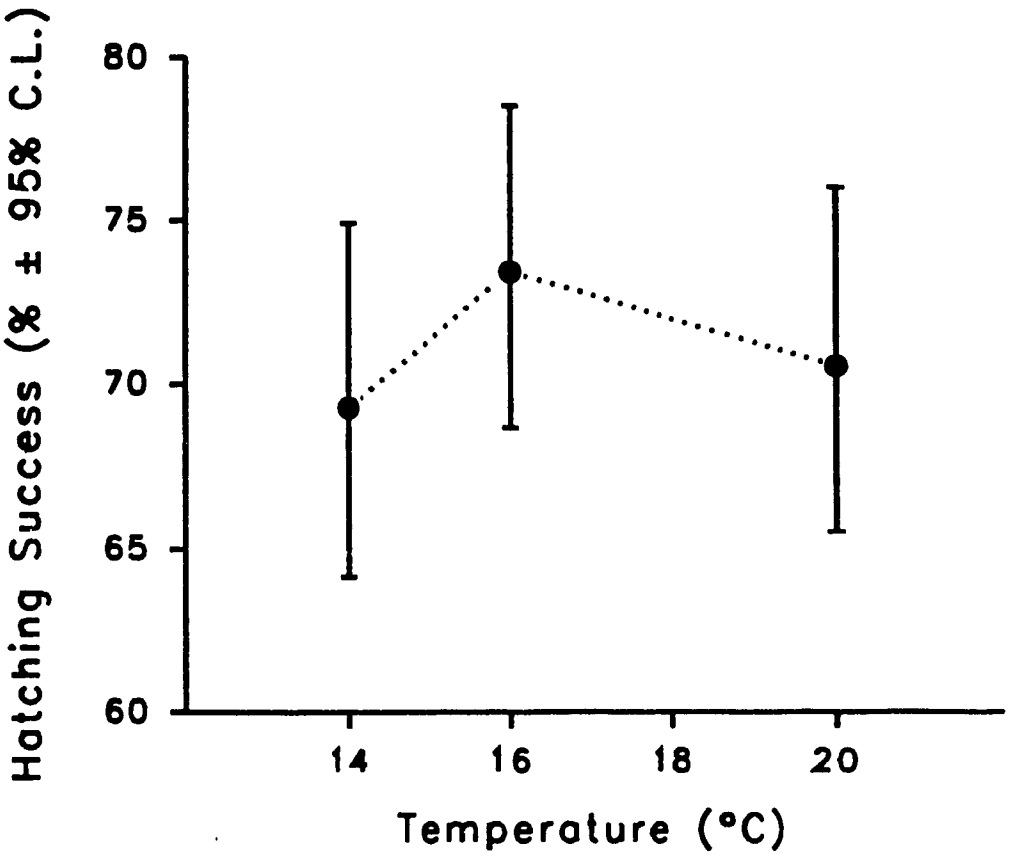

Hatching Success (% ± 95% C.L.)
80
75
70
65
60
14
16
18
20
Temperature (°C)

Figure 3.2. Hatching distribution of *Cyclocoelum mutabile* eggs incubated at 14, 16 and 20 °C. Arrows show the median of the distributions.

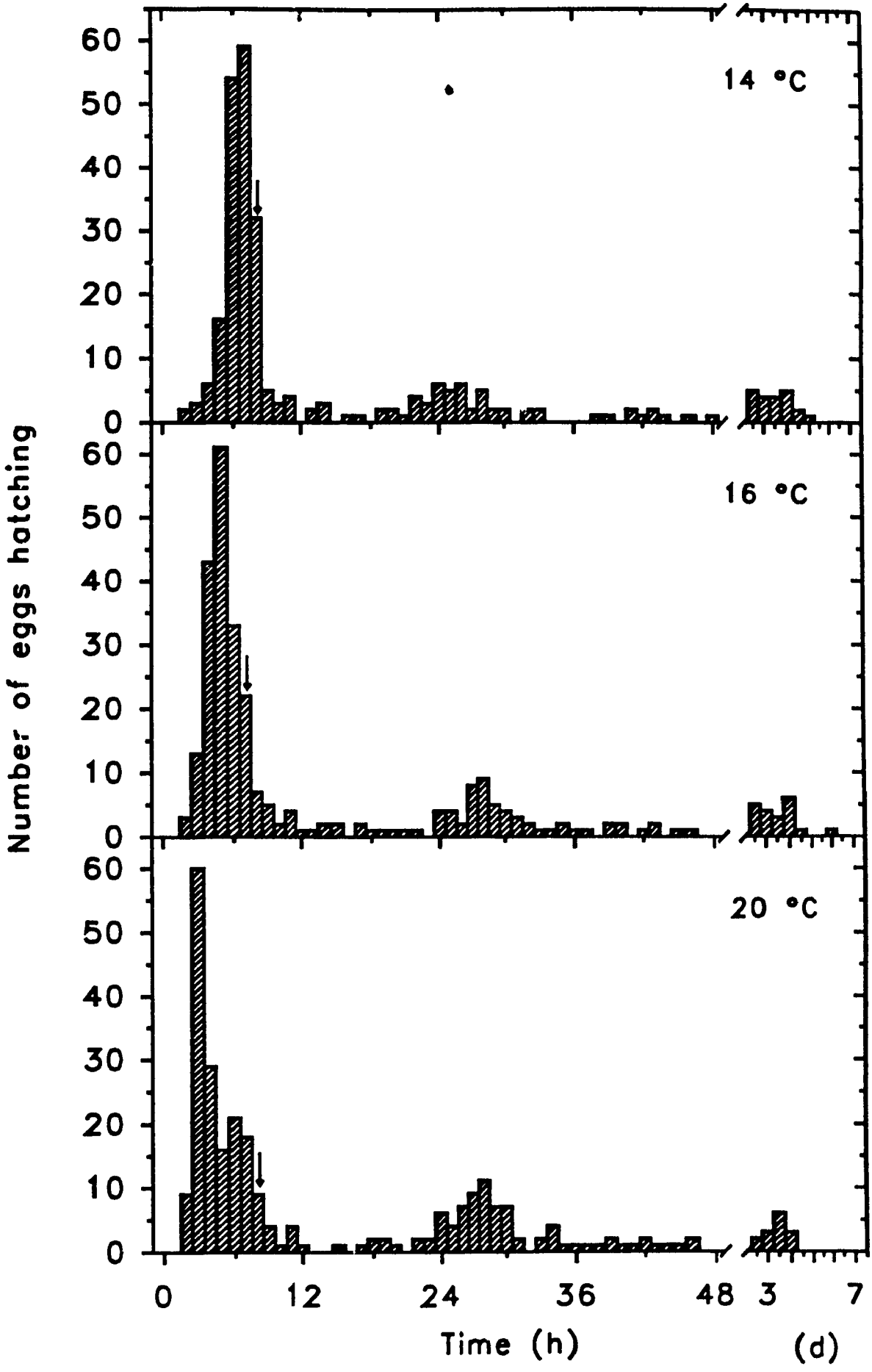
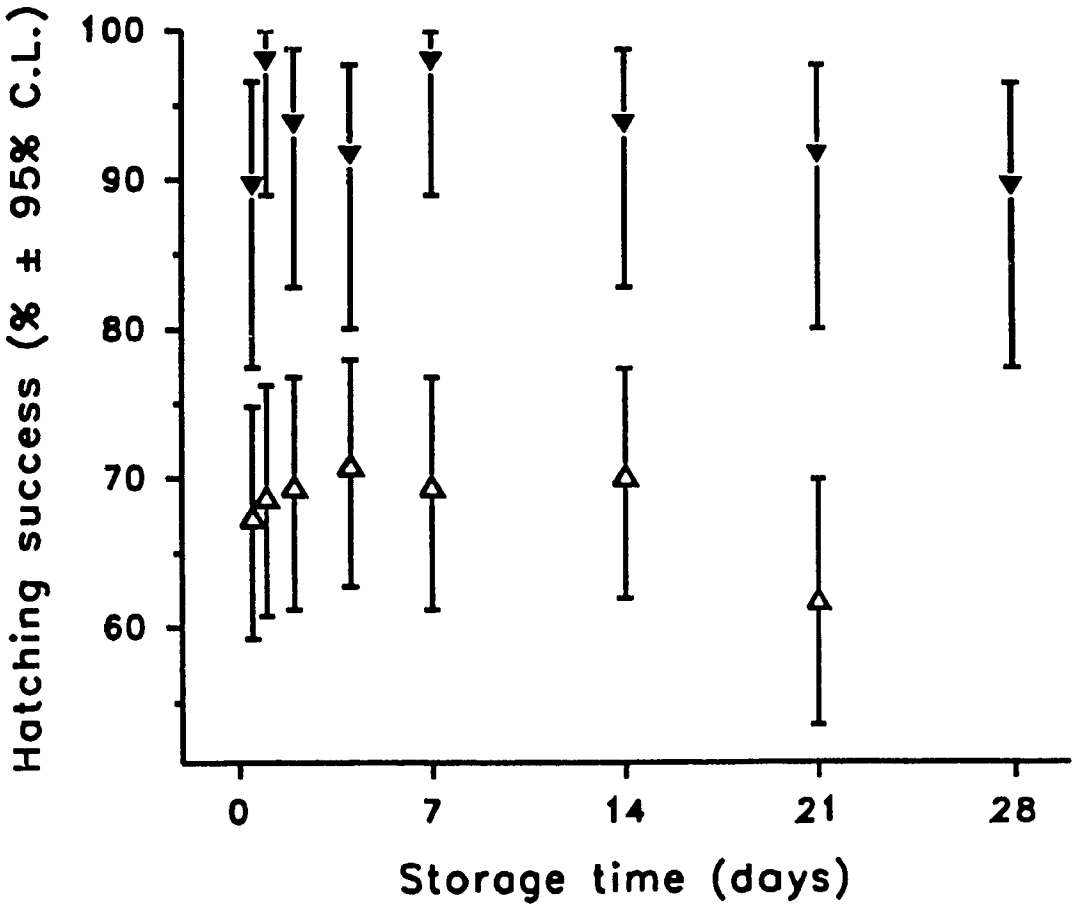

14 °C
16 °C
20 °C
Number of eggs hatching
0
10
20
30
40
50
60
0
12
24
36
48
3
7
Time (h)
(d)

Figure 3.3. Hatching success of *Cyclocoelum mutabile* eggs stored for varying lengths of time at 12 °C prior to being raised to 14 °C. (▲), eggs from coot feces. (▼), eggs dissected from worms.


Hatching success (% ± 95% C.L.)
100
90
80
70
60
0
7
14
21
28
Storage time (days)

difference in hatching success of eggs from coot feces kept in cold storage for 12 hours to 3 weeks prior to being raised to 14 °C (G = 4.22, P = 0.647) was found. The hatching success of eggs stored at 12 °C then raised to 14 °C was similar to the hatching success of eggs incubated at 14 °C in experiment 3.3a (G = 0.76, P = 0.382).

There were also no significant differences in the hatching success of eggs from dissected worms stored for between 12 hours and 4 weeks at 12 °C when warmed to 14 °C (G = 6.65, P = 0.466).

A G test of partial independence showed highly significant differences in the hatching success of eggs derived from the two sources (G = 115.93, $P < 0.0005$). The mean hatching success of dissected eggs at 12 °C was 93.75%, fully 25% higher than that seen in eggs isolated from coot feces and stored for the same period.

None (0/48) of the dissected eggs from control plates and very few of the control eggs from coot feces (one each at 12, 24 and 48 hours and 1 and 2 weeks of the total of 144 control eggs) hatched.

3.3c. Miracidial survival

The survivorship of the miracidia hatching from passed and dissected eggs is shown in Fig. 3.4 (data points represent the mean proportion of miracidia alive at time t, estimated from the two trials). Survival time depends on the temperature. At 20 °C survivorship begins to decline almost immediately whereas the

majority of miracidia at 16 and 14 °C were alive after 10 and 15 hours, respectively. Once mortality starts, the decline was exponential.

Anderson & Whitfield (1975) have developed a pair of widely used models that describe, respectively, the age - dependent survivorship and instantaneous death rates of larval digeneans such that:

$$P_t = \exp[(a/b)(1-\exp(bt))] \qquad \text{(eqn. 3.1)}$$

and

$$\mu_t = a\exp(bt)\,. \qquad \text{(eqn. 3.2)}$$

Here, P_t is the proportion of miracidia alive at age t, μ_t the age - dependent instantaneous death rate of the miracidia, and a and b are constants estimated by nonlinear regression of the survivorship data obtained from the separate trials pooled for each egg source / temperature combination. The constant a represents the instantaneous death rate of miracidia at hatching (t=0); b represents the magnitude of the increase in mortality as the miracidia age. The observed age-dependent instantaneous death rates at the different temperatures were calculated as

$$\mu_t = \ln N_t - \ln N_{t+1} \qquad \text{(eqn. 3.3)}$$

and are shown as data points in Fig. 3.5 (data points represent the mean instantaneous death rates of miracidia at time t, estimated from the two trials), where N_t is the number of miracidia alive at time t and N_{t+1} the number of miracidia alive

Figure 3.4. Survivorship of *Cyclocoelum mutabile* miracidia hatching from eggs obtained from two sources at three temperatures. (▲), eggs from coot feces. (▼), eggs dissected from worms. (———), best fit curves from the model (equation 3.1) described in the text. See Table 3.1 for parameter estimates and fit of the model.

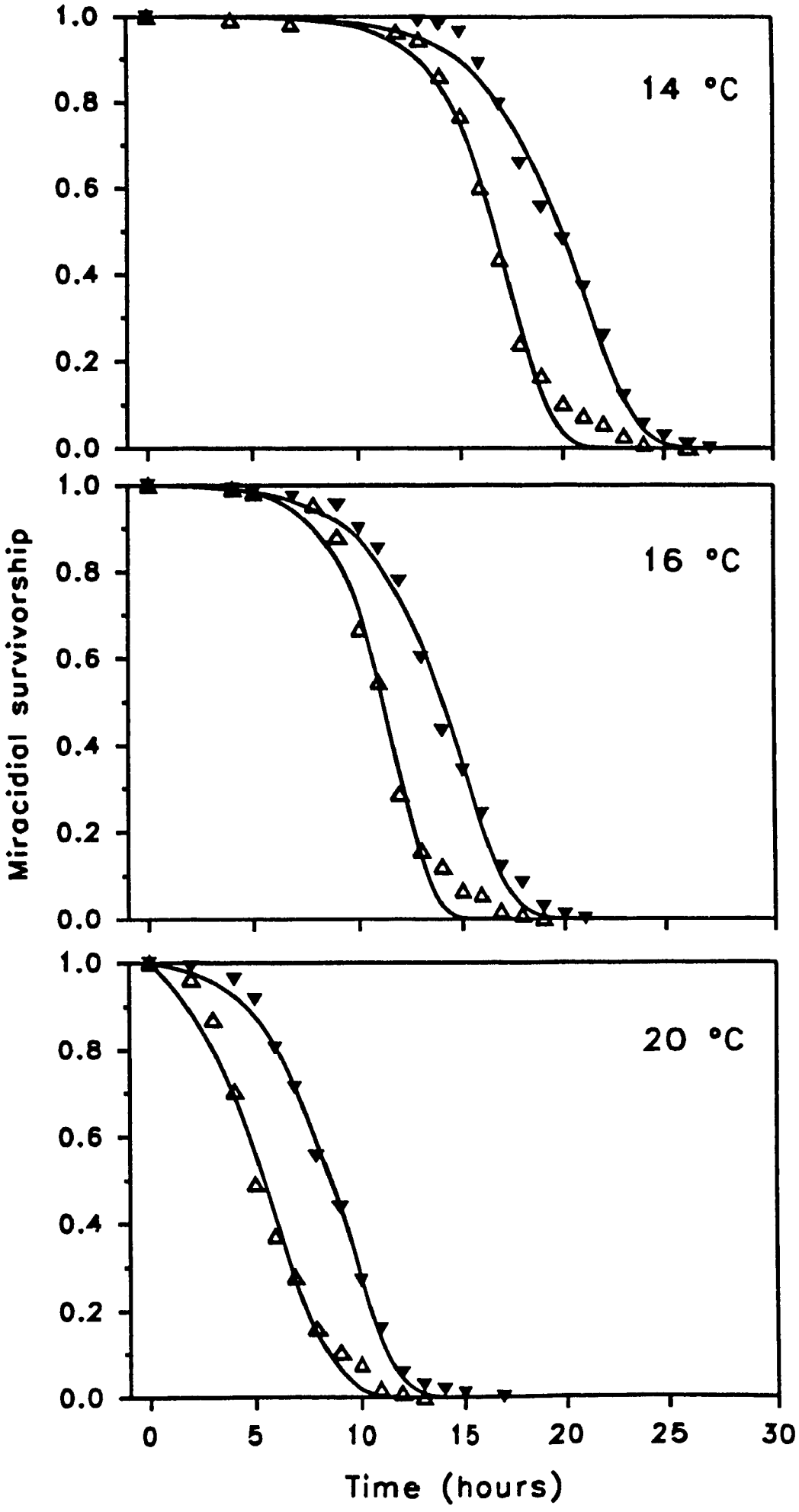

14 °C
16 °C
20 °C
Miracidiol survivorship
1.0
0.8
0.6
0.4
0.2
0.0
0
5
10
15
20
25
30
Time (hours)

Figure 3.5 Age - dependent instantaneous death rates of *Cyclocoelum mutabile* miracidia hatching from eggs obtained from two sources at three temperatures. (▲), eggs from coot feces. (▼), eggs dissected from worms. (———), curves from the model (equation 3.2) described in the text. See Table 3.1 for parameter estimates.

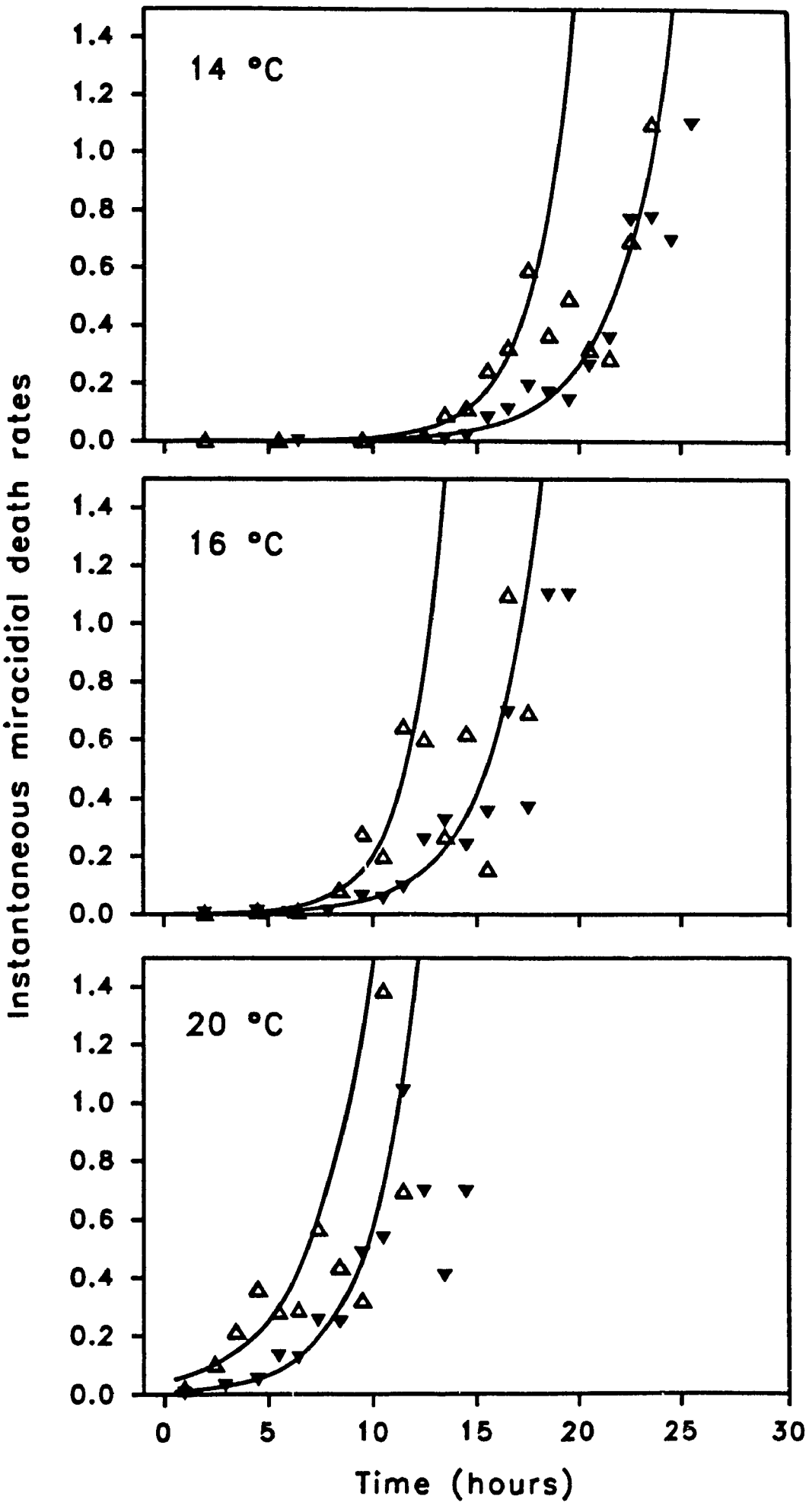

14 °C
16 °C
20 °C
Instantaneous miracidial death rates
Time (hours)
0.0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
0
5
10
15
20
25
30

Table 3.1. Constant values, maximum lifespans (T_{max}), calculated values of L (equation 3.4), and fit of the model (equation (3.2)) to the experimental miracidial survivorship data for *Cyclocoelum mutabile* eggs obtained from both dissected worms and isolated from coot feces and incubated at 14, 16 and 20 °C.

Egg Source	Temp (°C)	a (± 95% CL)	b (± 95% CL)	r^2	T_{max}	L (hrs)
feces	14	0.00009 (0.00009)	0.491 (0.071)	.993	26	16.36
feces	16	0.00061 (0.00068)	0.578 (0.114)	.990	20	10.86
feces	20	0.04161 (0.01765)	0.356 (0.099)	.986	16	5.30
worm	14	0.00015 (0.00012)	0.373 (0.047)	.992	24	19.40
worm	16	0.00096 (0.00054)	0.405 (0.047)	.994	20	18.41
worm	20	0.00722 (0.00201)	0.436 (0.040)	.997	14	8.25

at time t+1 (Anderson & Whitfield, 1975). Mortality was seen to increase much sooner at higher than at lower temperatures. These models (eqns. 3.1 and 3.2) provide a very good fit to the observed data for each experimental trial (Table 3.1 and Figs. 3.4, 3.5).

While miracidial longevity may be compared by determining either the maximum survivorship of a miracidium in a population (see Table 3.1) or any of the measures of central tendency commonly considered (i.e., the mean, mode or median), none considers the entire distribution of the survival data. Bundy (1981a) suggested considering the mean expected lifespan of the miracidia in a population, L, such that

$$L = \int_{t=0}^{T_{max}} P_t dt. \qquad \text{(eqn. 3.4)}$$

Here, T_{max} is the maximum lifespan of a miracidium observed under experimental conditions. The calculated values for L are reported in Table 3.1. However, computation of these values does not readily lead to an effective method of comparison. Further, even though the model provides an excellent fit to the observed data in all cases, these values imply that the decline in miracidial populations is a simple function of age at all points along the survivorship curve; an assumption that may not be correct due to the relevant biological characteristics of the parasite (Holland, 1990).

As a way to circumvent this problem, Holland (1990) suggests the application of statistics designed to take into account the

entire detailed shape of the survivorship curves. Such models incorporate differences between the various survivorship curves at each time point into the test statistic. These models, such as the Lee-Desu algorithm providing the *D* statistic (SPSS Inc., 1990), incorporate differences between the different survivorship curves at each time point into the test statistic. When this is done, highly significant differences in survivorship were apparent between the different treatments ($D = 497.75$, $P < 0.00005$). When miracidial longevity is compared between temperatures for miracidia derived from each egg source, we see that in both cases, miracidia at lower temperatures live longer than those incubated at higher temperatures ($D = 244.16$ and 242.64 for passed and dissected eggs, repectively, $P < 0.00005$). Comparison of the longevity of miracidia between egg sources at the same temperatures also differs significantly, those hatching from eggs dissected from worms living longer than those obtained from coot feces in all cases ($D = 50.98$, 50.13, and 55.72 for miracidia at 14, 16 and 20 °C, respectively, $P < 0.00005$).

3.3d. Miracidial Infectivity: Effect of Aging

The miracidia of *C. mutabile* inject a single redia into the snail host. As this is the only redial generation produced by each infection, the number of miracidia infecting a snail in multiple exposures can be determined by a direct count of the rediae that develop. The proportion of miracidia at different ages that were infective to snails at 14, 16, and 20 °C is shown

in Fig. 3.6. Stepwise logistic and simple linear regression were used to determine the relative significance of miracidial age, incubation temperature and the interaction between these two factors on the infectivity of the miracidia. The logistic regression was more appropriate for the model but the results of the linear regression are easier to interpret. Tabachnick and Fidell (1989) state that unless the responses (in this case a miracidium being infective or not infective) are split with less than 25% of the outcomes in either category, the results from either analysis should yield the same results.

Both analyses yielded similar results. Infectivity of miracidia was best explained by the interaction between age and temperature. However, both miracidial age and, to a lesser extent, environmental temperature, also explain significant amounts of the variance in the model (Table 3.2). Partitioning of the results showed that infectivity did not differ between temperatures at hatching or in the first two hours (*Wilks G* test, $0.60 < P < 0.90$). Infectivity of miracidia incubated at 20 °C began to decline after 4 hours ($G = 34.65$, $P < 0.0005$), whereas the infectivity at both 14 and 16 °C began to decline after only 2 hours ($G = 11.83$, $P = 0.008$; and $G = 25.38$, $P < 0.0005$, respectively). Once initiated, the decline in infectivity was greatest at 20 °C and lowest at 14 °C (ANCOVA, $F = 13.85$, $P < 0.0005$).

Figure 3.6. Age - dependent infectivity of *Cyclocoelum mutabile* miracidia to 3-5mm *Gyraulus parvus* at three different temperatures. Solid vertical lines show nonsignificant differences in infectivity of miracidia between temperatures at a given time (G test, $P < 0.05$).

% miracidia infective
0
20
40
60
80
Age of miracidia (h)
0
5
10
15
14 °C
16 °C
20 °C

Table 3.2. The influence of miracidial age and environmental temperature on the transmission of *Cyclocoelum mutabile* to snails: Value and significance of the coefficients of the model: *Miracidial infectivity = Constant + Temperature + Age + Temperature * Age*. Fit of the model: $r^2 = 0.343$.

Variable	Coefficient Value	Weighted Value	T	Significance
Interaction	- 0.007490	- 1.146435	- 17.226	0.0000
Temperature	0.018239	0.088713	5.901	0.0000
Age (h)	0.057149	0.591451	8.522	0.0000
Constant	0.513777		10.147	0.0000

The age dependent instantaneous rates of infection, β_t, may be calculated (Anderson, 1978) from the redial recovery data. In this case, this parameter is defined as:

$$\beta_t = -\ln(1-R/M_0)/E, \qquad \text{(eqn. 3.5)}$$

where R is the mean number of rediae recovered per snail, M_0 is the number of miracidia present in each well at $t=0$, and E is the exposure time. These rates are shown graphically in Fig. 3.7. Although a number of authors have developed various models to describe these types of data (e.g. Anderson, Whitfield & Mills, 1977; Evans & Gordon, 1983a), a model of the form

$$\beta_t = x + yt^z \qquad \text{(eqn. 3.6)}$$

provided the best, although strictly empirical, description of the data (see Fig. 3.7).

Although the instantaneous rates of infection appear to supply little more information than the proportion of miracidia successfully infecting snails, they permit calculation of a single summary statistic of infectivity, $\overline{\beta}$, the mean instantaneous rate infection of the miracidia over the infective lifespan of the miracidia (Evans, 1985). This is calculated by dividing the area under each infection curve in Fig. 3.7 by the corresponding infective lifespan of the miracidia. Calculation of these values for each incubation temperature (given in Table 3.3), reveals the mean instantaneous infectivity ($\overline{\beta}$) was highest at the highest temperature, 20 °C.

May & Anderson (1978) have shown that the transmission

Figure 3.7. Instantaneous rates of infection of *Cyclocoelum mutabile* miracidia to 3-5mm *Gyraulus parvus* at three different temperatures. (●), values derived from the model (equation 3.5) described in the text. (———), best fit curves from the model (equation 3.6) described in the text.

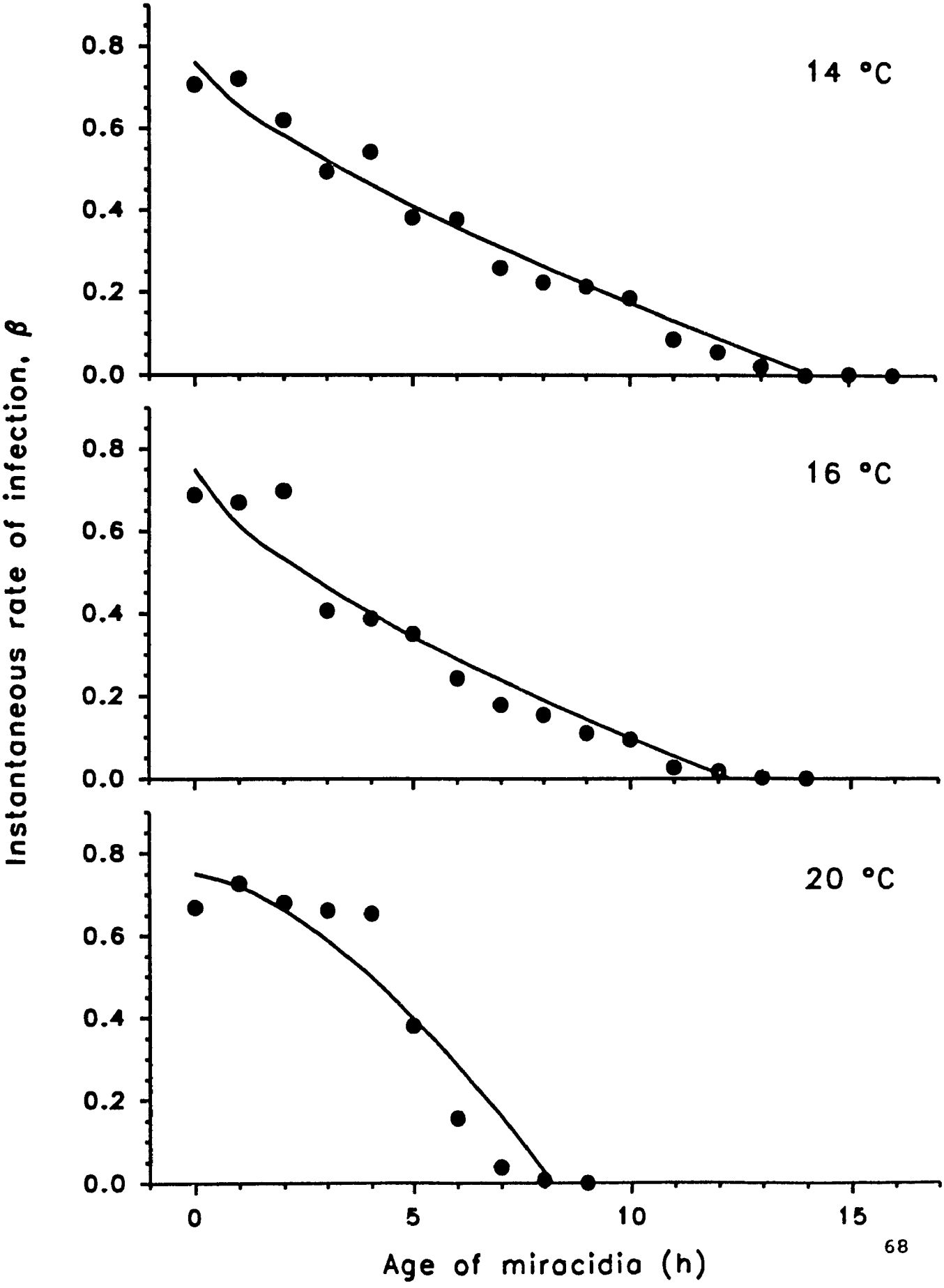

Instantaneous rate of infection, β
0.8
0.6
0.4
0.2
0.0
14 °C
16 °C
20 °C
0
5
10
15
Age of miracidia (h)

Table 3.3. The influence of temperature on the transmission parameters of *Cyclocoelum mutabile* miracidia.

Temperature	$\overline{\mu}$	$\overline{\beta}$	$\overline{\beta}/\overline{\mu}$
14	0.010	.284	28.728
16	0.049	.262	5.381
20	0.092	.408	4.446

efficiency of parasite infective stages, H_o, may be estimated by the ratio of the death rate of the infective stages, μ (in this case the miracidia), to the rate at which the infective stages invade the host, β (in this case the snail). This value varies inversely with the proportion of infective stages that establish in the host. A more intuitive value for the representation of transmission efficiency is the inverse of this value, $1/H_o$, which is estimated (Evans, 1985), as

$$\overline{\beta}/\overline{\mu}. \qquad \text{(eqn. 3.7)}$$

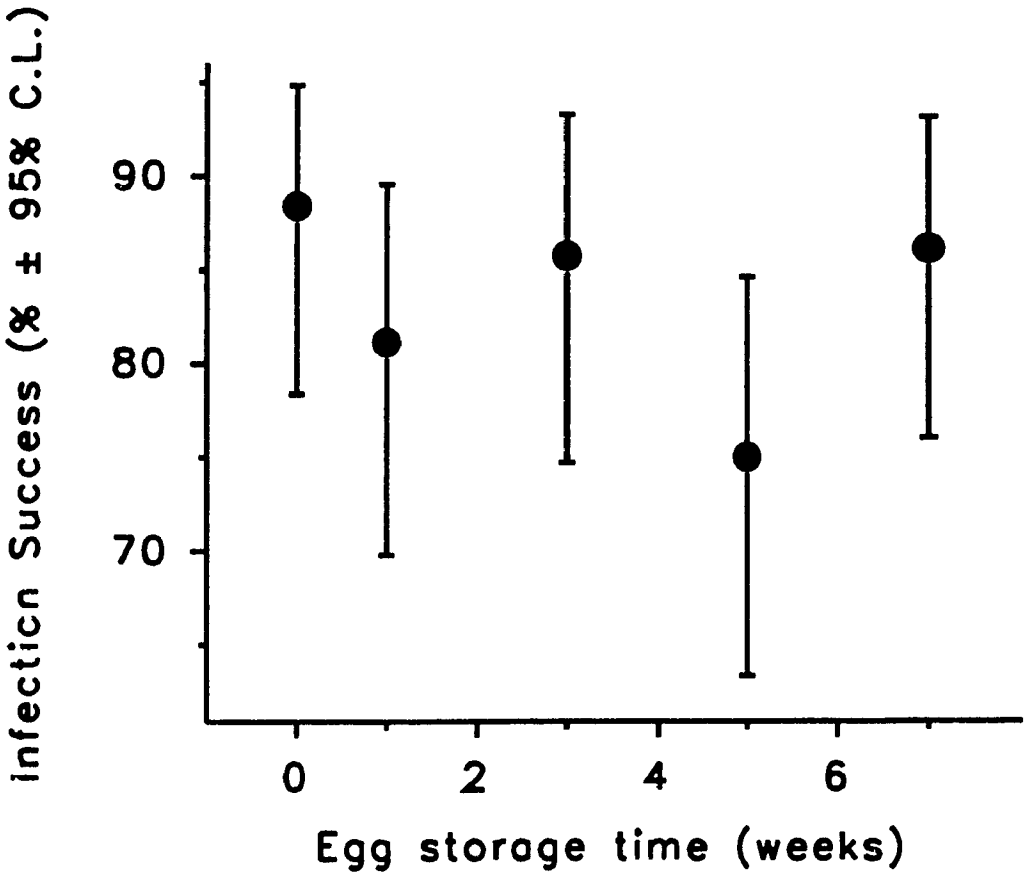
These values are presented in Table 3.3. The mean instantaneous death rate of the miracidia, $\overline{\mu}$, is calculated by division of the area under the various curves shown in Fig. 3.5 by the corresponding infective lifespan of the miracidia. Even though the mean instantaneous rate of infection was found to be the highest at 20 °C, the associated low survivorship of miracidia at this temperature acts to produce the lowest transmission efficiency (Table 3.3). Conversely, by virtue of miracidial survivorship being highest at the lowest incubation temperature, the transmission efficiency was highest at 14 °C, despite the comparatively low mean instantaneous rate of infection observed at this temperature.

3.3e. Miracidial Infectivity: Effect of egg age

The infection success of miracidia hatching from eggs stored for 0, 1, 3, 5 and 7 weeks at 7 °C prior to being raised to 14 °C did not differ significantly (G = 5.53, P = .237). Results are

shown in Fig. 3.8.

Figure 3.8. Infectivity of *Cyclocoelum mutabile* miracidia hatching from eggs stored for 0, 1, 3, 5 and 7 weeks at 7 °C prior to hatching when raised to 14 °C.


Infection Success (% ± 95% C.L.)
90
80
70
0
2
4
6
Egg storage time (weeks)

3.4. Discussion

When coots return to the breeding grounds in April and May, they arrive with patent infections of *C. mutabile* (Colbo, 1965; McLaughlin, 1986). At this time, water temperatures are low as is both the number and diversity of snail species. Because of its influence on both survival and infectivity, temperature is one of the most important factors influencing the transmission dynamics of free-living stages of larval parasites (Evans, 1985). When the infective stage considered is a miracidium, this assertion can be extended to include the effect of temperature on the viability of the precursor of the infective stage, the egg, as well as its effect on the timing of egg hatching.

In most digenean life cycles, eggs are shed in an unembryonated state. Thus, such eggs require a period of time in the external environment to embryonate before they hatch. Developmental rates of such eggs are temperature dependent. In many species development does not occur below a certain temperature (Erasmus, 1972). In others, embryonation may occur slowly, even at low temperatures (Campbell, 1961; McKindsey & McLaughlin, 1993). Eggs of most digeneans in migrant waterbirds are of this type and when passed simply accumulate, embryonate and hatch once a species-specific temperature threshold is reached.

Since the eggs of cyclocoelids are fully embryonated when shed, no temperature-mediated developmental delay occurs.

Temperature only affects the survival of the eggs or inhibition of hatching until a threshold temperature is reached. Results from this study revealed that a temperature threshold for *C. mutabile* egg hatching does exist. Eggs held at 12 °C failed to hatch. However, eggs held at 12 °C for up to 3 or 4 weeks hatched spontaneously and showed no decline in hatching success when the incubation temperature was raised to 14 °C.

A number of other researchers have examined the viability of digenean eggs following prolonged storage and found variable results. Eggs of *Schistosoma japonicum* may remain viable for one week in host feces (Garcia, 1976, in Jourdane & Théron, 1987). Eggs of *Dicrocoelium dendtriticum* may also display prolonged survival when on pastures (Alunda & Rojo-Vázquez, 1983, and references therein). Both these species have eggs that are embryonated when shed. Other studies examining the survivorship of embryonated eggs have used species that shed unembryonated eggs. These studies have examined eggs that were allowed to embryonate normally but some key factor required for hatching, such as exposure to light or an increase in incubation temperature, was not provided until some later date. Griffiths (1939) reported that eggs of *Fasciola hepatica* hatched normally and within minutes of being raised to room temperature following incubation at 2 - 4 °C for up to 37 days. The resulting miracidia appeared and behaved normally. Eggs stored for up to 14 months also hatched but the resulting miracidia were smaller and slower (Griffiths, 1939). When the incubation temperature of

embryonated *Hysteromorpha triloba* eggs kept at 8 °C for over a week was raised, many failed to hatch (Hugghins, 1954). Embryonated eggs of *Echinostoma liei* maintain their hatchability for at least three weeks at 4 °C but hatching success is reduced after six weeks storage (Christensen, Frandsen & Roushdy, 1980).

It may not be sufficient to use hatching success as the sole criterion for evaluating the impact egg age has on the potential infectivity of *C. mutabile* miracidia. *Echinostoma liei* miracidia hatching from eggs stored for 30 days or longer displayed reduced infectivity when compared to miracidia hatching from eggs stored for 23 days or less (Kuris, 1980). In contrast, this study has shown the infectivity of *C. mutabile* miracidia hatching from eggs stored for up to 3 weeks at 7 ° C to remain unchanged. The reduced viability of the *E. liei* miracidia may have been due in part to the high incubation temperature of the eggs prior to being stimulated to hatch (27 °C as opposed to 7 °C in this study).

In most digeneans with eggs requiring a period of embryonation outside the host, the hatching distribution is positively skewed. This results in a peak with most eggs hatching over a few days and the remainder hatching over a prolonged period. The eggs of *C. mutabile* are already embryonated when shed and hatching is initiated within hours, yet they too showed an extended hatching distribution. Peak hatching at all temperatures occurred in the first 12 hours and eggs continued to hatch over the next few days. What was not expected

was the second minor surge in hatching that began about 24 hours after the first.

Factors controlling this second surge are unknown, however, environmental factors including daylight cycle are known to influence the hatching of trematode eggs. Bundy (1981b) observed that eggs of *Transversotrema patialense* hatched almost exclusively in the daylight hours, even when incubated from the start of embryonation under 24 hr light conditions. He attributed this to endogenous factors with darkness acting as a negative feedback control, halting hatching. MacDonald (1975), working with three species of *Diclodophora* (Monogenea), found that a definite daylight-dependent hatching pattern existed for each of the species examined. Both these authors concluded that the hatching patterns they observed could be related to the daily behaviour of their next host, a snail in the case of *T. patialense*, a fish in the case of the *Diclodophora* spp.

A daylight - dependent mechanism does not explain the pattern observed in this study. The experiments in this study were initiated at different times of the day, and when hatching is plotted against the time of day, rather than the time the experiment was initiated (i.e., when the eggs were first placed in water), the bimodal distribution in hatching times becomes less clear. The hatching of *C. mutabile* eggs appears to depend on contact with water and once initiated continues regardless of the time of day. Light may have an enhancement effect on hatching. Schistosomes, like *C. mutabile*, hatch shortly after

contact with water and hatching success, while not dependent on light, is increased by it (Jourdane & Théron, 1987).

Miracidia of many digeneans, including *C. mutabile*, possess eyespots and may respond either positively or negatively to light. In either case, the initial response acts to concentrate miracidia in regions where they are likely to encounter the snail host (Smyth & Halton, 1983). Most of the snails that serve as intermediate hosts for *C. mutabile* are aquatic pulmonates that reside near the surface so there would be some advantages to hatching during the day. Coots are primarily diurnal and presumably begin to defecate when they become active at dawn. As the second peak occurs about 24 hours following the first, Eggs hatching both days would do so in the daylight.

Alternatively, the adult fluke may regulate the timing of release of eggs on a circadian basis, as has been shown to occur in a number of schistosome species (see review by Hawking, 1975) and, in this way, regulate the time of day the eggs hatch. Unfortunately, coots in captivity do not defecate as regularly as they would in the field and this aspect could not be addressed further.

There are both theoretical and empirical reasons to expect animals living in unpredictable environments to have phenotypically variable offspring (see Shostak & Dick, 1989). Variability in the timing of egg hatching increases the chances of at least some of the miracidia hatching in an environment with snails present. The bimodal hatching distribution seen in these

experiments would increase the temporal dispersion of the miracidia in the environment, yet still allow a great majority of the miracidia to hatch at a time of day when transmission to the snail host should be most efficient, i.e., in daylight hours. As coots had multiple infections from different sources, we cannot attribute the observed bimodal hatching distribution to differences in eggs among worms or to differences between eggs from the same individual. Studies on eggs from single fluke infections, preferably from different sources, could resolve the issue.

Miracidial survival studies showed that survivorship was not only a function of temperature, as expected, but was also dependent on the source of the eggs (see Fig. 3.4). This may help explain the heterogeneity in the survivorship of miracidia of the same species reported in the literature and should be considered when comparing studies. The reasons why miracidia from dissected eggs survive longer than those from passed eggs are not readily apparent. Weina (1986) has reported source-dependent developmental times for eggs of *Paragonimus kellicotti*. Eggs from cat feces required at least ten days longer to embryonate than eggs obtained from the lung but no explanation for this observation was offered. Eggs dissected from the worm are slightly younger than those obtained from the feces, and lower survival may be the result of older miracidia (in passed eggs) having consumed a greater portion of their limited endogenous energy reserves while still in the bird host.

Alternatively, factors associated with passage through the gut may have had an effect. The adults of both species infect the respiratory system and eggs must pass through intestine as well as the acid conditions in the proventriculus. This observation also suggests that when hatching or infection studies are performed using eggs that are not passed by the normal route (as is the case in this study, and often the case in studies of schistosomes), the results should be interpreted with caution.

The observation that miracidia do not remain infective over their entire lifespan is in agreement with results reported for other species (e.g. Cherin, 1968; Christensen, Nansen & Fradsen, 1976a; Anderson, 1978; Prah & James, 1977; Evans & Gordon, 1983a; Anderson, Mercer, Wilson & Carter, 1982; Smith & Grenfell, 1984; Evans, 1985; Waadu, 1991). Many of these studies were conducted by allowing miracidia to age and then selecting actively swimming individuals of a given age to challenge snails. Some of the other studies involved placing snails in containers with large numbers of aged miracidia and then examining them at some later date for infections. Neither of these methods provides information on the entire population of miracidia in question, only of the longest lived or most infective miracidia in that population.

Results from this study show that environmental temperature has no effect on the infectivity of newly hatched *C. mutabile* miracidia. It does, however, have an effect on the length of time miracidial infectivity remains maximal. Miracidia incubated

at 20 °C exhibited a decline in infectivity after four hours while those incubated at 14 and 16 °C exhibited a decline in infectivity after only two hours. This observation may be explained by miracidial invasiveness being related to activity (Anderson, 1978). Miracidial activity is positively correlated with environmental temperature and negatively with its age (Wilson & Denison, 1970; Prah & James, 1977). Thus, miracidia hatching at higher temperatures may start and remain above a certain activity threshold required for successful penetration of a snail for a longer period than miracidia incubated at lower temperatures. However, once below this threshold, a sharp decline in infectivity is observed at 20 °C. In contrast, miracidia at lower temperatures may start at lower levels of activity (but still above the threshold) with many remaining above this threshold for a longer period as is seen by the slow decline in the infectivity curves. Other factors may also act to decrease the infectivity of older miracidia. Prechel & Nollen (1979), for example, have shown that older miracidia of *Megalodiscus temperatus* do not respond to stimuli given off by snails.

Temperature also had a pronounced effect on the overall transmission efficiency. Transmission efficiency was much higher at 14 °C than at either 16 or 20 °C. The greater longevity at 14 °C offsets the comparatively low mean instantaneous rate of infection observed at this temperature. Although the mean instantaneous rates of infection were greatest at 20 °C, the

higher rates of miracidial mortality lowered transmission efficiencies. This finding is in contrast to similar studies on miracidia (Anderson *et al.*, 1982) and cercariae (Evans, 1985). These studies have shown that high instantaneous rates of transmission observed at higher temperatures are offset by lower survival rates, producing a wide temperature range over which transmission efficiencies are high. The temperature range, over which transmission is optimal, is also one that is optimal for the snail hosts (Anderson *et al.*, 1982; Evans, 1985).

In this study, maximal transmission efficiency occurred at lower temperatures just above the threshold level required for hatching. Thus, efficiency is greatest early in the year when young snails are appearing. The prolonged searching ability of miracidia at this time is important in the transmission of the parasite as snail populations are not as dense at this time as later in the season. The emergence of new snail populations is important as Anderson (1978) and Wilson & Taylor (1978) have shown the net rate of infection of the snail host is proportional to the density of miracidia and snail hosts present in a system. Also, Anderson *et al.* (1982) have demonstrated that transmission efficiencies may only be expressed maximally under high host densities. Although miracidial transmission efficiency would be lower when temperatures are higher in late spring, this would be offset as snail densities increase throughout the spring.

In summary, this study has shown that 1) although the distribution of hatching times of eggs is influenced by

temperature, hatching success is not. 2) A temperature threshold exists between 12 and 14 °C, below which hatching does not occur. 3) Eggs kept below this threshold remain viable for at least 3 weeks (4 weeks if the eggs are dissected from worms) and 4) miracidia hatching from eggs stored at 7 °C for up to 7 weeks remain viable and may infect snails upon hatching. Thus *C. mutabile* eggs deposited in early spring would accumulate until water temperatures rose to 14 °C, then hatch spontaneously and successfully infect snails.

Pulmonate snails in temperate regions typically begin oviposition once water temperatures reach about 7 - 11 °C and die soon after (Duncan, 1975; McMahon, 1983). Surface water temperatures warm much sooner than bottom temperatures (Wetzel, 1983) (see also Fig. 5.5 for temperature regime recorded in the study area used in this thesis). Thus, young snails would be available by the time the bottom temperatures reached 14 °C and the fluke eggs begin to hatch. Such a delay would prevent hatching into an environment with a low density of snails, many of which will die as soon as they reproduce. As expected, miracidial longevity was found to be a function of incubation temperature but it was also found to be a function of the source of the eggs from which the miracidia hatched. Temperature also had a significant influence on the infective life span of miracidia. Differences in infective lifespans and mortality rates at different temperatures showed that maximal transmission efficiencies occurred at 14 °C, the hatching threshold required

of *C. mutabile* eggs.

Taken together, these results suggest that *C. mutabile* is extremely well adapted for transmission to snails in the spring. Eggs deposited by coots in early spring would collect until bottom temperatures reached 14 °C. These would then hatch *en masse*. Most of these miracidia would be infective as no loss in infectivity was apparent in miracidia hatching from eggs stored for up to 7 weeks at 7 °C. The low temperatures would allow the miracidia to search for widely dispersed snails before infectivity declined greatly. Eggs deposited later in the spring when temperatures are higher would also contribute to the development of the *C. mutabile* infective pool in the snail community as higher snail densities at this time would offset lower transmission efficiencies. The reduction in the number of intramolluscan stages in the life cycle would result in the development of a new metacercarial generation by mid summer, when coots begin to acquire new infections.

Chapter 4.

Experimental observations on the specificity of *Cyclocoelum mutabile* miracidia toward intermediate snail hosts

4.1. Introduction

The majority of digeneans exhibit a high degree of specificity for the first intermediate host in their life cycle (van der Knaap & Loker, 1990). Thus, snails harbouring sporocyst or redial infections are usually aggregated in the environment (Kennedy, 1976). As a consequence, snails harbouring sporocysts or rediae are rarely encountered. This aggregated distribution is offset by a low specificity of the cercarial stage. Typically, the cercariae emerge from the snail and, during a brief free-swimming existence, locate and encyst in a broad range of second intermediate hosts. Broader specificity at this level results in a wider spatial distribution of the parasite within the environment and increases the probability of transmission of the parasite back to the definitive vertebrate host (Evans, Whitfield & Dobson, 1981).

Cyclocoelum mutabile, a parasite of coots (*Fulica americana*), differs from most flukes in that it has no free swimming cercariae in its life cycle. Instead, cercariae encyst as metacercariae within the same snail in which they are produced. Since there is no dispersal of the parasite in space, *via* a free-swimming cercariae, we would expect an aggregated

distribution of the parasite in the snail population, which would also be reflected in the definitive coot host. However, this does not seem to occur. Up to 80% of juvenile coots become infected each year (McLaughlin, 1986), suggesting the parasite is well distributed in the environment.

This suggests two possible transmission processes. Either the parasite may infect a large number of individuals (including individuals of different sizes) of a particular snail species, or it may infect a variety of snail species. While the former option would work well in some potholes (ponds), different potholes may have very different snail communities (Pip, 1978; personal observations) and the requisite snail species may not always be present. The second option, low first host specificity, could accomplish the same thing. McLaughlin (1976) infected a small number of several snail species during studies on the life cycle of this fluke and found all to be susceptible. Thus low specificity for the snail host could result in metacercariae of *C. mutabile* being widely dispersed in a snail community, regardless of its composition.

Metacercariae of *C. mutabile* are apparently unavailable to returning coots in the spring (McLaughlin, 1986). As adult coots loose their infections by late spring (McLaughlin, 1986), the miracidia responsible for the infections seen later in the summer would necessarily have hatched from eggs deposited earlier in the spring. Transmission of the parasite back to coots depends on the successful establishment of new infections in whatever snails

are present at the time the fluke eggs hatch.

The factors underlying infectivity of miracidia are complex. Infectivity is known to be a function of not only the species of snail challenged (e.g. Boray, 1966; Mamo & Redda, 1989), but also of the size (or age) of the snail being challenged (e.g. Huffman & Fried, 1990; Niemann & Lewis, 1990), the environmental temperature (e.g. Chu, Massoud & Sabbaghian, 1966; Christensen, Nansen & Frandsen, 1976a), as well as combinations of these factors (e.g. Gold & Goldberg, 1979; Anderson *et al.*, 1982).

In Chapter 3, I have shown that eggs of *C. mutabile* only begin to hatch when water temperatures rise above 12 °C and that temperature influences survival time, longevity, and through these, transmission efficiency under lab conditions. Water temperatures of shallow ponds such as those used by coots generally reflect ambient air temperatures (McMahon, 1983). Thus, water temperatures of potholes tend to be variable in the spring. The snail community structure is also variable at this time; a large proportion of the overwintered adult snails die off and many young of the year appear at the same time (Duncan, 1975; McMahon, 1983). The success of miracidia in infecting different species and sizes of snails under the temperature regimes encountered in the spring will have a great influence on the development of the infective pool of metacercariae available to coots later on each summer.

The objectives of this study were (1) to determine what effect snail species and snail size has on the compatibility

(infection success and production of metacercariae) of *C. mutabile* with the different snails, and (2) to determine what effect temperature has on the infection success of the miracidia to the different snails examined. (3) The study also addresses patterns of transmission when a choice of two snails is available to miracidia in the laboratory.

4.2. Materials and Methods

4.2a. Parasite and host material

The general procedures for obtaining parasites and snail hosts are given in Chapter 2. Briefly, the *C. mutabile* used were originally collected from wild coots, passed through lab-raised *Stagnicola elodes* and then established in a group of lab-reared coots. Miracidia used for infection studies were obtained by dissection of the anterior third of the uterus of worms from these lab-infected coots. The eggs were hatched at the same temperature required by the infection protocol. Miracidia were used as soon as they hatched.

Snails were either from populations maintained in the lab since 1990, populations raised from egg packets collected in the field, or were wild-caught. The snails used in all infection studies originated from the Delta Marsh, Manitoba, from a pond in nearby Portage la Prairie, Manitoba, or from a creek just south of Portage la Prairie. With the exception of *Lymnaea stagnalis*, all snails of a given species were from a single source; *L. stagnalis* were raised from egg packets collected from both the Delta Marsh and the creek near Portage la Prairie. These eggs were pooled and no attempt was made to differentiate between the two populations of snails used. Some species were wild-caught. These were kept in the lab for a minimum of one week prior to being exposed to *C. mutabile* miracidia. This allowed the snails to become adjusted to the standardized lab diet and for any

extraneous digenean infections to be detected. Each of the wild-caught snails was checked for the presence of sporocysts or redia and isolated to detect cercarial shedding prior to being exposed to *C. mutabile* miracidia. Most of the wild-caught snails were planorbids and digenean infections are easily seen through the shell of these species. No infections of *C. mutabile* were observed in any of the wild-caught snails.

4.2b. Infection of single host species

General procedures

Single snails were placed in wells of 24-well tissue culture plates (3ml water/well) and a single freshly-hatched (<1 hour old) miracidium was added to each well. Snails were exposed to miracidia for 12-18 hours, removed from the tissue culture plates and placed in aquaria maintained at 20 °C. Snails were dissected at 28 days pi. At dissection, the presence or absence of infection was noted in all snails. The number of metacercariae produced within infected snails was also recorded. Sample sizes of the different snail species, size class and temperature combinations examined are given in Table 4.1. All infections and dissections were performed using a stereomicroscope.

Species effects

A total of nine species from three families of pulmonate snails was exposed to *C. mutabile* miracidia. These included the lymnaeids *Lymnaea stagnalis jugularis* (Say, 1817) and *Stagnicola*

Table 4.1. Sample sizes used in single species infection studies of *Cyclocoelum mutabile* in four size classes of 9 species of snails at three different temperatures. Except for *H. trivolvis*, numbers separated by a comma represent the number of snails surviving and considered in each trial (from initial samples of 192 snails). For *H. trivolvis*, the denominator indicates the initial number of snails exposed to miracidia for each trial carried out, numerators the number snails surviving the 4 week incubation period and examined for *C. mutabile* infection. Multiple numbers separated by a comma indicate multiple trials, single numbers indicate single trials.

Snail species	Size class*	Temperature		
		14°C	16°C	20°C
S. elodes	1	116, 133	145, 112	112, 125
	2	156, 142	176, 151	186, 191
	3	115, 148	173, 155	83, 187
	4	93, 138	179, 127	186, 152
L. stagnalis	1	162, 137	126, 157	107, 135
	2	130, 164	166, 126	164, 130
	3	94, 153	186, 160	177, 147
	4	69, 102	109, 115	117, 91
P. exacuous	1	137, 168	186, 153	186, 183
	2	102, 93	144, 122	133, 122
A. crista	1	165, 182	123, 136	174, 161
G. circumstriatus	2	65, 88	81, 105	116, 110
G. parvus	1	115, 134	177, 173	108, 161
H. trivolvis	2	85/96	62/72	76/96
	3	109/120	116/144	109/120
P. jennessi	1	121, 131	167, 139	141, 102
	2	153, 131	166, 132	181, 128
	3	105, 108	146, 124	148
	4	186	-	-
P. gyrina	1	110, 155	179, 162	175, 159
	2	148, 153	176, 160	172, 156
	3	129, 129	153	153, 129
	4	151	168	125

* size classes are as follows: 1; 0-0.0025g tissue mass
2; 0.0026-0.01g tissue mass
3; 0.02-0.05g tissue mass
4; 0.06-0.10g tissue mass

(*stagnicola*) *elodes* (Say, 1821), the physids *Physa gyrina gyrina* Say, 1821 and *Physa jennessi skinneri* Taylor, 1953, and the planorbids *Armiger crista* (Linnaeus, 1758), *Gyraulus circumstriatus* (Tryon, 1866), *Gyraulus parvus* (Say, 1817), *Helisoma* (*Pierosoma*) *trivolvis subcrenatum* (Carpenter, 1856), and *Promenetus exacuous exacuous* (Say, 1821). These snails are among the most abundant species found in the Delta Marsh (E. Pip, personal communication; personal observations).

Size effects

The relationship between the infectivity of miracidia to different sizes of various snail species was also examined. In order to compare the susceptibility of a certain size class of one species to that of a different species, a common measurement was needed. Given the widely differing shape of the species used, the length cannot be used as a common basis for comparison. Similarly, the different shell constructions (from thin and fragile to very thick and robust) precludes the use of total snail mass as a criteria for comparison. It was decided to use the wet tissue weight of the snails as the criteria for grouping snails of different species with similar size classes for comparison.

Snail sizes (maximum lengths or diameters) were measured using a micrometer under a dissecting microscope and were recorded to the nearest 0.1mm. Depending on the species, the length was taken to be either the furthest distance from the apex

to the outer lip (families Lymnaeidae and Physidae) or as the maximum diameter (family Planorbidae) (Clarke, 1973). Wet tissue weights were determined by carefully crushing the shell between two Petri dishes under water, dissecting away all the shell fragments, gently blotting the tissue dry of any excess moisture and then weighing it to the nearest 0.001 or 0.0001g with a Mettler BasBal BB240 or a Mettler AJ100 scale, respectively.

Forty to 90 snails for each species were measured to ensure that a range of sizes was represented more or less evenly. A series of nonlinear regression equations of the form

$$T = x Y^z \qquad \text{(eqn. 4.1)}$$

were constructed to allow the estimation of the snail tissue weight. Here, T is the wet tissue mass of a snail in grams, Y the length of the snail in millimetres, and x and z are constants estimated by nonlinear regression. In all cases, the estimated models provided an excellent fit to the data (see Table 4.2). Regression equations were not constructed for *A. crista*, *G. parvus* and *G. circumstriatus*. Preliminary measurements indicated only one size class of each species was available.

Four size classes were defined, these were: (1) < 0.0025g; (2) 0.0026 - 0.01g; (3) 0.02 - 0.05g; and (4) 0.06 - 0.10g. Although the larger species grew larger than the upper limit of size class #4, snails of this size were rarely encountered in the field and were not tested. When choosing snails of a certain size class for infection, snails at either end of the size class

Table 4.2. Values and fit of equation (4.1) for *Lymnaea stagnalis*, *Stagnicola elodes*, *Promenetus exacuous*, *Helisoma trivolvis*, *Physa gyrina* and *Physa jennessi*.

Species	n	size range examined (mm)	*a*	*b*	r^2
S. elodes	67	1.6-17.9	0.000232	2.374	0.977
L. stagnalis	76	2.4-23.8	0.000006	3.541	0.985
P. exacuous	90	2.7- 7.4	0.000063	2.812	0.992
H. trivolvis	40	2.2-20.1	0.000238	2.532	0.988
P. gyrina	63	2.8-12.3	0.000054	3.105	0.983
P. jennessi	35	2.6-10.5	0.000270	2.371	0.971

were found and used as guides for choosing the remaining snails. Care was taken to ensure the majority of the snails selected were from the middle of each size class.

Temperature effects

Most snail species - size class combinations were tested at three different temperatures: 14, 16, and 20 °C, encompassing the temperatures examined in egg hatching and miracidial studies (Chapter 3). The number of trials and sample sizes of the different snail species, size and temperature combinations are given in Table 4.1.

4.2c. Infection of simulated multi-species host communities

This experiment investigated patterns of host snail invasion by miracidia of *C. mutabile* when presented with a choice of different snail species. The species considered were *S. elodes*, *P. jennessi*, and *P. exacuous*. These species were chosen for two reasons. Firstly, they were the most prevalent in the ponds studied in the field aspect of my research (see Chapter 5). Secondly, these species showed differing susceptibility to infection by *C. mutabile* and I wanted to determine if the presence of one species may reduce the potential effectiveness of the other as a potential host (i.e. one species acting as a sink for otherwise viable miracidia).

Infection procedure involved placing 5 freshly hatched miracidia into plastic cups containing 2 snails (a single snail

of each of two different species or two snails of the same species) in 200ml water at 20 °C. The snails were left in the cups for 3-4 weeks at 20 °C and then dissected. The number of infections in each snail was recorded. Each trial consisted of 72 experimental pairs (12 for each possible two-species combination and for each same species control). Six trials were performed.

4.2d. Statistical analysis

Infection rates (%) were tested for differences between treatments (snail species, size and temperature effects). The production of metacercariae was analyzed only between snail species and size classes; the mean number of metacercariae produced by each size class and species at each temperature were the data used for comparison. Initially, both infection rates and metacercarial production were examined by the regression approach to ANOVA to determine if overall differences were present between treatments. Subsequent analysis of infection rates was performed using either ANOVA or logit analysis. The further analysis of metacercarial production was performed using ANOVA. When ANOVA was used, the analysis was followed by a Tukey test to detect where differences existed. The relationship between snail susceptibility and metacercarial production was examined by Pearson product moment correlation. In the section examining the pattern of transmission where miracidia are given a choice of two snails, the prevalence and intensity of infection

between treatments for different species was compared using ANOVA. A series of T-tests were employed to determine if the expected overall transmission success of miracidia in the two species choice experiments differed from what would be expected by chance. Statistical methods used are discussed further in those sections in which they are employed in the results.

4.3. Results

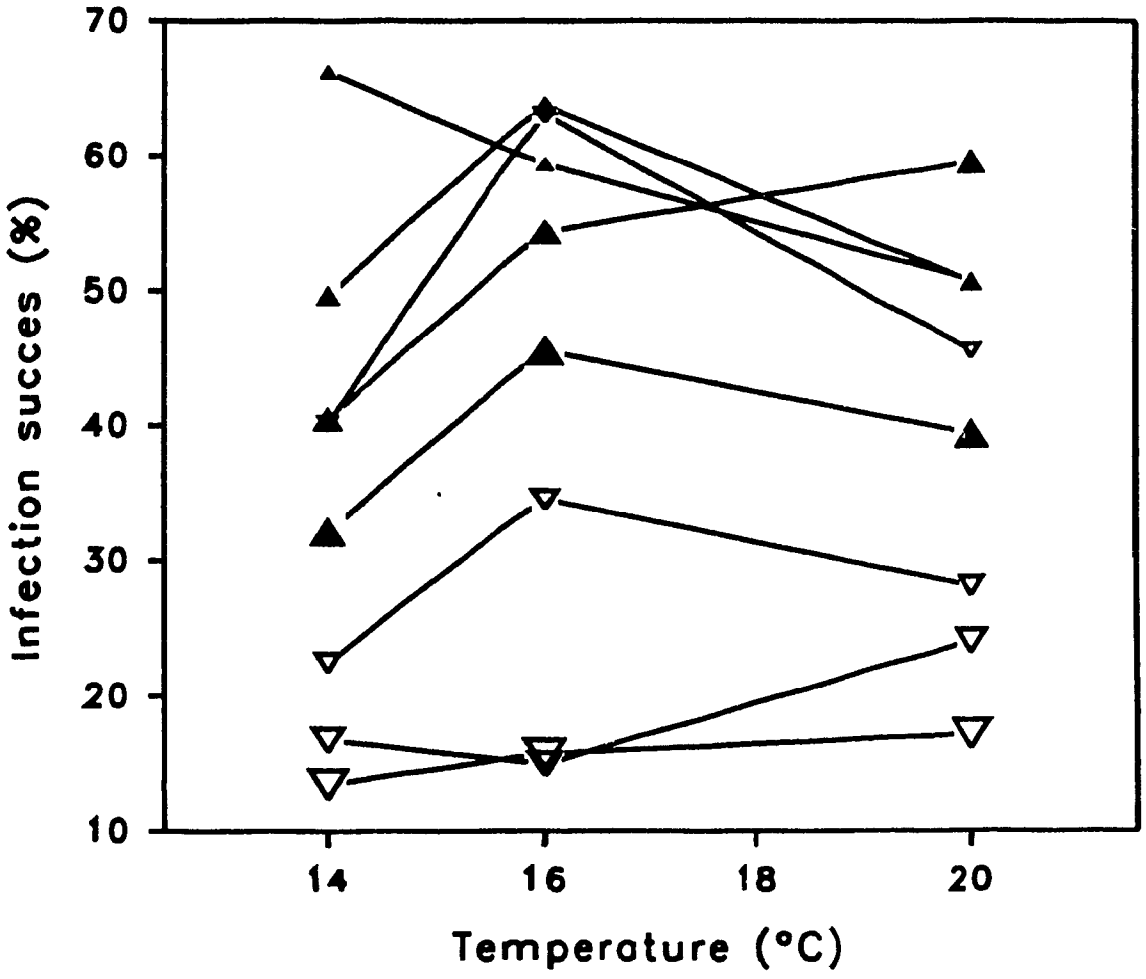
4.3a. Infection of single host species

The presence of many empty cells in the experimental design precluded the use of MANOVA for the detection of differences between treatments (snail species, snail size and temperature) on snail susceptibility and metacercarial production. Similarly, the use of ANOVA to test each of these responses separately was also precluded. Accordingly, the data were coded and tested for main effects by the regression approach to ANOVA. Significant differences for both the percentage of snails infected (F = 622.37; df = 13,16998; $P < 0.00005$) and the number of metacercariae produced (F = 11.19; df = 10,66; $P < 0.00005$) under the different treatments were detected. The percentage of snails infected and the production of metacercariae were subsequently considered separately. Each of these variables was examined for size and, if applicable, temperature effects for each snail species under separate analyses.

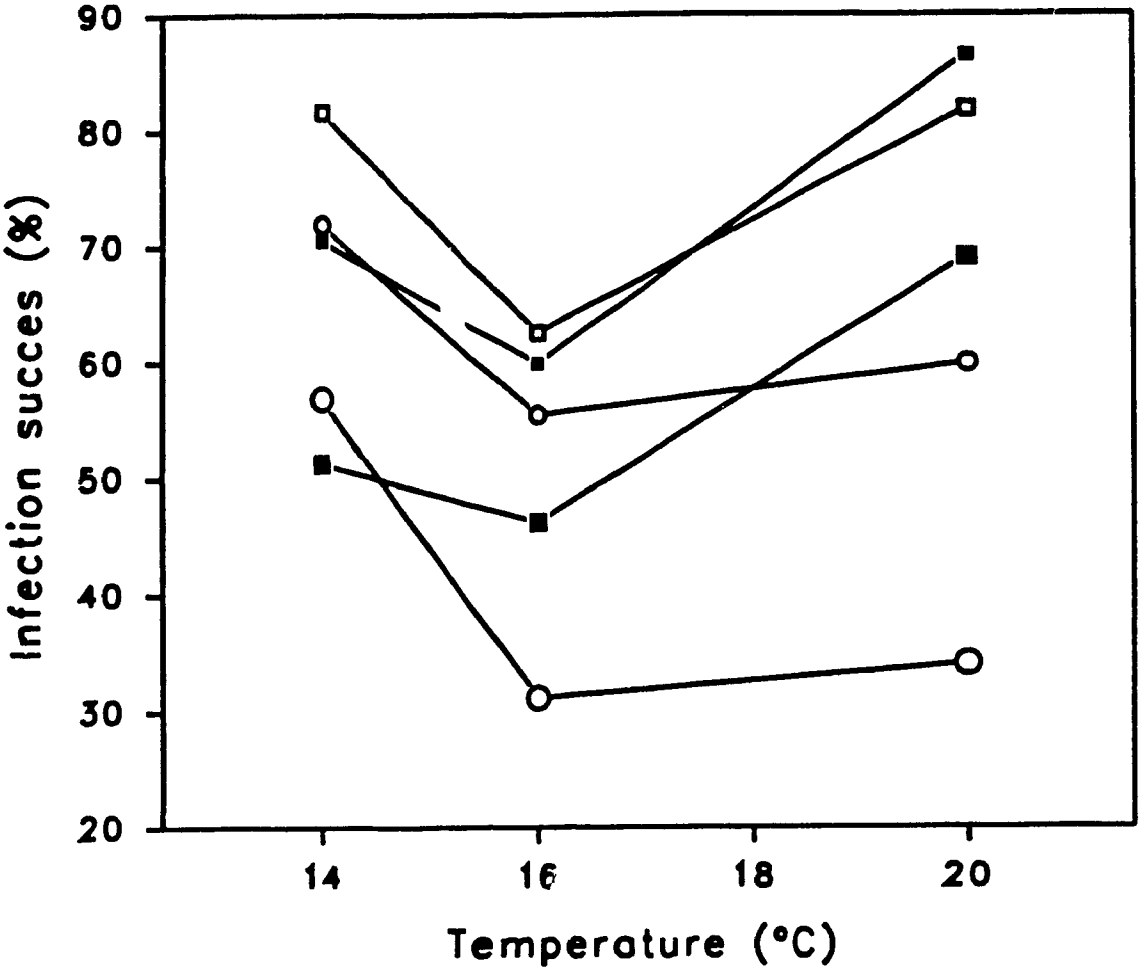
Snail susceptibility

The percentage of each species and size class that became infected at each temperature is given in Appendix 1 and shown in Figs. 4.1a-c. Of the 9 species examined, only *P. gyrina* failed to become infected. For the purpose of analysis, each snail species was classified as susceptible (overall infection success $\geq$ 25%), refractive (infection success < 25%) or insusceptible

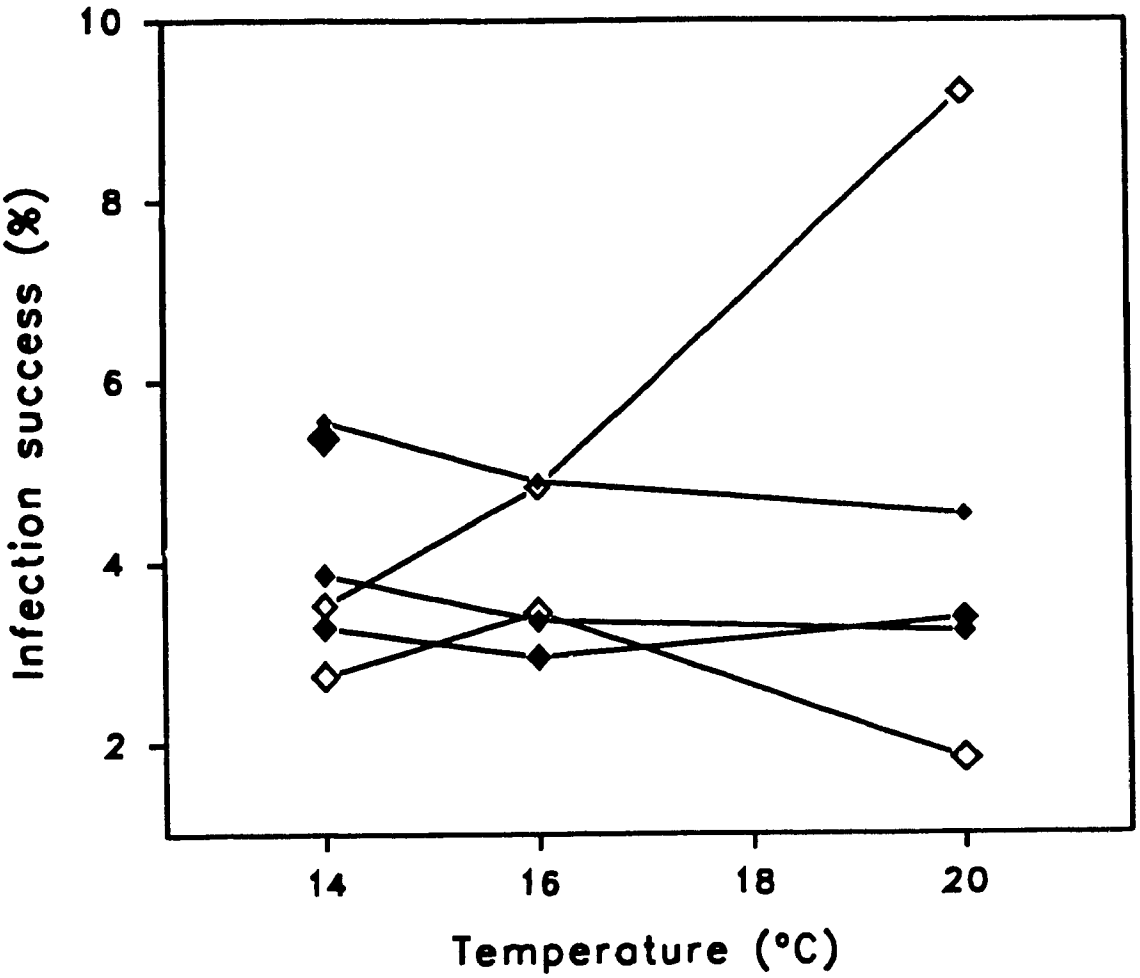
Figure 4.1. Infectivity of *Cyclocoelum mutabile* to eight species of snails. (A), susceptible lymnaeids (▲, *Lymnaea stagnalis*; ▼, *Stagnicola elodes*). (B), susceptible planorbids (■, *Promenetus exacuous*; □, *Armiger crista*; ●, *Gyraulus parvus*; ○, *Gyraulus circumstriatus*). (C) refractive species (♦, *Physa jennessi*; ◇, *Helisoma trivolvis*). Symbols of the same type but different size represent snails of the same species but of different size classes, the largest symbols representing the largest size class, the smaller ones the smaller size classes.



(A)


90
80
70
60
50
40
30
20
Infection succes (%)
14
16
18
20
Temperature (°C)

(B)



(C)

(infection success = 0%). Except *P. gyrina* (that species found to be insusceptible), each species was tested separately for the effect of exposure temperature and, if appropriate, the effect of size and the interaction between these two effects.

Refractive species (*H. trivolvis* and *P. jennessi*) were analyzed by two-way stepwise logit analysis. For *H. trivolvis*, neither snail size, temperature nor the interaction between these two factors were needed to provide a good fit of the model to the observed infection level ($G = 6.376$, $P = .271$). As size class #4 for *P. jennessi* was tested only at 14 °C, this resulted in an incomplete table for analysis and the results were pooled with those obtained for size class #3 for analyses. Again, an adequate model was found with no effects included ($G = 4.101$, $P = .842$). Neither snail size nor temperature had any effect on the observed infection rate.

For ease of interpretation, susceptible species (*S. elodes*, *L. stagnalis*, *P. exacuous*, *A. crista*, *G. circumstriatus*, and *G. parvus*) were analyzed by one- or two-way analysis of variance (ANOVA), depending on whether there were 1 or more size classes to consider. Separate analyses for each species showed that temperature, snail size, and usually the interaction between these two factors had a significant effect on the percentage of snails that became infected (Table 4.3). Judging by the magnitude of the mean square errors calculated for each analysis, snail size had a greater influence on infection levels than temperature (Table 4.3). The effect of snail size was much

Table 4.3. ANOVA table for infection success of *Cyclocoelum mutabile* miracidia to susceptible snail species: species effects.

Source of variation					
Group	Factors	df	MS	*F*	*P*
S. elodes	Size	3	18.50	102.91	< 0.0005
	Temperature	2	2.20	12.26	< 0.0005
	Size x Temperature	6	1.16	6.44	< 0.0005
	Error	3469	0.18		
L. stagnalis	Size	3	4.81	19.92	< 0.0005
	Temperature	2	2.00	8.29	< 0.0005
	Size x Temperature	6	1.75	7.25	< 0.0005
	Error	3212	0.24		
P. exacuous	Size	1	11.56	55.72	< 0.0005
	Temperature	2	9.34	45.00	< 0.0005
	Size x Temperature	2	0.11	0.54	0.585
	Error	1713	0.21		
A. crista	Temperature	2	3.35	19.15	< 0.0005
	Error	874	0.18		
G. circumstriatus	Temperature	2	3.28	14.38	< 0.0005
	Error	562	0.23		
G parvus	Temperature	2	2.24	9.50	< 0.0005
	Error	1033	0.24		

Table 4.4. ANOVA table for infection success of *Cyclocoelum mutabile* miracidia to susceptible snail species: size effects.

Source of variation					
Group	Factors	df	MS	*F*	*P*
Size class #1	Species	4	9.04	42.21	< 0.0005
	Temperature	2	1.40	6.52	< 0.0005
	Species x Temperature	8	3.80	17.76	0.001
	Error	4478	0.21		
Size class #2	Species	3	14.67	64.72	< 0.0005
	Temperature	2	0.07	0.30	0.744
	Species x Temperature	6	3.20	14.12	< 0.0005
	Error	3141	0.23		
Size class #3	Species	1	47.28	238.66	< 0.0005
	Temperature	2	2.41	12.16	< 0.0005
	Species x Temperature	2	0.93	4.68	0.009
	Error	1772	0.20		
Size class #4	Species	1	19.55	111.61	0.018
	Temperature	2	0.70	4.00	< 0.0005
	Species x Temperature	2	0.38	2.15	0.117
	Error	1472	0.18		

simpler to interpret within a species: larger snails were infected less frequently than smaller snails in all three species. Although the interaction between size and temperature was usually significant, no trends were apparent.

A comparison of the susceptible species by size class and temperature showed both significant main and interaction effects for all effects and interactions except for size classes #2 and #4 where the temperature effect and the interaction, respectively, were not significant. Comparison of the calculated mean square errors for each analysis showed the effect of snail species was much more influential than that of temperature and the interaction between the two effects (Table 4.4) and only this effect was analyzed further. The data for each species at each temperature were pooled and compared for each size class by ANOVA, followed by a Tukey test. Results are shown in Table 4.5. Where both lymnaeids and planorbids could be compared, the planorbids were generally more heavily infected. Also, the order of susceptibility to infection remained constant between the different size classes.

In half of the species - size class combinations of the two lymnaeid species examined (*S. elodes* and *L. stagnalis*), infection success was higher at 16 °C and lower at the other temperatures; the other combinations showed no clear trend (Appendix 1 and Fig. 4.1a). For the planorbids (*A. crista*, *G. circumstriatus*, *G parvus* and *P. exacuous*), infection success was lowest at 16 °C for all the species - size class combinations examined (Appendix

1 and Fig. 4.1b).

Species and size-dependent production of metacercariae

The results of the metacercarial production studies are shown in Table 4.6. While no size effects were found, there were significant species effects. Infections in the lymnaeids resulted in more metacercariae per snail than in either the planorbids or the refractive physid (*P. jennessi*).

The relationship between snail susceptibility and production of metacercariae was examined. As the experimental design included many empty cells, the use of ANOVA to test the effects of snail size and species and the interaction between the two was precluded. The mean number of metacercariae produced by each susceptible species and size combination (from Table 4.6) was found to have a significant negative correlation with the mean infectivity levels observed in the same snails (from Table 4.5) (Fig. 4.2): rediae in more susceptible snails generally produced fewer metacercariae (Pearson product moment correlation coefficient = -0.603, $P = 0.029$).

4.3b. Infection of simulated multi-species host communities

The pattern of miracidial infections when presented with a choice of two snails is shown in Tables 4.7 and 4.8. No significant differences were seen between the percentage of each snail species that became infected in either treatment (ANOVA, $F = 2.492$ and 2.606; $P = 0.116$ and 0.107 for *G. parvus* and *S.*

Figure 4.2. The relationship between the susceptibility of snails to *Cyclocoelum mutabile* and the production of metacercariae within infecteds. Plot symbols represent observed values for the different size classes of the different snail species.

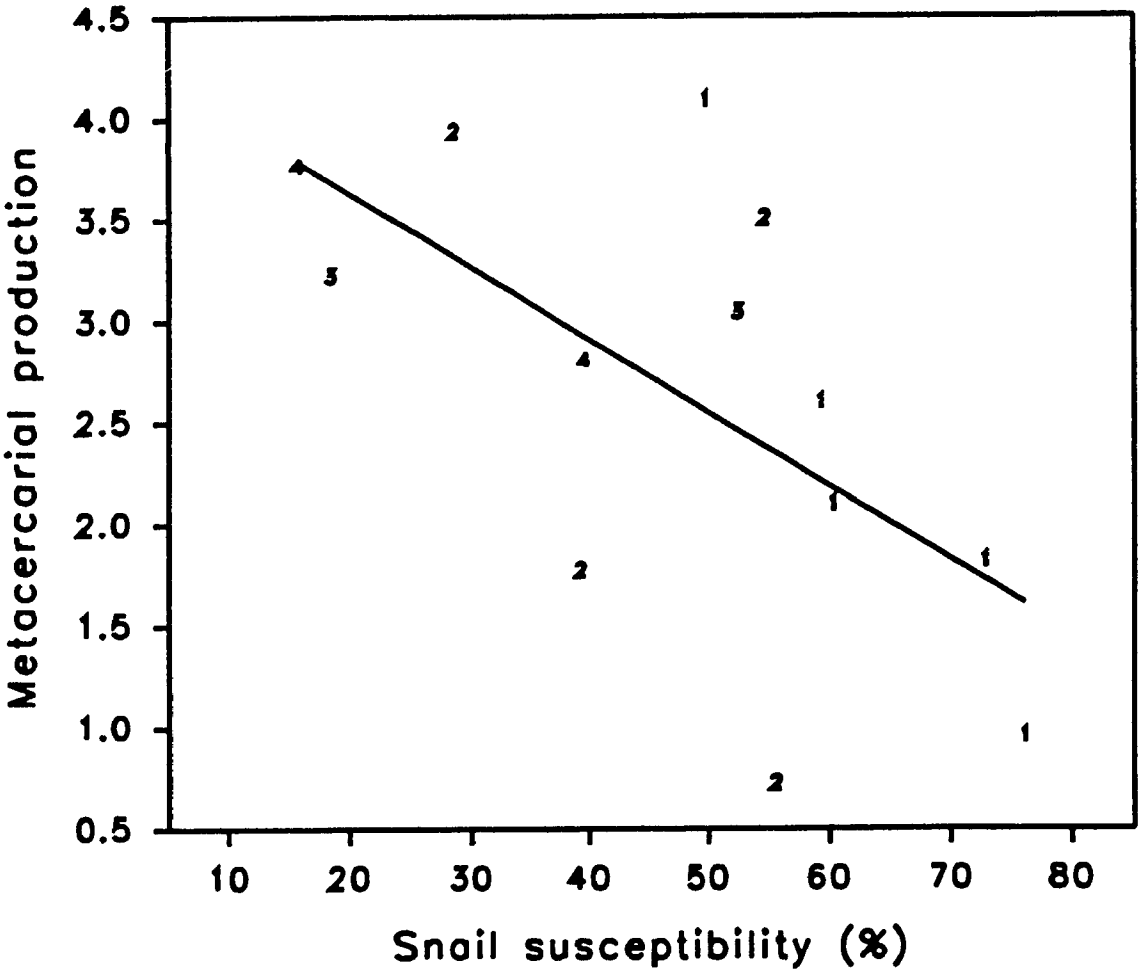

Metacercarial production
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
10
20
30
40
50
60
70
80
Snail susceptibility (%)

Table 4.5. Mean infectivity (%) of *Cyclocoelum mutabile* miracidia to susceptible snail species. Results for each size class-species combination are pooled from trials carried out at 14, 16 and 20 °C. Different letters in columns indicate significant differences between species (Tukey test). Different numbers in rows indicate significant differences between sizes (Tukey test). Dashes represent size class-species combinations that were not tested.

Snail Species	Size class			
	1	2	3	4
A. crista	76.05 a	-	-	-
P. exacuous	72.75 a 1	55.52 a 2	-	-
G. parvus	60.33 b	-		
L. stagnalis	59.34 b 1 2	54.66 a 1 2	52.45 a 2 3	39.64 a 3
S. elodes	49.80 c 1	28.54 b 2	18.35 b 3	15.66 b 3
G. circumstriatus	-	39.29 c	-	-

Table 4.6. Production of metacercariae by snails infected with single *Cyclocoelum mutabile* miracidia following incubation for 4 weeks at 20 °C. Results for each size class-species combination are means of means (± S.E.) from trials carried out at 14, 16 and 20 °C. Different letters in columns indicate significant differences between species (Tukey test). Different numbers in rows indicate significant differences between sizes (Tukey test). Dashes represent size class-species combinations that were not tested.

Snail Species	Size class			
	1	2	3	4
A. crista	0.95 (0.20) a	-	-	-
P. exacuous	1.81 (0.48) a 1	0.72 (0.24) a 1	-	-
G. parvus	2.09 (0.76) a b	-	-	-
L. stagnalis	2.59 (0.59) a b 1	3.50 (0.77) b c 1	3.21 (0.31) b 1	2.80 (0.18) b 1
S. elodes	4.08 (0.55) b 1	3.93 (0.56) c 1	3.21 (0.72) b 1	3.76 (0.38) b 1
G. circumstriatus	-	1.77 (0.75) a b	-	-
H. trivolvis	-	0.76 (0.45) a 1	0.69 (0.28) b 1	-
P. jennessi	1.45 (0.16) a 1	1.34 (0.30) a 1	1.50 (0.32) a b 1	0.90 (--) a 1

Table 4.7. Infection of three species of snails exposed to *Cyclocoelum mutabile* miracidia in 6 different 2-snail communities.

Community structure	No. miracidia	#replicates /#trials	Mean percent snails infected (± S.E.)		
			S. elodes	*G. parvus*	*P. jennessi*
2 *S. elodes*	5	12/6	59.7 (6.9)		
2 *G. parvus*	5	12/6		71.3 (5.7)	
2 *P. jennessi*	5	12/6			0.0
1 *S. elodes* 1 *G. parvus*	5	12/6	58.0 (5.7)	72.2 (5.7)	
1 *G. parvus* 1 *P. jennessi*	5	12/6		73.7 (4.5)	0.0
1 *P. jennessi* 1 *S. elodes*	5	12/6	45.0 (9.5)		0.0

Table 4.8. Transmission success of *Cyclocoelum mutabile* miracidia to three species of snails in 6 different 2-snail communities.

Community structure	Mean # redia/snail (± S.E.)			Overall Transmission success (%) (± S.E.)
	S. elodes	*G. parvus*	*P. jennessi*	
2 *S. elodes*	1.10 (0.18)			44.2 (7.1)
2 *G. parvus*		1.37 (0.10)		54.6 (3.8)
2 *P. jennessi*			0.0	0.0
1 *S. elodes* 1 *G. parvus*	1.36 (0.26)	1.08 (0.15)		48.8 (5.4)
1 *G. parvus* 1 *P. jennessi*		1.36 (0.13)	0.0	27.2 (2.6)
1 *P. jennessi* 1 *S. elodes*	0.81 (0.23)		0.0	16.2 (4.5)

elodes, respectively) (Table 4.7). There were also no significant differences in the mean number of redial infections found in snails from different treatments within each species (ANOVA, $F = 1.728$ and 1.511; $P = 0.211$ and 0.253 for *G. parvus* and *S. elodes*, respectively) (Table 4.8).

The expected overall transmission success for each two snail species combination was calculated by averaging the mean infection success observed for each of the single species communities comprising the two species community in question. For example, the expected overall transmission success of miracidia in the *S. elodes* - *G. parvus* community was calculated as:

$$\frac{Mean_{Se} + Mean_{Gp}}{2} = \frac{44.2 + 54.6}{2} = 49.4. \qquad \text{(eqn. 4.2)}$$

where the mean values used are from Table 4.8. In order to compare observed and expected proportions, this value was similarly calculated for each of the six trials. Using these values as replicates, a series of *t*-tests was then performed for each snail community. None of the comparisons showed significant differences between the observed and expected transmission success values ($0.035 \leq t \leq 1.015$; $0.335 \leq P \leq 0.973$ for all comparisons). Thus, miracidia do not seem to choose one snail species over another.

4.4. Discussion

My results confirm that *C. mutabile* can infect a wide range of snail species from three separate families. This is in general agreement with the results of other authors working with this species (McLaughlin, 1976) or with other members of the family Cyclocoelidae (e.g. Scott, Rau, & McLaughlin, 1982; Taft, 1972, 1974, 1975, 1986; Taft & Heard, 1978; Timon-David, 1955). All of these species display similar life cycles.

My results are in general agreement with those in preliminary studies by McLaughlin (1976), although some notable differences were observed. The two major differences involve *H. trivolvis* and *P. gyrina*. In this study, a maximum of 9.2% of *H. trivolvis* became infected whereas McLaughlin (1976) found over 50% infected. This may be due to a size difference in the snails used in the two studies. Snails used in this study were of size classes #2 and #3 whereas McLaughlin (1976) used snails that would have been of size class #1 in this study (McLaughlin, personal communication). The greater susceptibility of the smallest size classes in this study makes this suggestion plausible. If this is the case, then *H. trivolvis* becomes refractive to infection by *C. mutabile* at a young age. However, Scott (1980) found the size of *H. trivolvis* had no affect on the infectivity of either *Typhlocoelum cucumerinum cucumerinum* and *T. c. sisowi* in this snail. Fried, Scheuermann & Moore (1987) have also found susceptibility of *H. trivolvis* to *Echinostoma*

revolutum to remain fairly constant over a broad range of sizes.

None of the over 3000 *P. gyrina* exposed and examined in this study became infected whereas McLaughlin (1976) found an infection rate of 25% for this species. This cannot be explained by different susceptibility of the different size classes. Also, preliminary experiments conducted one year earlier revealed that *P. gyrina* was susceptible to infection with *C. mutabile*. As these were only preliminary tests, the data were not recorded.

A number of studies, mainly pertaining to the schistosomes, have emphasized the importance of host and parasite genotypes to the levels of prevalence observed (e.g. Rollinson & Southgate, 1985; Bayne & Loker, 1987; Mulvey & Vrijenhoek, 1982). Genetic drift and founder effects are usually suggested as the primary causative agents of this heterogeneity in infectivity and susceptibility (e.g. Mulvey & Vrijenhoek, 1982; Jarne & Delay, 1991). Some authors (e.g. Lively, 1989; Lie & Heyneman, 1979) have suggested that the parasites themselves drive snail populations to develop resistance to the parasites present in a system. These effects are also of importance in the lab as obtaining strains of snails and parasites that show consistent susceptibility and infectivity, respectively, is difficult (Lie, Heyneman & Richards, 1979) and the genetic heterogeneity of the species used should be considered both within studies and when comparing studies, otherwise any conclusions drawn may be incorrect (Richards, 1976).

The *P. gyrina* used in this study were obtained from a pond

in Portage la Prairie, some 25km south of the Delta Marsh. Those used by McLaughlin (1976) and in last year's preliminary trials were obtained from the Delta Marsh. Thus, differing genotypes may well explain the differences reported between the two studies for *P. gyrina*. This may also help account for the differences observed for *H. trivolvis* and for other less pronounced differences in susceptibility noted for the other species. The differences in the infectivity of miracidia to *S. elodes* (40-63% in this study, 11% in the other) may also be explained in part by the low sample size of this snail used by McLaughlin (1976) (9 snails).

The susceptibility of snails to miracidial infection and the subsequent development of sporocyst or redial generations has often been found to be a function of the size (or age) of the snail in question (for reviews of some of the more medically and economically important species, see Boray, 1969; Loker, 1983; and Huffman & Fried, 1990). Older snails may be more refractive to infection as their immune systems are more fully developed or for mechanical reasons (their body wall is thicker and presumably harder to penetrate). However, this is not always the case. The trend is often reversed or intermediate sized snails may be the most or least susceptible to infection. This may occur because other factors, such as the ability of miracidia to locate and identify snails may vary with host size (Anderson & Crombie, 1984), as may the physiological suitability of the snail for development of the parasite.

In this study, a negative correlation was observed between the size and susceptibility. Although different snail species in the same size class are not necessarily at the same developmental stage (nor presumably, at the same level of immunocompetence), comparison of different species of the same size class showed the same order of infectivity between size classes. Studies have shown that the immune system of *L. stagnalis* becomes more efficient with increasing snail size (Dikkeboom, van der Knapp, Meuleman & Sminia, 1985). We have no evidence to conclude this causes the size-dependent decline in susceptibility to *C. mutabile* in *L. stagnalis*, or any of the other species examined, but it remains a possibility.

As pointed out by Cheng (1968), determination of compatibility of snail-trematode associations should not focus solely on rates of susceptibility of the snail to the parasite, but also on the ultimate production of cercariae by the sporocyst or redial generation within the host. Studies in the past have demonstrated that more susceptible strains of two *Biomphalaria* species infected with *Schistosoma mansoni* also yield higher numbers of cercariae (Barbosa, 1975). Unfortunately, many studies have involved exposure of snails to several miracidia and although cercarial output is generally not correlated with the number of miracidia penetrating a snail (see review in Christensen, 1980), Ward, Lewis, Yoshino & Dunn (1988) have found that production of *S. mansoni* cercariae is correlated with the number of primary sporocysts infecting *B. glabrata*. Thus studies

reporting differences in cercarial output following exposure of snails to numerous miracidia may actually be reporting differences in the number of miracidia that successfully establish within the snail, not the cercarial production of a single miracidial infection. As this study used single miracidia to establish infections and all the cercariae produced encyst within the same snail, production of metacercariae may be used as an additional criterion for determining the compatibility of *C. mutabile* in different snail species. Although the results from the metacercarial productivity studies were highly variable, it was apparent that metacercarial productivity was higher in the lymnaeids than in the other families. This finding would not be expected if the susceptibility to infection were correlated positively with metacercarial production as suggested above. In fact, when only susceptible species (infection success > 25%) are considered, there is an overall negative correlation between the two parameters.

It was also apparent that size had little effect on productivity within species. This is in contrast with other studies examining the production of cercariae. These studies have generally found cercarial output to increase with snail size (e.g. Jourdane & Théron, 1987; Loker, 1983; Niemann & Lewis, 1990). However, these studies deal with species that produce a number of asexual multiplicative stages prior to the production of cercariae and larger snails may produce more daughter sporocysts or rediae (Lim & Lee, 1969), which would in turn

result in larger numbers of cercariae. That size had no effect on the production of metacercariae in this study may simply reflect the fact that there is but a single redia in the life cycle. However, Scott (1980) working with two subspecies of *Typhlocoelum* (also a cyclocoelid with a single redial generation) has shown shell length to be positively correlated with metacercarial production in most parasite-host combinations. Alternatively, as metacercarial production was just starting within the snails when they were examined, size-dependent differences may simply not have been apparent at this time. Perhaps, given time, differences in metacercarial production would have become more apparent.

Whether or not a miracidium will attempt to penetrate unsusceptible species depends on the parasite species (see reviews by Saladin, 1979; Christensen, 1980). Although miracidia of some species may ignore unsuitable hosts, others may invade the snail but perish. Although only three species of snails were used in the simulated multi-species host community studies, the results revealed that miracidia of *C. mutabile* do not distinguish between refractive (*P. jennessi*) and susceptible species (*S. elodes* and *G. parvus*). They appear to attempt to infect whatever species they encounter. For a species with a wide range of potential hosts, this is a logical strategy. This is particularly true for a species like *C. mutabile* where transmission to the snail host occurs in the spring when snail densities are low. Although miracidia of a number of species may

attempt to infect most snails they encounter in lab studies (e.g. Cherin, 1968; Cherin & Perlstein, 1969; Christensen, Nansen & Frandsen, 1976b), Saladin (1979) warns against extrapolating too much from laboratory studies. Miracidia in nature may rely on a number of other factors such as differential location of the various species of snails, as well as the olfactory cues utilized by miracidia to find snails in lab studies. As all the snails used in this study commonly occur together in the same micro-environments (personal observations), this warning can be disregarded for these experiments.

Pip (1978) made a comprehensive study of the submerged macrophyte - snail communities of southern Manitoba and the adjacent areas. *L. stagnalis*, *P. gyrina*, *H. trivolvis*, *S. elodes*, and *G. parvus* were found most often, each species accounting for between 13.3 and 9.3% of all snails found. The other species used in this study accounted for between 1.9 (*P. jennessi*) and 0.6 % (*A. crista*) of the total number of snails recorded. This last number may be low as these snails are very small (< 4mm (Clarke, 1973)) and I have found them wherever I have looked in southern Manitoba.

Adult coots feed primarily on vegetation (e.g., Jones, 1940; Fredrickson, 1970). Juveniles feed exclusively on animal matter for the first few days of life (Gullion, 1954), consuming greater amounts of vegetation as their ability to break down fibrous materials increases with age (Jones, 1940). This study and that by McLaughlin (1976) suggest that a good proportion of the snails

potentially encountered by *C. mutabile* miracidia may become infected. All of these species are associated with the vegetation used by coots as food (Jones, 1940; Pip, 1978) and may be important in the transmission of the infection back to coots in southern Manitoba.

In a field study of the distribution of *Echinoparyphium recurvatum* metacercariae in seven species of molluscs, Evans, Whitfield & Dobson (1981) suggested that the importance of any species in the overall transmission of the parasite was dependent on a number of factors. The most important were the abundance of each mollusc species in the environment and the susceptibility of the different species to infection by the parasite. While this study showed temperature had a significant effect on the susceptibility of most species, the effect was variable and infectivity of *C. mutabile* miracidia remained high at all temperatures. Thus, temperature is not as important in the transmission of the parasite, compared to snail size and species.

As this study found significant but variable temperature-dependent infection levels for the different snail species-temperature combinations examined, the effect of temperature seems to be mostly on the snails, rather than the miracidia. If temperature had a greater effect on the miracidia, then I would have expected to see the same pattern of infectivity between snails at the different temperatures.

Other studies examining the effect of temperature on the infectivity of miracidia (or susceptibility of snails) have found

variable results. Studying the infectivity of *Fasciola hepatica* to four species of lymnaeids at temperatures ranging from 12 to 24 °C, Gold & Goldberg (1979) found the infection rate to increase to a maximum at 20 or 24 °C and then decline. Purnell (1966) found the infection rate of *Schistosoma mansoni* to *Biomphalaria sudanica tanganyicensis* to increase linearly with temperature to a maximum at 39 °C. However, also working with *S. mansoni*, Anderson, Mercer, Wilson & Carter (1982) found that temperatures in the range of 15-35 °C had little effect on the infectivity of miracidia to *B. glabrata*.

In *C. mutabile*, the production of metacercariae within the different snail species should also be considered in the transmission of the parasite back to the coot host. However, the relevance of the observed metacercarial production to the transmission of the parasite to the coot assumes metacercariae produced within each species are viable. This assumption is not necessarily valid as Scott, Rau & McLaughlin (1982) have shown that metacercariae of a related digenean, *T. cucumerinum*, show differing viability, depending on the snail species from which they were formed. This aspect of the transmission of *C. mutabile* will be covered in Chapter 6.

The broad specificity of the miracidia of *C. mutabile* found here conveys the same advantages as the broad specificity reported for cercariae of *E. recurvatum* and *Pseudechinoparyphium echinatum* reported by Evans & Gordon (1983b) and McCarthy & Kanev (1990), respectively. These include an increased density of

potential intermediate hosts in the environment, regardless of the structure of the local snail community, and maintenance of genetic variability of the parasite by exposing different subpopulations to different selective pressures. This first point may be of particular importance in the spring as snail densities are low and quite variable (Russel-Hunter, 1978) and may differ widely between potholes (personal observations). However, if a snail community is composed principally of refractory or insusceptible species, the wide host range and apparent non-selective choice of snail hosts by *C. mutabile* miracidia may actually reduce the local transmission of this parasite. To estimate the importance of any snail species to the overall transmission of *C. mutabile* parasite, longitudinal studies of natural systems are needed.

Chapter 5.

Seasonal field studies on the transmission and survival of *Cyclocoelum mutabile* infections in natural snail populations in southern Manitoba, Canada

5.1. Introduction

The digenean *Cyclocoelum mutabile*, a parasite of coots (*Fulica americana*), appears to undergo a yearly extinction in both its bird and snail hosts. Adult coots returning to the breeding grounds in the spring harbour patent infections but these are soon lost and recruitment of new infections does not begin until later that same summer (Colbo, 1969; McLaughlin, 1986). Since other digenean species transmitted by the same snail species as those used by *C. mutabile* are acquired during this period, it appears that *C. mutabile* infections do not overwinter in the snails or if they do, they are not accessible to the coots. Thus, flukes in coots returning in the spring apparently reseed the environment, establishing the infections seen later each summer.

C. mutabile has a life cycle that utilizes a single intermediate host. The single redial generation produces cercariae that encyst as metacercariae within the same snail host. The absence of a free-swimming cercarial stage poses some restrictions on the potential spatial distribution of the metacercariae. This seems to have been overcome by the

parasite's ability to utilize a wide range of snail species as intermediate hosts (McLaughlin, 1976; Chapter 4, this study). How the parasite is transmitted through the environment depends on the abundance of the different sizes and species of snails present at different times of the year.

The objectives of this study were twofold: (1) to monitor the seasonal development of the infective pool of *C. mutabile* in natural snail communities in an attempt to corroborate the experimental studies on hatching dynamics and host specificity described in Chapters 3 and 4; and (2) to determine whether *C. mutabile* infections are capable of surviving the winter in the snail hosts.

5.2. Materials and Methods

5.2a. Study sites

The study sites consisted of four separate ponds in the Delta Marsh, Manitoba, a large lacustrine marsh along the southern edge of Lake Manitoba (50°11'N, 98°19'W). A total of 38 ponds located throughout the Delta Marsh was examined to determine their appropriateness for the study. Factors considered were the length, width and depth of the pond, vegetation type, bottom substrate, whether the ponds were flooded by the marsh in the spring, and the abundance and diversity of the snail community. Those selected for study in the summer of 1991 were first examined in mid May of that same year and checked every two weeks thereafter until the start of the experiment to ensure a varied and large snail community was present. Those used in the 1992 portion of the experiment were monitored throughout the 1991 field season for the same reason as above and were set up the following spring.

Three of the ponds (ponds 1, 6, and A) were part of a series of 28 created by Hoffman (1970) on a 78 acre peninsula between First and Second Bays on Claire Lake. These were made by blasting with ammonium nitrate and diesel fuel and were originally used for waterfowl utilization studies. Each pond was roughly oval in shape with well delimited margins and had maximum depths of 1.20, 1.10 and 0.95m (ponds 1, 6, and A, respectively). Lengths and widths, as well as the distribution of the

predominant vegetation in each pond are shown in Figs. 5.1a-c.

Ponds 1, 6 and A were surrounded by a mixture of vegetation, comprised predominantly of whitetop (*Scolochloa festucacea* (Willd.)), phragmites (*Phragmites australis* (Cav.)), and cattail (*Typha glauca* Godr.) Emergents within these ponds included the latter two species as well as bulrush (*Scirpus lacustris* ssp. *glaucus* (Sm.)). Floating species included star duck-weed (*Lemna trisulca* L.) and filamentous green algae. The only submergent species present was sago pondweed (*Potomageton pectinatus* L.). The floating vegetation was associated only with the emergents in ponds A, 1 and 6 and was most dense in pond A. *P. pectinatus* occurred as a discreet clump in pond 6. Identification and nomenclature of all plants was made following Scoggan (1978). Each pond had a mud bottom.

The fourth pond (pond WS) was located on the grounds of the Research Station. Unlike the other ponds, it was roughly circular, had no well defined margins and the substrate consisted of soft, deep mud. The pond was bordered by a wide (>5m) edge of cattail on the south and west sides and by a narrow (<2m) edge on the others. The diameter of the open area was approximately 8m. Aside from the cattail borders, vegetation consisted predominantly of duck-weed (*Lemna minor* L.) with lesser amounts of both star duck-weed and water meal (*Wolffia punctata* Griseb.) These species formed a continuous mat over the surface of the entire pond from June through the summer. None of these ponds had sufficient surface area to attract wild coots, thus natural

contamination of the snail community was not a problem.

5.2b. Seasonal studies

The purpose of studying whole ponds was two-fold. Firstly, I wanted to determine the structure of the snail communities throughout the seasons. Although numerous studies exist on snail-community structure, these have usually been based on data from a single sampling date (e.g. Beckett, Aartila, & Miller, 1992). Those that include many sample dates have concentrated on one or a few species, not the entire community (e.g. Hunter, 1975; Boag & Pearlstone, 1979). By dissecting each of the snails collected in the sampling, I could then see if the distribution of infections in the snails was consistent with what would be expected based on the findings in Chapters 3 (temperature effects) and 4 (temperature, snail species and size effects) and the snail communities observed in the different ponds.

General procedures

Three large cages (4.3 x 1.0 x 1.25m) were constructed and placed on each of the experimental ponds in 1991. Cages were constructed with chicken wire on three sides and half-inch weld-wire on the bottoms. Each had large (1.5 x 1.25m) doors at the ends to facilitate the addition and removal of coots. The cages were positioned over the water on an angle to allow the coots free access to water and yet also provide a dry area for roosting. A 0.75 x 1.0m shelter was provided to protect the food

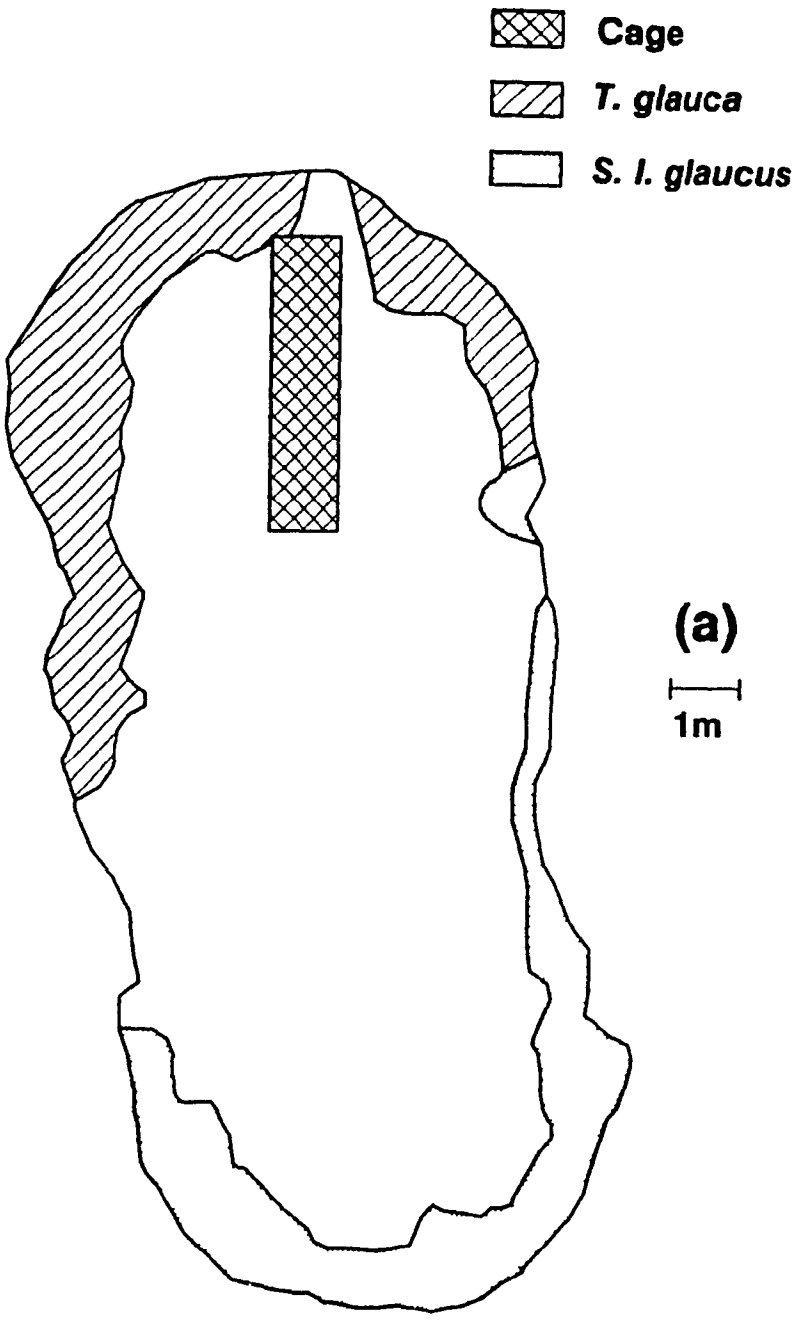
for the coots. Food was provided *ad libitum*. The ponds were contaminated with eggs passed in the bird's feces.

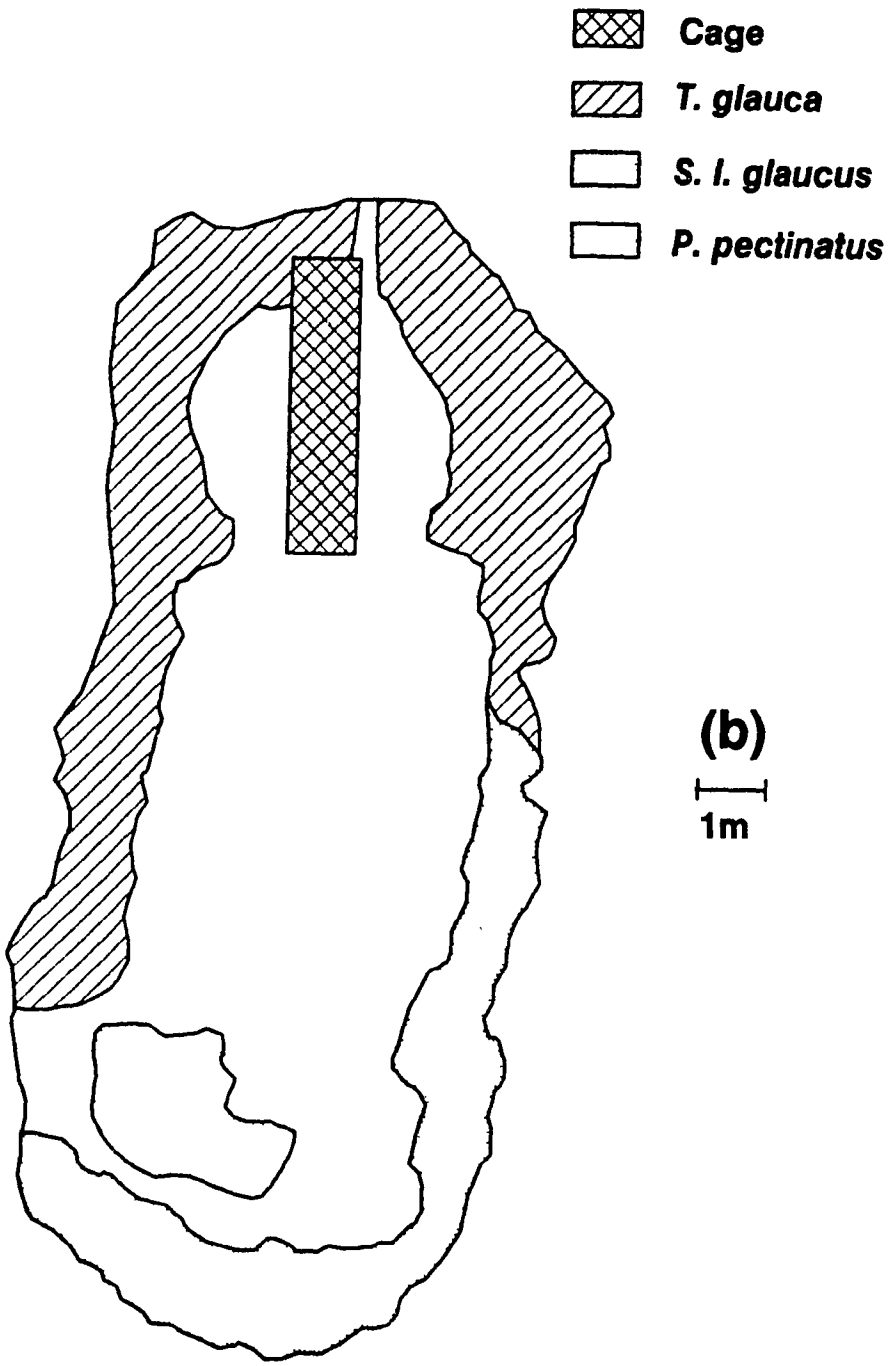
In early August of 1991, 6 juvenile lab-infected coots (infecti · confirmed by visual detection of *C. mutabile* eggs in each bird's feces) were placed in cages on ponds 1, 6 and WS to simulate infected family groups. The coots were removed from the cages in late September, when they would normally leave wetlands of this type (Hochbaum, 1944; P. Ward, personal communication).

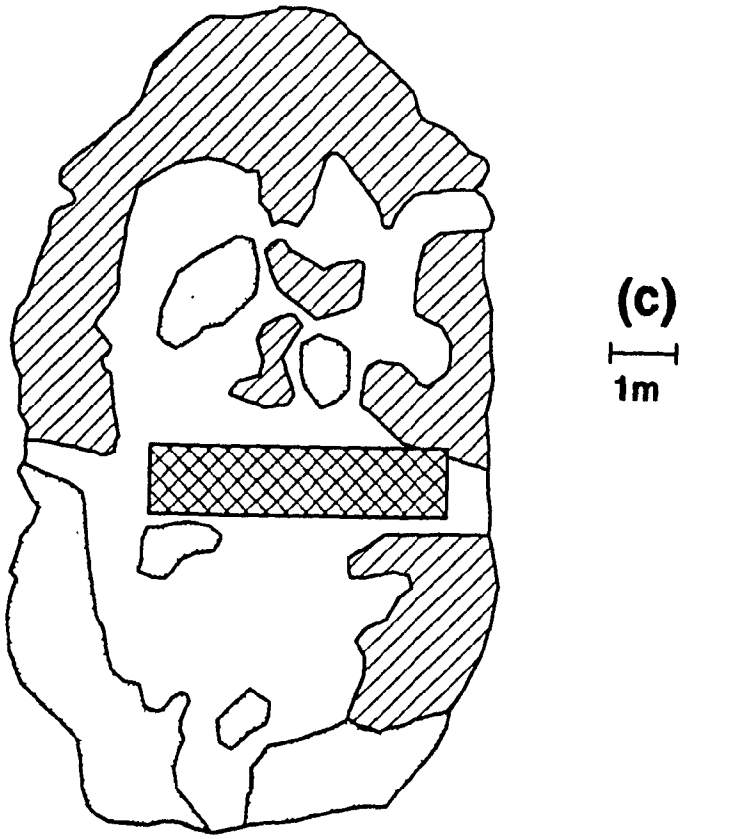
These ponds were sampled to estimate snail community structure and to monitor the buildup of the *C. mutabile* infective pool. By documenting the presence of *C. mutabile* infections in the snails, I could also determine if infections overwinter by sampling the ponds in the early spring of 1992.

In early spring of 1992, two lab-infected coots were placed in cages on three ponds. They were left until mid June when they were removed to simulate the presence and subsequent loss of infections in a nesting pair of coots. Unfortunately, the snail populations in two of the ponds disappeared in early summer before the second sampling was done. These ponds were therefore deleted from the study, leaving only pond A. Six juvenile coots were placed on pond A in early August 1992 and left until late September. Pond A was sampled throughout the spring, summer and autumn to determine the snail community structure and to monitor the development of the *C. mutabile* infective pool.

Figure 5.1. An outline of three of the ponds used (a, pond 1; b, pond 6; c, pond A) showing the placement of the cages and the major macrophyte stands.


Cage
T. glauca
S. l. glaucus
(a)
1m


Cage
T. glauca
S. l. glaucus
P. pectinatus
(b)
1m


Cage
T. glauca
S. l. glaucus
(c)
1m

Sampling protocol

Sampling dates and sizes for 1991 and 1992 are given in Fig. 5.2a and b, respectively. Briefly, in 1991, sampling was initiated the day coots were placed in cages on the ponds and was repeated every three weeks thereafter until late September. In 1992, sampling was first done one week prior to placing the coots and cages on the ponds in May, then again 6 weeks later (when the coots were removed), every three weeks for the next 4 sample dates and then 5 weeks later in late September.

Sampling of snail populations may involve the use of either direct or indirect methods (Hairston, Hubendick, Watson & Olivier, 1958). Indirect methods are generally used to estimate snail population sizes and involve the capture, marking, release and subsequent recapture of the snails, or the use of traps. None of the indirect methods were practical in this study as dissection of the snails retrieved was part of the protocol. Most direct methods involve the use of samplers with hinged or sliding jaws (i.e. Ekman dredges, Peterson grabs, or weed-box grabs), corers, dipnets, or drag scoops. Unfortunately, these methods are not suitable for sampling in emergents such as the cattail and bulrush encountered in ponds 1, 6 and A.

A preliminary sampling trial using a modification of a box sampler (Madsen, 1982) was tested. This sampler is a simple box with no bottom. The box is pushed into the substrate and all the plants, etc. are gathered and sorted at a later date. For the purposes of this experiment, the rigid box was found to be more

Figure 5.2. Flowcharts of the sampling dates and protocol carried out in the ponds in (a), 1991; and (b), 1992. duckweed, ct, br, and sago represent samples of *Lemna* spp and *Wolffia punctata*, *Typha glauca*, *Scirpus lacustris glaucus* and *Potomageton pectinatus*, respectively

(a)

Date	Event
Aug 4, 1991	Place 6 coots on ponds
	Sample ponds WS: 2 duckweed
	1: 2 ct, 2 br
	6: 2 ct, 2 br, 2 sago
Aug 25, 1991	Sample ponds WS: 2 duckweed
	1: 2 ct, 2 br
	6: 2 ct, 2 br, 2 sago
Sept 15, 1991	Remove coots from pond
	Sample ponds WS: 2 duckweed
	1: 2 ct, 2 br
	6: 2 ct, 2 br, 2 sago
May, 1992	Intensive sampling of all ponds

Date	Event
May 3, 1992	Sample pond: 4 ct, 3 br
May 10, 1992	Place 2 coots on pond
June 21, 1992	Remove coots from pond
	Sample pond: 2 ct, 2 br
July 12, 1992	Sample pond: 6 ct, 6 br
Aug 2, 1992	Place 6 coots on pond
	Sample pond: 2 ct, 2 br
Aug 23,1992	Sample pond: 2 ct, 2 br
Sept 28,1992	Remove coots from pond
	Sample pond: 11 ct, 5 br

(b)

cumbersome than helpful with such an uneven substrate and a simple square quadrat was eventually employed.

A 1x1m grid was constructed over each of ponds 1, 6 and A with baling string. The ponds were then drawn to scale on a map, detailing the vegetation in each. Each 1x1m quadrat was further subdivided into four 0.5x0.5m quadrats and assigned a number. Quadrats chosen by the use of a random numbers table were then sampled from each of the ponds at each sampling date. A minimum of two quadrats of each vegetation type (shown in Figs. 5.1a-c) were selected at each pond at each sampling date (sample numbers are given in Fig. 5.2). Although sampling with larger numbers of smaller quadrats would have allowed a more precise estimate of the snail populations (Pringle, 1984), the dense, tough vegetation made the use of smaller quadrats cumbersome. Also, quadrats of the size chosen should be large enough to provide reproducible results by minimizing the possible effects of a heterogenous distribution of the snails (Pip & Stewart, 1976).

The actual sampling involved placing a 0.25m² square quadrat over the vegetation, cutting off all emergent vegetation at the water level, discarding it, and then cutting off all the submergent vegetation as close to the substrate as possible and placing it in strong, labelled plastic bags. All the dead plants and the upper layers of the mud substrate within the quadrat were then collected and placed in the same bag and transported to the lab for sorting and counting.

All vegetation was examined by hand in the lab and all

snails found placed in water. The vegetation was then rinsed in a series of three pails and the pails searched for snails after each rinsing. The mud and dead vegetation were similarly rinsed in a series of pails. The water from these rinsings was then passed through a series of sieves, the smallest mesh size being 0.35mm. That part of the sample retained by the sieves was rinsed into white enamel tray and any remaining snails retrieved.

Due to different conditions (floating vegetation), sampling of pond WS was done by taking a 0.175m² (0.35x0.50m) sample 1m east and west of the midpoint of the cage. The emergent vegetation in this pond were not sampled as it went dry periodically. The snails were collected by vigorously mixing small samples of the plants in water in a series of three buckets and then examining them in a white enamel tray. Preliminary trials found further rinsings to yield very few further snails. After each rinsing, the snails from the buckets and tray were removed and placed in separate containers for each sample.

Snails found were identified to the species level, measured to the nearest 0.1mm with a micrometer under a dissecting microscope and then dissected and examined for digenean infections. Only infections of *C. mutabile* were identified to the species level, all others were recorded only as infection by digenean sp. When snails were discovered with *C. mutabile* infections the number of metacercariae present, if any, was recorded.

The temperature regime of pond A was monitored in 1992 by

recording both surface and bottom temperatures at the same time (16:00) on the same day each week.

5.2c. Overwinter survivorship of infections in snails

The survivorship of *C. mutabile* in snails overwinter was examined in two ways. The first method was to simply search the ponds used in the 1991 studies (ponds 1, 6 and WS) for infected snails in the spring of 1992. Beginning on May 6, each pond was searched every other day until the end of the month. Searching was done by two people, starting at the same place and working around the pond in opposite directions. Each person examined all the floating debris (dead vegetation) they encountered, then discarded it to make searching easier the following day. Vegetation was also pulled up from the substrate, examined for snails and then discarded. All snails found were measured and examined for *C. mutabile* infection as above.

The second method involved infecting snails in the lab and placing them in cages in the ponds. The cages were made of wood, measured 0.4 x 0.4 x 1.5m in height and were covered with 1mm plastic screen. The bottoms were reinforced with two layers of half-inch weld-wire. Two cages were placed in each of ponds 1 and 6. In the fall of 1991, the cages were dug approximately 15cm into the substrate and filled with mud to the same level. A complete clump of cattail, some sago pondweed and some lettuce (both boiled and fresh) was added to each as a source of food for the snails overwinter (E. Pip, personal communication). The

cattail and sago pondweed were thoroughly searched for snails before being added to the cages.

Equal numbers of infected and uninfected snails (size class #1 from Chapter 4) were added to each cage in late September when surface water temperatures had reached 8 °C. Snails were allowed to acclimate to this temperature over 2 days in an incubator and then added to the cages. One hundred infected and 100 uninfected *G. parvus*, an equivalent number of *P. exacuous* and 80 infected and 80 uninfected *S. elodes* were placed in each cage. Snail cages were retrieved the following spring and all plants and mud in each cage were examined as outlined above in the sampling protocol.

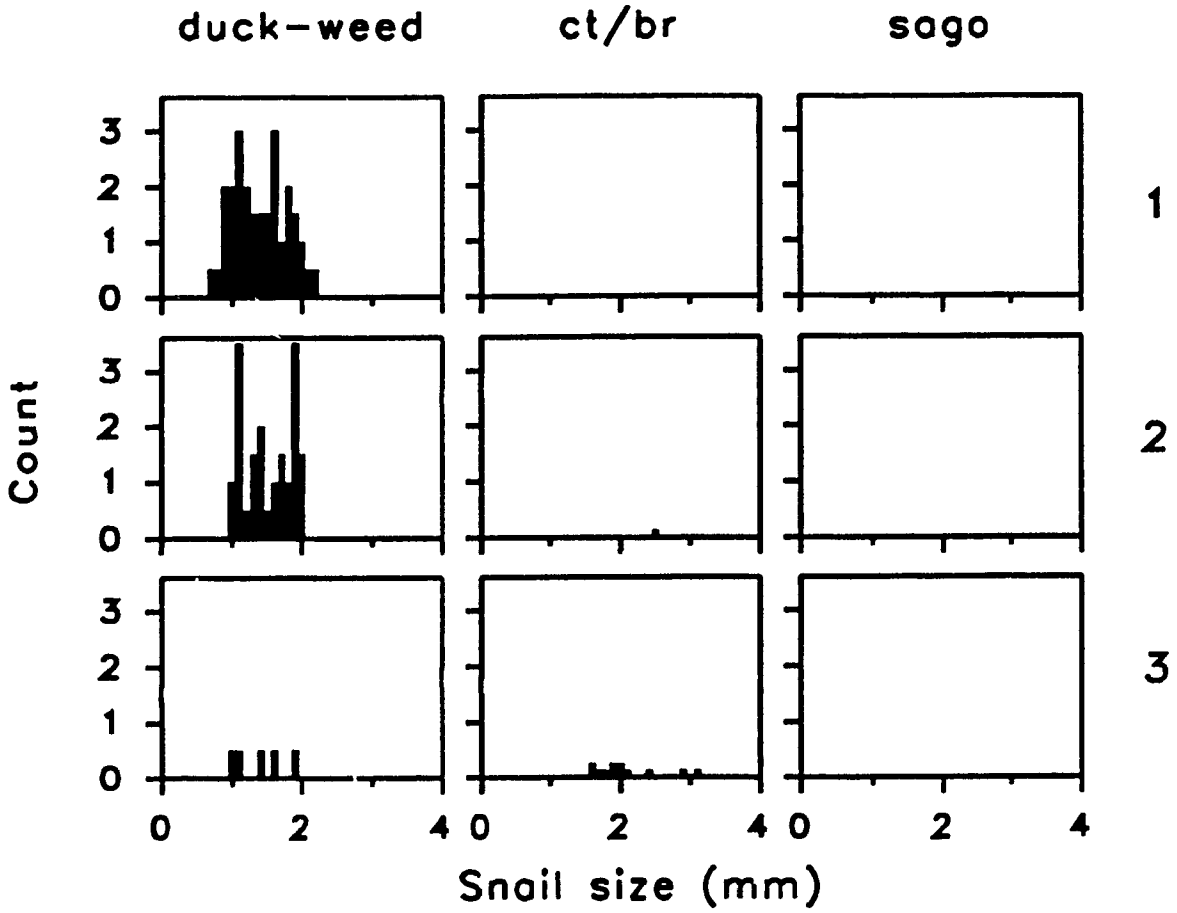
5.3. Results

5.3a. Snail communities

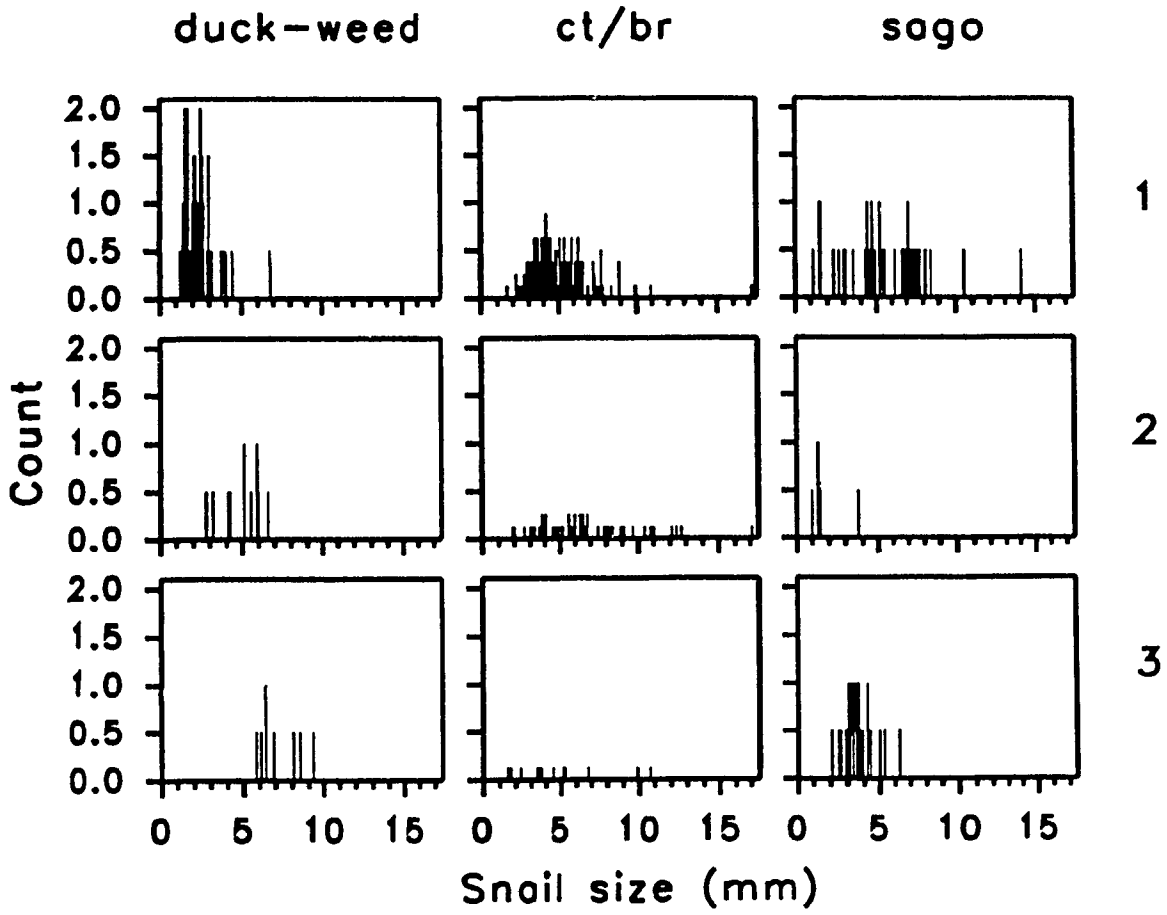
In August and September 1991, six species were found in the three ponds examined: *Physa jennessi*, *Promenetus exacuous*, *Stagnicola elodes*, *Armiger crista*, *Gyraulus parvus*, and *Helisoma trivolvis*. Preliminary examination of the data suggested that the number and species of snails recovered from cattail and bulrush samples from both ponds 1 and 6 did not seem to differ substantially between ponds or vegetation type. Samples of *P. pectinatus* from pond 6 were also quite similar, as were the *Lemna* spp./*W. punctata* samples from pond WS. Accordingly, data from the cattail and bulrush samples (ct/br) were combined at each sample date, as were both samples of sago pondweed (sago) and *Lemna* spp./*W. punctata* (duck-weed). Not all substrates were present in each pond.

Both *G. parvus* and *H. trivolvis* were rarely encountered (a total of 10 *G. parvus* and 4 *H. trivolvis*, most of which were found in the later ct/br samples). These will not be considered further. Size frequency distributions of the other four species from each substrate on the three sampling dates are shown in Figs. 5.3a-d. To facilitate comparison, the numbers in each figure represent the mean counts of snails/quadrat found in the pooled samples (ie, ct/br, sago or duck-weed) from all ponds at each sampling date. In order of predominance, *P. jennessi* was the most common snail found, followed by *S. elodes*, *P. exacuous*

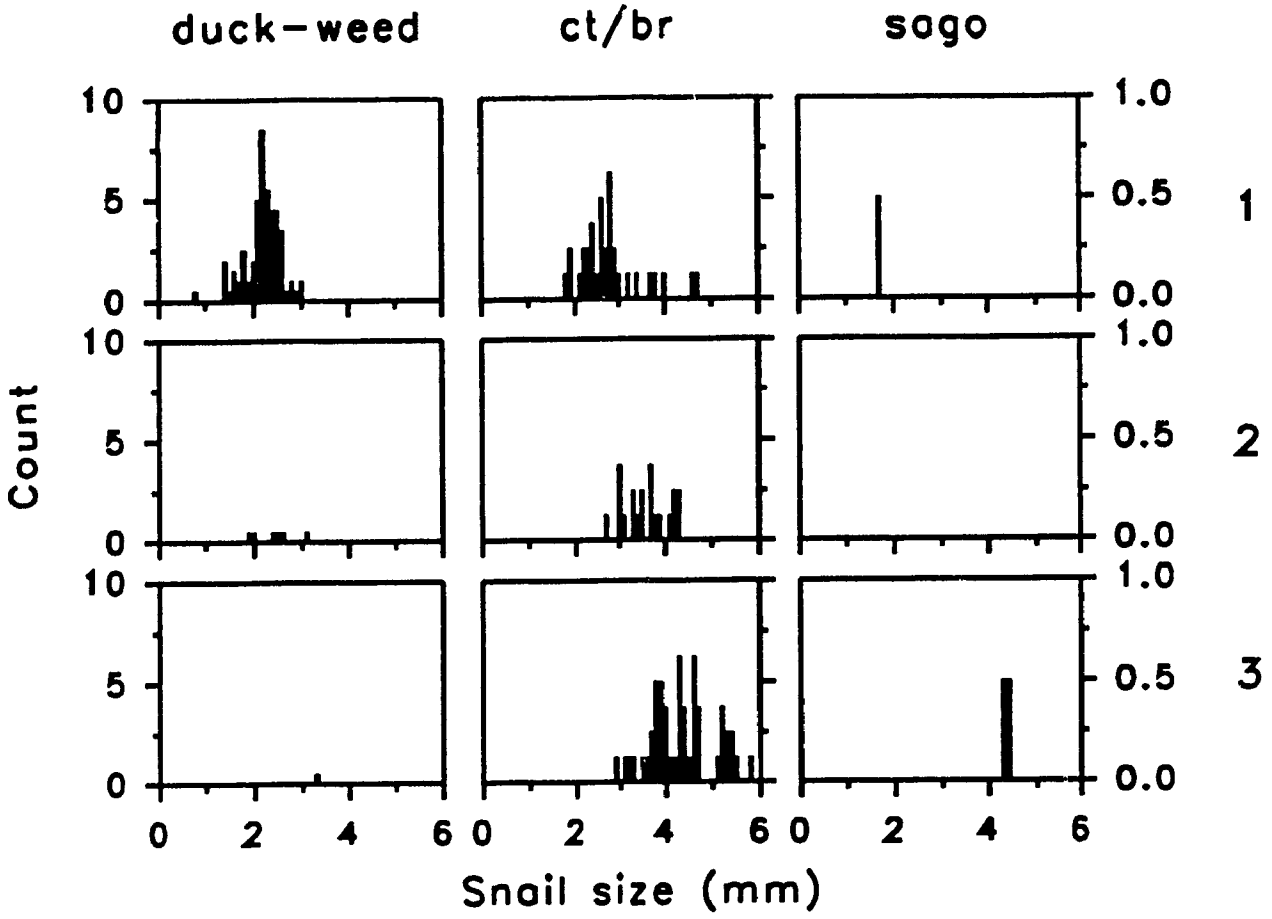
Figure 5.3. Seasonal size-class distribution of four species of snails (A, *Armiger crista*; B, *Stagnicola elodes*; C, *Promenetus exacuous*; D, *Physa jennessi*) from three different habitat types: duck-weed, cattail & bulrush (ct/br), and sago pond-weed (sago). Values are calculated as means of all quadrats sampled at each sampling date. Numbers along the right axis represent different sampling dates: (1), August 4, 1991; (2), August 25, 1991; (3), September 15, 1991.



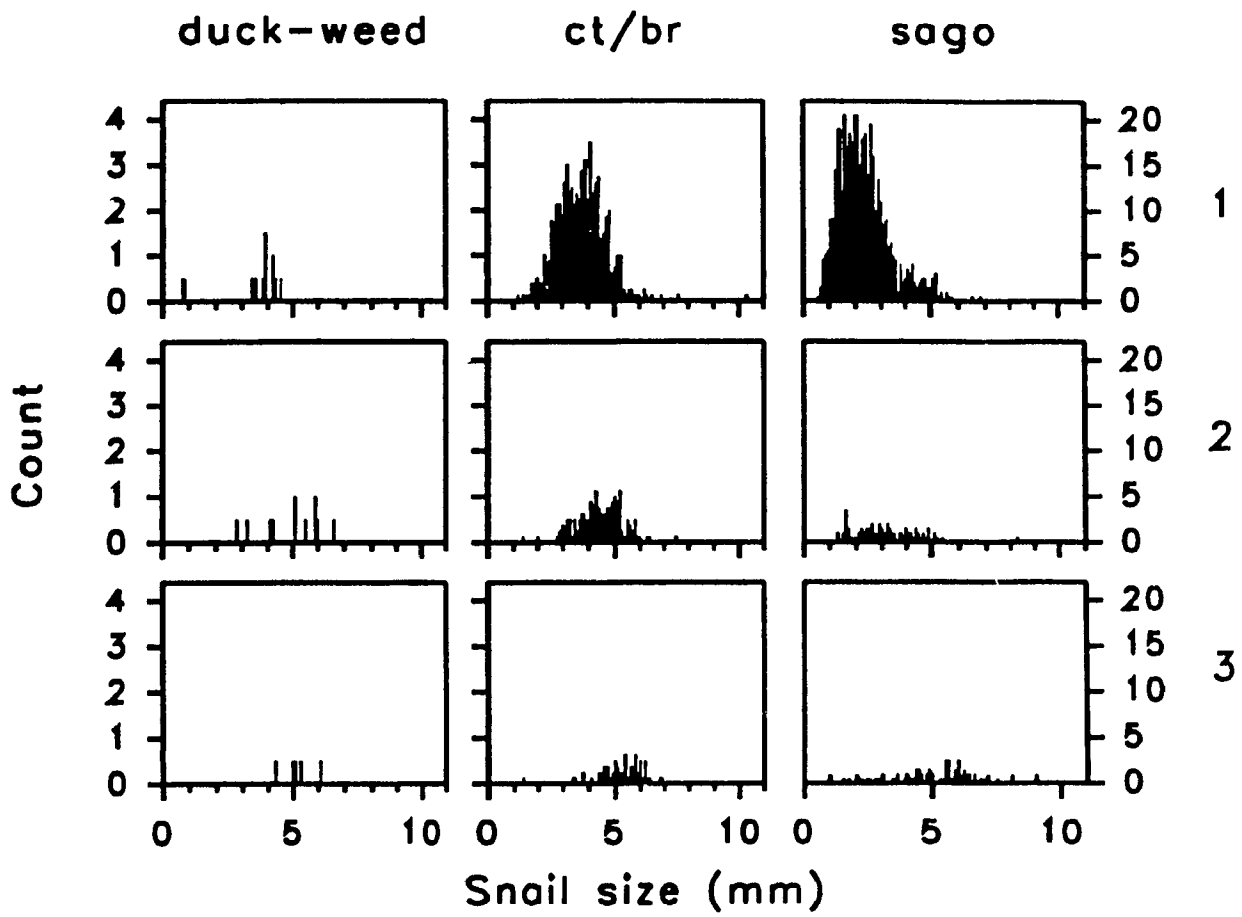
(A)


duck-weed
ct/br
sago
Count
2.0
1.5
1.0
0.5
0.0
1
2
3
0
5
10
15
Snail size (mm)

(B)



(C)



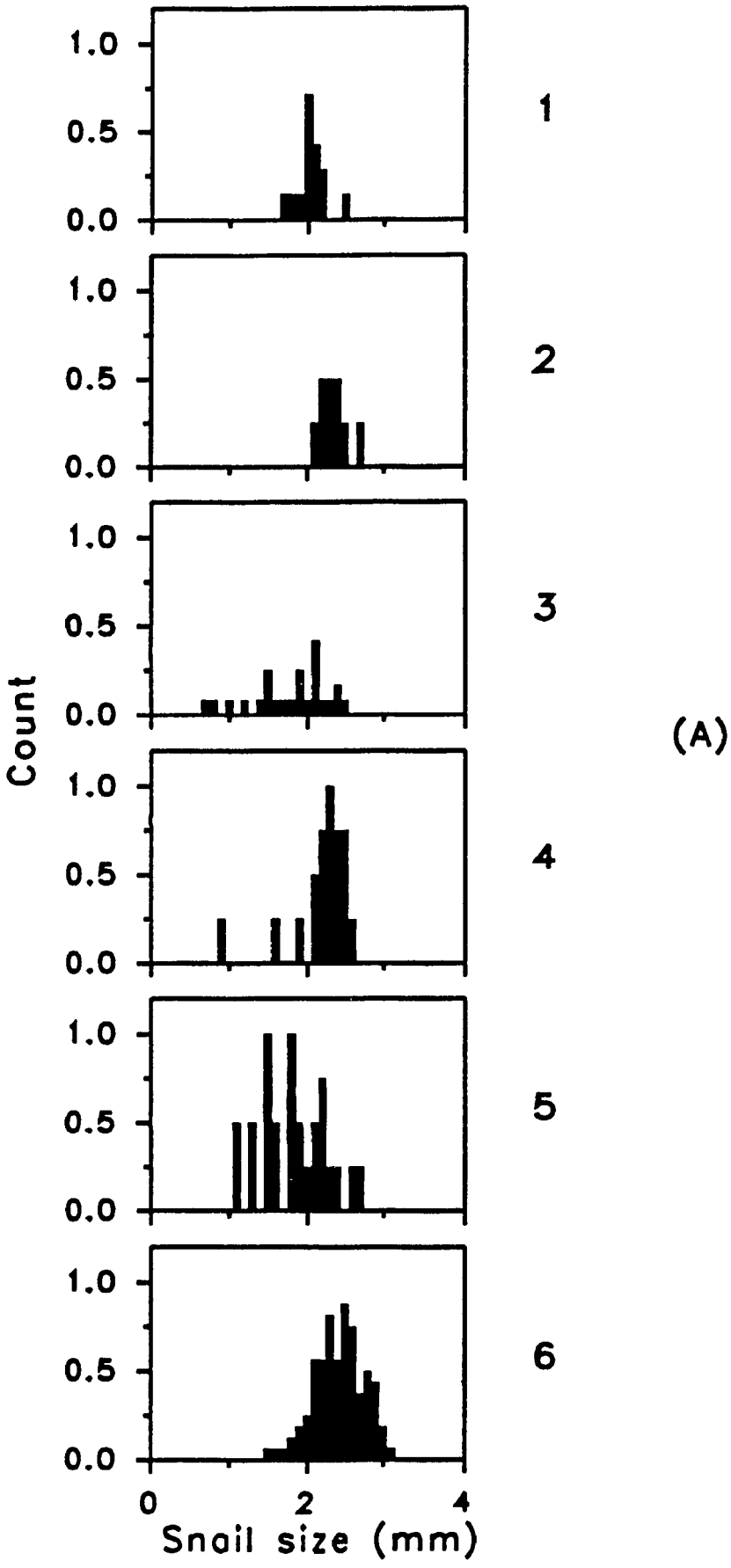
(D)

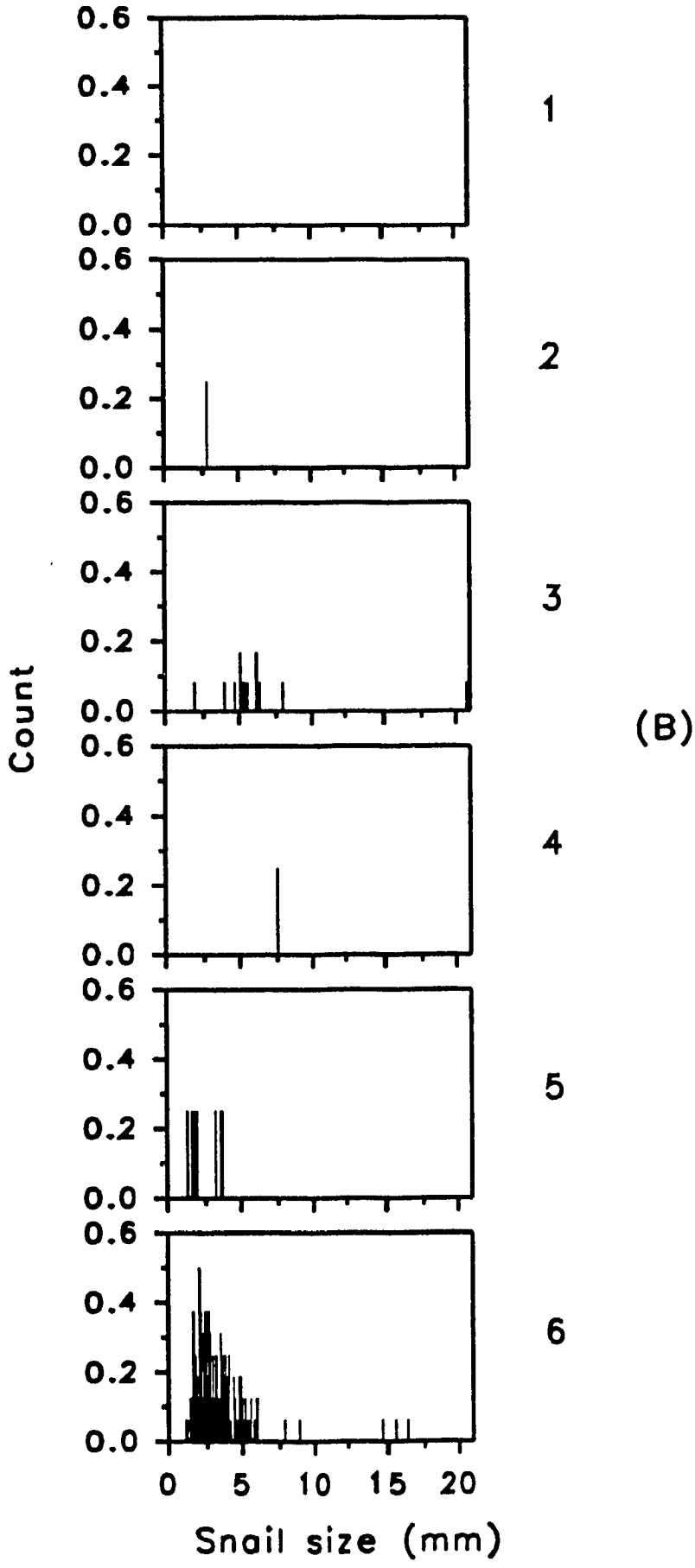
and *A. crista*, respectively. Generally, the number of each species encountered in each substrate declined over time. The one exception was *P. exacuous* (Fig. 5.3c), whose numbers dropped in the duck weed sample but were stable in the ct/br sample. The tendency to associate with a particular substrate varied among snail species and to some extent, with time. *A. crista* was found almost exclusively associated with duck-weed and *P. exacuous* with both duck-weed and ct/br. *S. elodes* was found associated with all vegetation types but *P. jennessi* was found only rarely in duck-weed, commonly in ct/br and was very abundant in the sago samples.

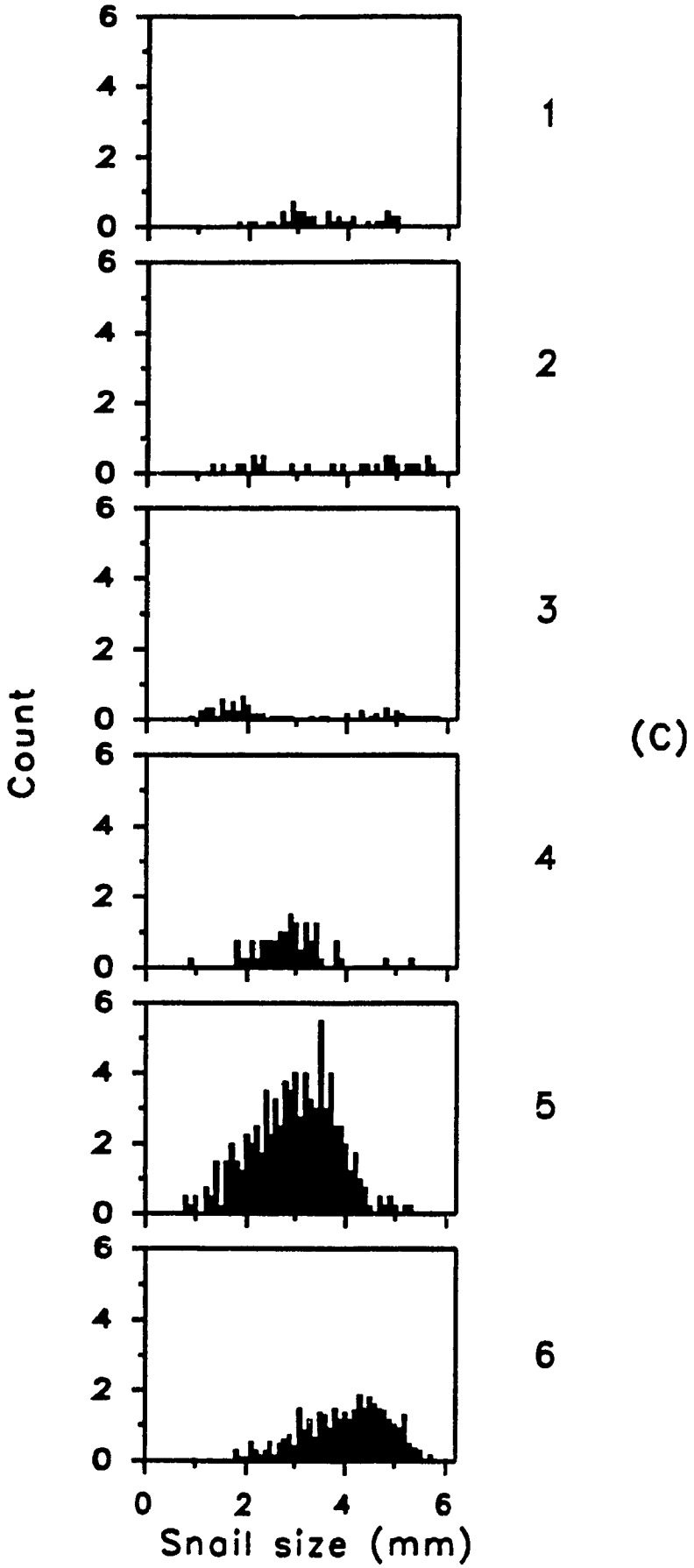
The size frequency distribution of snails also differed with time. *A. crista* showed no trends in any substrate type. In all ponds, *P. exacuous* increased in size throughout the sample season. The mean size of *S. elodes* increased with sampling date only in the duck-weed samples. No trends in the seasonal size frequency distribution of *S. elodes* were apparent in the other substrate types. Also, most of the *S. elodes* were in the size range of 1-8mm with only a few large (>10mm) individuals. *P. jennessi* showed no clear trend in the duck-weed sample but tended to increase in size with subsequent samplings in both of the other vegetation types.

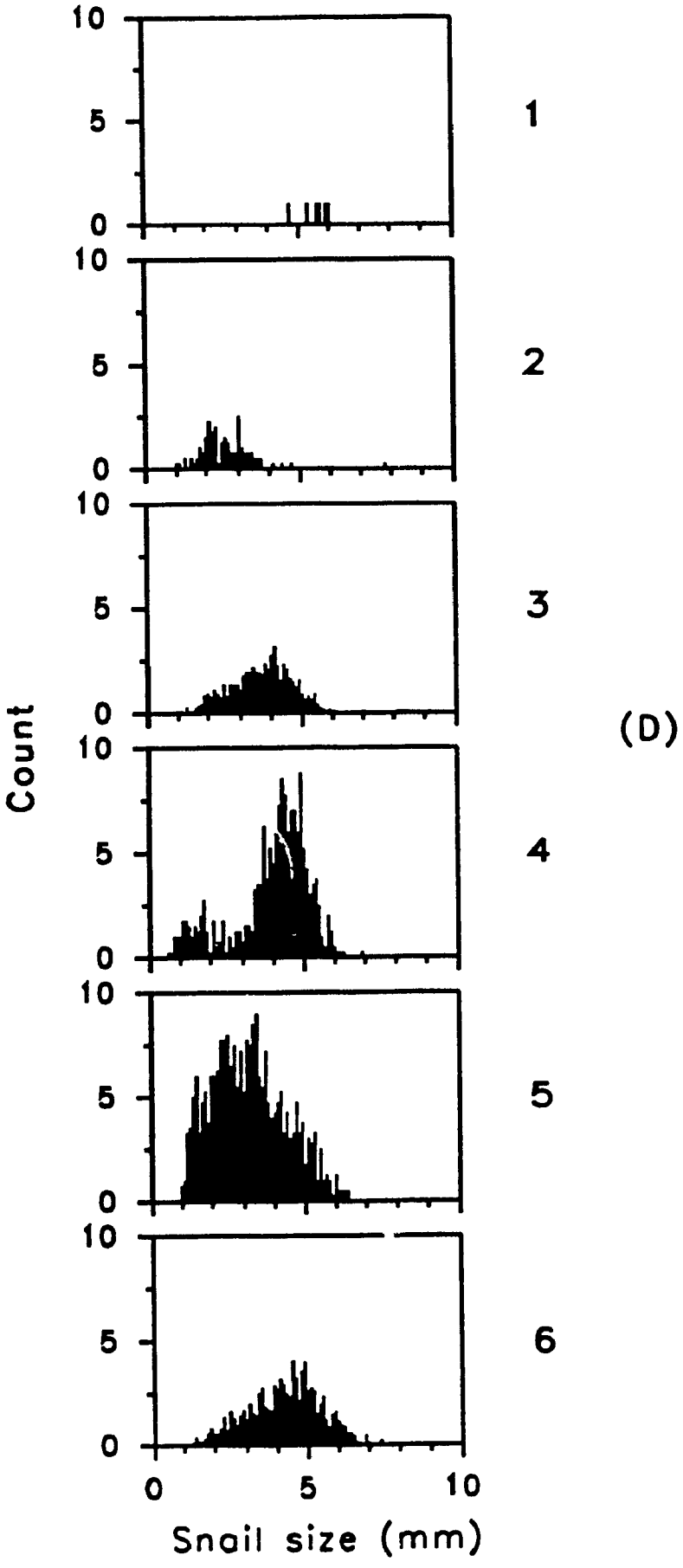
The results from the 1992 are based entirely on the collection of snails from pond A, and are shown in Figs. 5.4a-e. Except for an increase in numbers through time, the *A. crista* population showed no clear trends between sampling dates and will

Figure 5.4. Seasonal size-class distribution of five species of snails (A, *Armiger crista*; B, *Stagnicola elodes*; C, *Promenetus exacuous*; D, *Physa jennessi*; E, *Gyraulus parvus*) from pond A. Values are calculated as means of all quadrats sampled at each sampling date. Numbers along the right axis represent different sampling dates: (1), May 10, 1992; (2), June 21, 1992; (3), July 12, 1992; (4), August 5, 1992; (5), August 23, 1992; (6), September 27, 1992.

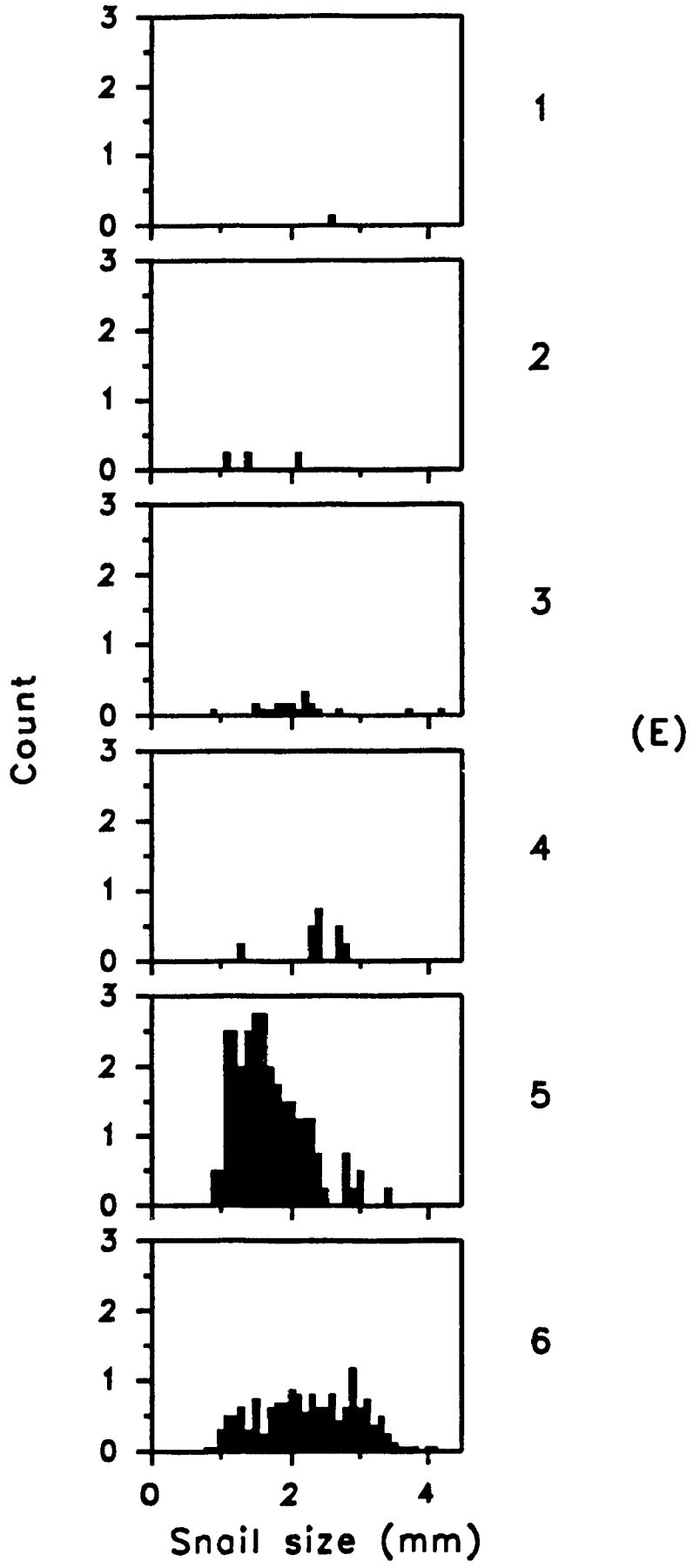

1.0
0.5
0.0
1
2
3
4
5
6
Count
(A)
0
2
4
Snail size (mm)


0.6
0.4
0.2
0.0
1
2
3
4
5
6
Count
(B)
0
5
10
15
20
Snail size (mm)


Count
Snail size (mm)
1
2
3
4
5
6
(C)



(D)



(E)

not be discussed further. Few snails of any species were found on the first sampling date (May 3). No *S. elodes* and only a single *G. parvus* were found. The *P. exacuous* and *P. jennessi* were typically large and the size frequency distribution of the *P. exacuous* sample was quite wide. By the second sample date (June 21) *S. elodes* and *G. parvus* were still quite rare. The number of *P. exacuous* recovered remained about the same but the size frequency distribution was wider still. Almost all large *P. jennessi* had disappeared by this time but a new cohort of this species was evident by the large increase in snail numbers in the smaller size classes. Thus, oviposition in this species must have occurred a couple of weeks prior to this sampling date. By early summer (July 12), the *P. jennessi* population had increased both in number and in mean snail size. Both *S. elodes* and *G. parvus* were still quite rare. The density of *P. exacuous* was slightly higher and a new cohort of snails was evident by a slight increase in the number of snails observed in the smallest size classes. The initiation of hatching for *P. exacuous* observed in the July 12 sample was reflected by a doubling of that species' population size and an increase in the mean shell size by the August 2 sampling. The population of *P. jennessi* had also more than doubled and a second generation of this species was evident by the slight bimodal distribution of the size frequency histogram. *S. elodes* and *G. parvus* were still quite rare. By the August 23 sample, the density of *S. elodes* had increased slightly but that of *G. parvus* had increased

dramatically. The distribution of *G. parvus* was skewed towards the larger size classes, suggesting a possible late summer hatch for this species. The size distribution of *P. exacuous* had shifted further towards the larger size classes and the population density had again increased over five-fold. The *P. jennessi* population increased further but a large portion of the larger cohort from the previous sample had disappeared, apparently replaced by a younger cohort. By the last sample date, the number of *G. parvus*, *P. jennessi* and *P. exacuous* had all decreased by at least 50%, suggesting that many snails had died. The size distributions of all these species had shifted further towards the larger size classes. The number of *S. elodes* in this sample increased greatly and most of those were in the smaller size classes, suggesting an early fall hatch.

The weekly temperature regime of pond A shows a slow increase of water temperatures to a maximum in mid-August then a rapid decline through autumn (Fig. 5.5). Bottom water temperatures remained above the hatching threshold for *C. mutabile* eggs (14 °C) for about two months, between the end of June and end of August.

5.3b. Parasite infections

Twenty six snails were found infected with *C. mutabile* over the course of the two field seasons in the experimentally contaminated ponds (Table 5.1). Using the data on infectivity determined in Chapter 4, together with the data on the different

Figure 5.5. Seasonal surface and bottom temperatures for pond A in 1992. Dotted horizontal line represents the temperature threshold below which hatching of *Cyclocoelum mutabile* eggs does not occur.

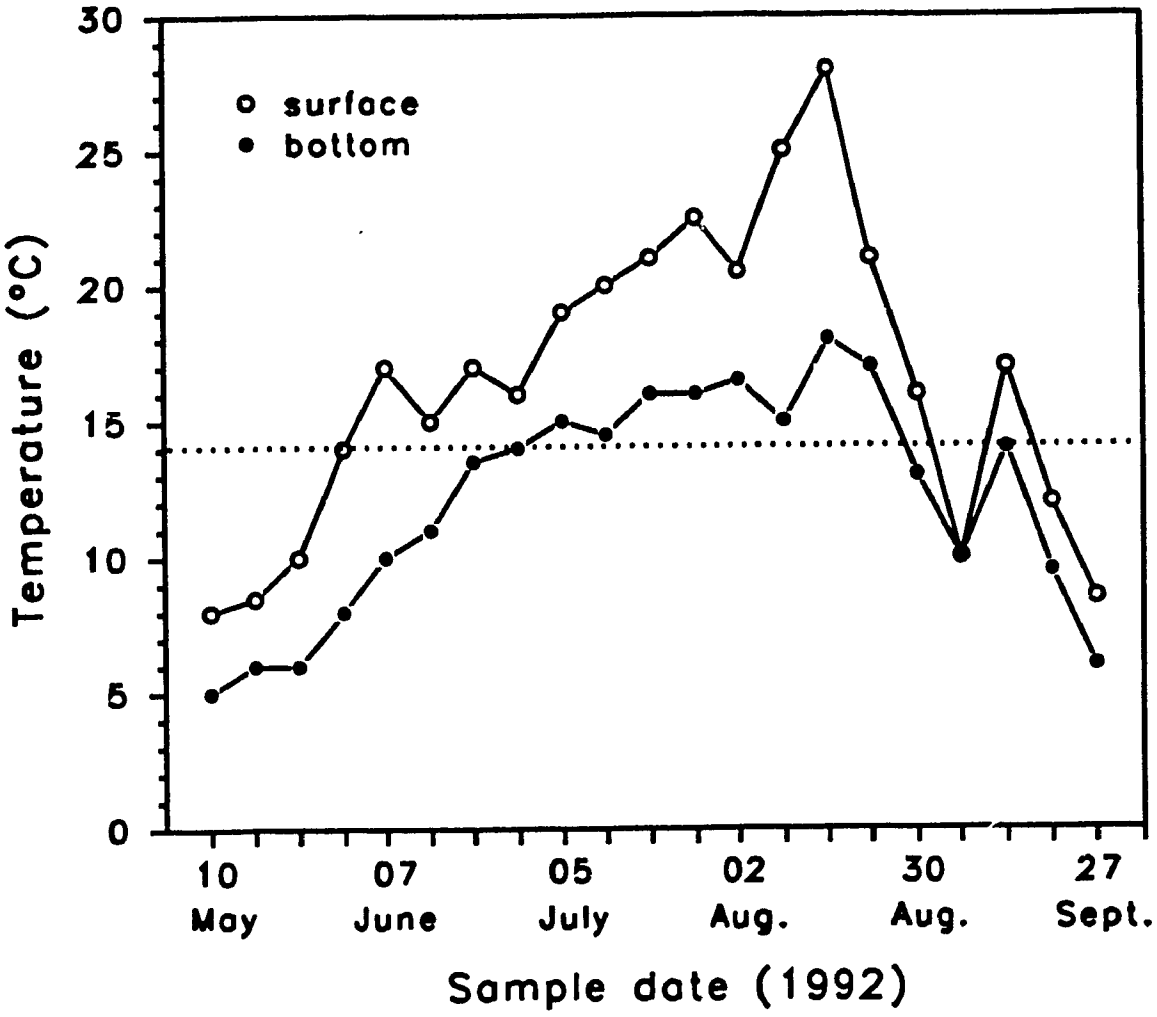

30
25
20
15
10
5
0
Temperature (°C)
surface
bottom
10 May
07 June
05 July
02 Aug.
30 Aug.
27 Sept.
Sample date (1992)

Table 5.1. Summary of all *Cyclocoelum mutabile* infections recovered from snails collected in the 4 ponds throughout the 1991 and 1992 field seasons.

Sample date	Pond	Snail species	Snail size (mm)	No. meta-cercariae
25/08/91	WS	*S. elodes*	4.2	0
	WS	*A. crista*	1.7	1
	WS	*A. crista*	2.0	2
	6	*S. elodes*	6.0	1
	6	*P. jennessi*	4.0	0
15/09/91	1	*S. elodes*	3.4	0
	1	*P. exacuous*	3.7	0
	6	*P. exacuous*	4.5	17
12/07/92	A	*S. elodes*	5.1	0
	A	*P. exacuous*	4.8	0
	A	*P. exacuous*	4.3	1
	A	*P. exacuous*	5.0	0
02/08/92	A	*P. exacuous*	5.3	14
23/08/92	A	*P. exacuous*	3.6	1
	A	*P. exacuous*	2.8	0
27/09/92	A	*S. elodes*	5.8	2
	A	*S. elodes*	4.8	11
	A	*P. exacuous*	3.4	8
	A	*P. exacuous*	2.8	6
	A	*P. exacuous*	4.7	6
	A	*P. exacuous*	3.6	8
	A	*P. exacuous*	2.4	5
	A	*P. exacuous*	3.6	3
	A	*G. parvus*	2.8	2
	A	*G. parvus*	3.0	1
	A	*G. parvus*	2.9	7

snails recovered in the samples, I estimated the expected number of infections that would be found in each species. Sample dates considered were those in which infections were recovered (i.e. each 1991 sample and those taken after June in 1992). Estimates were made using the mean of mean susceptibilities of the snails determined for each replicate at each temperature. The snails were separated by species and size classes to estimate the number of infections expected in each species and size class and the values added together for each size class of a given species. The calculated and observed values are given in Table 5.2. The observed and expected numbers were compared by a multinomial test (*H. trivolvis* was not included in the analysis). No significant differences were found between observed and expected frequencies ($G = 2.44$, $P = 0.786$).

Examination of Table 5.1 reveals three points. Firstly, infected snails with metacercariae were found in the second sample collected in 1991 (August 25). Thus, metacercariae may be produced within three weeks under field conditions in the late summer. Secondly, the first infected snails retrieved in 1992 were in the mid-July sample. A single metacercariae was recovered from 1 snail, suggesting that all were recent infections. Lastly, all infected snails recovered following the August 23 samples taken in 1992 were old infections (all had metacercariae).

Table 5.2. Observed and expected number of infected snails observed in ponds in 1991 and after June, 1992.

Snail species	% total abundance*		# infected	
	1991	1992	obs.	exp.
P. jennessi	72.5	67.4	1	3.1
P. exacuous	8.4	19.1	14	12.5
S. elodes	14.3	3.0	6	3.3
A. crista	4.2	3.2	2	3.2
G. parvus	0.4	7.3	3	3.9
H. trivolvis	0.2	-	0	0.0

* n = 2383 and 5450 for 1991 & 1992, respectively.

Table 5.3. Placement and recovery (=survivorship) of *C. mutabile* - infected and uninfected snails from four cages left overwinter in two ponds.

Pond	Position	Snail species	#retrieved/#placed	
			infected	uninfected
1	North	*S. elodes*	0/80	1/80
		G. parvus	0/100	0/100
		P. exacuous	0/100	0/100
	South	*S. elodes*	0/80	1/80
		G. parvus	0/100	0/100
		P. exacuous	0/100	4/100
6	North	*S. elodes*	0/80	4/80
		G. parvus	0/100	0/100
		P. exacuous	0/100	1/100
	South	*S. elodes*	0/80	3/80
		G. parvus	0/100	0/100
		P. exacuous	0/100	0/100

5.3c. Overwinter survivorship of infections in snails

None of the 3155 snails recovered in May of 1992 from ponds 1, 6 and WS had *C. mutabile* infections.

None of the 1120 infected snails left in the cages overwinter survived. However, 14 of the 1120 uninfected snails kept in the same cages survived and were recovered (Table 5.3). Examination of this data by Wilcoxon signed rank test showed significantly fewer infected snails survived the winter as compared to uninfected snails ($Z = 2.20$, $P = 0.028$).

5.4. Discussion

The various snail species found in the experimental ponds were generally associated with vegetation used by coots. In particular, species associated with the sago pondweed and the floating vegetation are likely to be ingested by coots as these plant species comprise a large portion of their diet (Jones, 1940; Driver, 1988).

Data on the diversity and population structure of snails in the experimental ponds revealed that comparatively few, mostly larger individuals, were present in the early spring. Populations of the various species increase over the summer, albeit at different rates, with the recruitment of different cohorts and then generally exhibit a decline in number in late summer and early fall. There appears to be a heavy overwinter mortality based on the number and population structure of each of the species in the spring, relative to that seen in the fall.

Although number and recruitment rates in the experimental ponds varied among species, a variety of species and size classes were present early in the year when infection of snails occurs.

The actual number of infected snails found in the experimental ponds was disappointingly low. While it is possible that some newly acquired infections were undetected, the rediae grow rapidly and are easily observed by the time the infections are one week old (personal observation). Recovery of infections from 5 of the 6 species found in the experimental ponds further

validates the conclusion that a broad degree of host specificity exists in *C. mutabile* (McLaughlin, 1976; Chapter 4) and confirms that these species are susceptible under natural conditions. Thus, each of these snail species may contribute to the transmission of this fluke from one coot to the next.

The proportion of infections from snails in the experimental ponds corresponded to the numbers of the dominant snail species in the different ponds. In 1991, the snail community of pond WS was dominated by *A. crista*, *P. exacuous*, and *S. elodes*; ponds 1 and 6 were dominated by *P. jennessi*, *P. exacuous* and *S. elodes* during the sampling period. In 1992, the dominant species in pond A were *P. jennessi*, *P. exacuous*, and *G. parvus*, which became abundant only later in the summer. In 1991, infections were found in all of the dominant snail species. The same was true in 1992 with the number of infections in both *G. parvus* and *S. elodes* increasing with the number of those species in late summer and fall. Although *P. jennessi* was the most abundant species, only one infected individual was found. This species was also found to be refractive to infection in lab studies (Chapter 4). On the other hand, *P. exacuous*, a highly susceptible species (McLaughlin, 1976; Chapter 4), was the second most abundant and 14 of the 26 infected snails recovered were of this species. Thus, the recruitment of infections by the snail populations seems to be a function of the density and susceptibility of the different species of the snail community. Evans, Whitfield & Dobson (1981) and McCarty & Kanev (1990) have reported comparable

findings for the transmission of cercariae of both *Echinoparyphium recurvatum* and *Pseudechinoparyphium echinatum*, respectively, to their second intermediate mollusc hosts.

Despite the large number of *P. jennessi* examined, only a single infected individual was found. This, and the low susceptibility of this species in lab studies (Chapter 4), suggests that *P. jennessi* contributes little to the transmission of *C. mutabile*. However, a large population of *P. jennessi*, such as the one observed in pond 6, may have a great negative effect on the overall transmission of *C. mutabile*. The apparent non-selective behaviour of the miracidia when presented with a choice of two snail species (Chapter 4) suggests that *P. jennessi* may limit the distribution and number of *C. mutabile* infections in the snail community by acting as a sink. This may also help account for the low prevalence of infection in the experimental ponds. These results suggest that the composition of the snail community can influence the transmission of *C. mutabile* if the dominant species is a poor host.

On a seasonal basis, infected snails were not recovered until the July 12 sample date in 1992. None were recovered in the June 21 sample, despite the fact that infected coots had been placed on the pond some five weeks prior to this sampling. Moreover, the infections recovered on the July 12 sampling date all appeared to be recent infections; one of these snails had a single metacercariae; the rest had only rediae. In Chapter 3, I demonstrated that a temperature threshold of 14 °C must be

reached in order for *C. mutabile* eggs to hatch. The bottom of this pond reached 14 °C the week of June 28. Eggs hatching at this time would have had 2-3 weeks to develop within the snail host by the July 12 sampling date, enough time to produce a limited number of metacercariae. Thus, metacercariae from infections established in the spring would be available by mid July when infection begin to appear in young coots (Colbo, 1965; McLaughlin, 1986).

With 1 exception, all infected snails found in subsequent samplings in 1992 harboured metacercariae. One *P. exacuous* encountered in early August had only a redia present and other snails had few metacercariae, suggesting comparatively recent infections. Metacercarial numbers were mostly higher in the September sample, suggesting older infections, although a few individuals harboured small numbers. However, the total lack of young infections (redial infections with no metacercariae produced) in the late September sample, despite the fact that six infected coots were on the pond, suggests that transmission had not occurred on this pond for some time. The recorded temperature regime of this pond shows the bottom temperature had not risen above 14 °C since the beginning of the month.

Although not conclusive, these results suggest that recruitment of new infections does not occur prior to the end of June or after late August in wetlands with a similar temperature regime. Thus transmission of *C. mutabile* to snails seems to occur during a very short transmission window: the two month

period between the end of June and end of August when temperatures allow the hatching of the eggs.

As bottom temperatures continue to decline following the beginning of September, no further hatching of the eggs was likely. Thus, it would seem that a large number of eggs produced in late summer and fall would not hatch in northern wetlands. However, the benefits of delaying hatching in the spring until water temperatures increase above 14 °C, when snail abundances are higher, probably outweigh the loss of potential infections established in late summer. Eggs deposited late in the year may possibly survive the winter and hatch the following spring as has been suggested to occur for the eggs of other digeneans of waterfowl such as *Hysteromorpha triloba* and *Sphaeridiotrema pseudoglobulus* by Hugghins (1954) and McKindsey & McLaughlin (1993), respectively. This aspect of the ecology of *C. mutabile* remains to be investigated.

The results from the caged snail experiments suggest that infection by *C. mutabile* lowers the overwinter survivorship of snails. Although not conclusive, the recovery of over 3000 uninfected snails from ponds 1, 6 and WS when infected snails were known to be present in these ponds in the fall of 1991 supports this idea of differential mortality between infected and uninfected snails. Additional stress added by a parasite infection may account for the observed differential survivorship. A number of authors have suggested that snails infected with digenean larvae may have a reduced tolerance to adverse

environmental conditions (e.g. Brown, 1978; Reader, 1972; Sousa & Gleason, 1989), as may the larvae themselves (e.g. Goater, Shostak, Williams & Esch, 1989; Fernandez & Esch, 1991).

The high overwinter mortality seen in the caged snails may be representative of what occurs in natural snail populations. Over 3000 snails were recovered from ponds 1, 6 and WS in the spring of 1992. This represents only a small fraction of the number of snails that were present in these ponds in the fall of 1991. A further pond selected in the fall of 1991 for use in the 1992 field season for its high abundance of snails also suffered a severe decline in snail numbers: the early spring sample yielded only 2 snails from eight 0.25m² quadrats. Thus, overwinter mortality of all snails in the ponds studied appears to be very high.

The importance of any of the snail species found in the ponds studied to the transmission of *C. mutabile* back to coots depends not only on the seasonal prevalence of the infections in the different snail species, but also on the biology of the coots. In the wild, many coots gather in large flocks away from the nesting sites in late summer (Ryder, 1963). Infections established in the fall may not produce metacercariae before coots start migrating south. Thus, the significance of the increase in prevalence of *C. mutabile* infections in *G. parvus* is not clear. In the ponds manipulated in this study, it would seem that *P. exacuous* plays the most important role in the transmission of *C. mutabile* to coots each year.

The results of this study corroborate the temperature threshold reported in Chapter 3 of this study and show this to be an important factor in the development of the infective pool of *C. mutabile* in a natural snail community. This temperature threshold acts to limit the width of the transmission window available to *C. mutabile*. The results also show the wide specificity of the miracidia reported in Chapter 4 to underlie the distribution of the infections in the snail community of the ponds studied.

Chapter 6.

Infectivity of *Cyclocoelum mutabile* metacercariae from three species of snails and the effect of coot age on susceptibility to infection.

6.1. Introduction

The digenean *Cyclocoelum mutabile* displays a bimodal seasonal prevalence in coots on their summer breeding grounds (Colbo, 1965; McLaughlin, 1986). Adult birds arrive each spring with patent infections that are lost by early summer. Recruitment of new infections begins later in the same summer. Once initiated, recruitment continues until the coots migrate south in the fall with the prevalence of infection increasing throughout this period. The conclusions regarding the role of various snail species in the transmission of *C. mutabile* in the preceeding three chapters have had the underlying assumption that metacercariae from the different snail species are equally infective to coots. This is not necessarily the case. Scott (1980) has shown that metacercariae of another cyclocoelid, *Typhlocoelum cucumerinum* sspp., differ widely in their infectivity to ducks and levels of *C. mutabile* in coots are greater in juvenile coots than in adults. Thus heterogeneity in the infectivity of metacercariae in the snail community or in the susceptibility between different cohorts of coots may influence the distribution of this fluke in coots.

Metacercariae of both *Typhlocoelum* subspecies from different snail hosts differ widely in their infectivity to ducks (Scott, 1980). Recently, Goring (1993) found that the cellular response in *Stagnicola elodes* to eight-week old *C. mutabile* infections was slightly stronger than that exhibited by either *Promenetus exacuous* or *Gyraulus parvus* with infections of the same age. He also noted that although similar proportions of metacercariae from each species excysted *in vitro*, the distribution of excystment times differed. A large proportion of metacercariae from each species excysted within the first 60 minutes but those from *S. elodes* required on average longer to excyst. As the passage rate for gut contents is generally high in birds (Duke, 1986), this raises the possibility that a larger proportion of metacercariae from *S. elodes* may not excyst soon enough to establish in the host. Thus, metacercariae from these species may not be equally infective to coots, thereby influencing the transmission dynamics of *C. mutabile*.

Adult coots remain less heavily parasitized than juveniles (Colbo, 1965, McLaughlin, 1986). The difference between young and adult coots may be a function of one or a combination of three factors. The first possibility is that the higher prevalence of *C. mutabile* in juveniles reflects a higher proportion of snails in the diet of young coots. The latter two possibilities are related to differences in the immunocompetence of the birds. Young coots may simply have a less efficient immune system than the adults. Alternatively, adults, most of

which have had previous exposure to the parasite (McLaughlin (1986) has found up to 80% of young coots to be infected each fall), may have acquired a resistance to the parasite.

The first objective of this study was to determine if metacercariae from the three different species of snails used by Goring (1993) vary in infectivity. Secondly, I wanted to determine if juvenile and adult coots differ in their susceptibility to infection by *C. mutabile*. The last objective of this study was to determine whether prior infection by *C. mutabile* alters the susceptibility of coots to homologous infection.

6.2. Materials and Methods

Thirty lab-reared juvenile coots (about 2 months old) were randomly divided into three equal groups. Each bird in each group received, via oral intubation, 25 *C. mutabile* metacercariae from either *S. elodes*, *P. exacuous* or *G. parvus*. All metacercariae used were from 7 to 8 week-old infections that were initiated at the same time in the various snails. For each infection, enough snails were dissected to obtain the required number of metacercariae which were pooled and subdivided into doses as needed. Eight naive adult coots and 10 that had previously been exposed and infected by *C. mutabile* metacercariae four months prior to reinfection attempts (infections confirmed by observation of *C. mutabile* eggs in the bird's feces) were fed an equivalent number of metacercariae from *G. parvus*. Adult and juvenile coots were exposed to metacercariae from *G. parvus* on the same day; juvenile coots exposed to metacercariae from *S. elodes* and *P. exacuous* were exposed on the subsequent 2 days. Birds were necropsied at 4-5 weeks pi and the number flukes that established in each bird determined.

A series of three contrasts were carried out on the same data set: among juveniles (challenged with metacercariae from three snail species), among adults (naive and previously infected), and among naive adults and juveniles receiving metacercariae from the *G. parvus* same snail species. A Bonferroni adjustment ($P \leq 0.05/(3x2) = 0.008$) was used for these

comparisons to keep the overall alpha level at 0.05 (Neter, Wasserman & Kutner, 1985).

6.3. Results

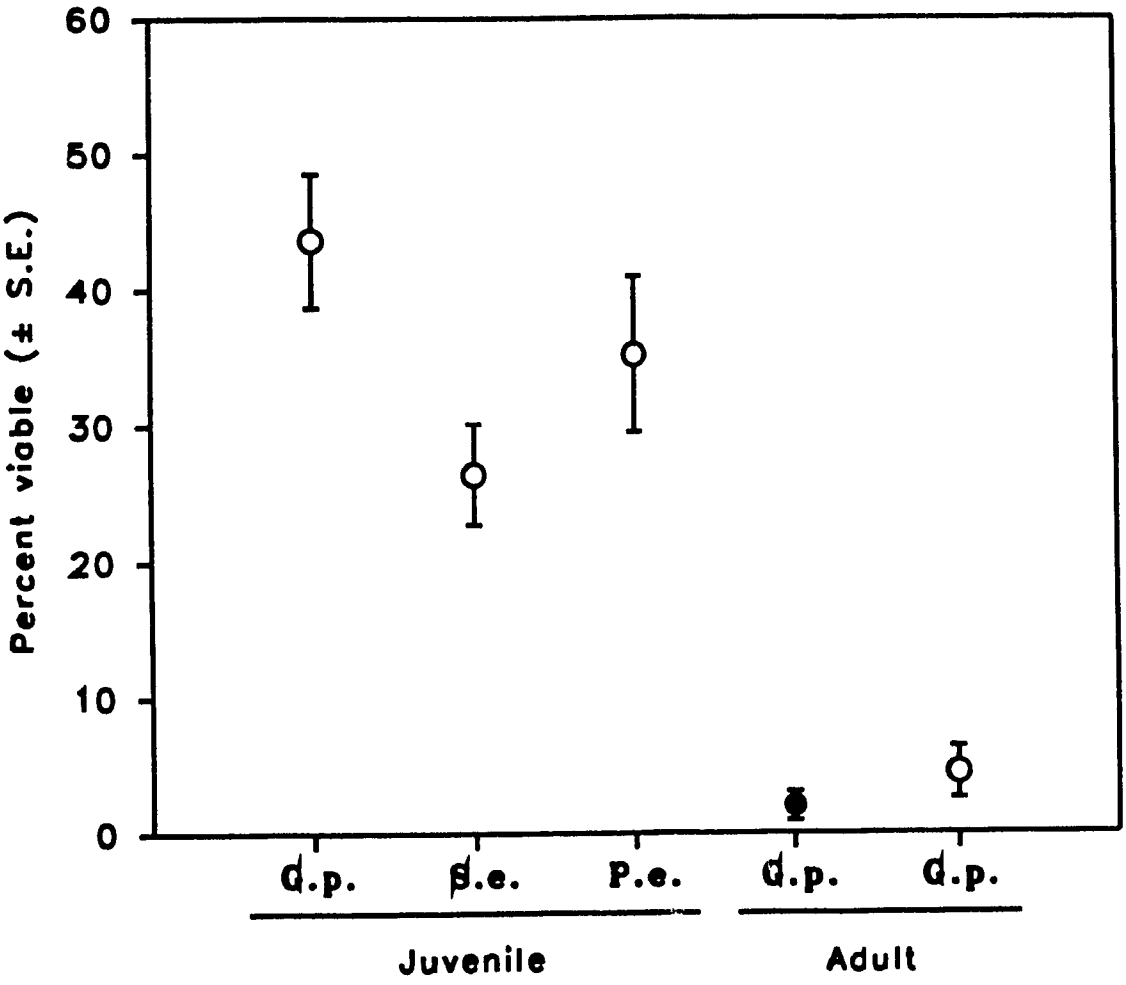
All of the juvenile coots exposed to metacercariae became infected. However, only 3 of the 10 previously infected adult coots became infected (30%) and only 2 of the 8 naive adult coots became infected. A significantly higher percentage of the metacercariae established as flukes in juvenile coots (43.6% of the initial dose) than did in adult coots (2.0%) (Mann-Whitney *U* test, $Z = 3.780$, $P = 0.0002$).

The proportion of metacercariae successfully infecting the coots is shown in Fig. 6.1. The mean infectivity of metacercariae to juvenile coots ranged from 26.4% in *S. elodes* to 43.6% in *G. parvus*. However, these differences were not statistically significant (ANOVA: $F = 3.145$; $P = 0.059$).

As the distribution of infections in adult coots did not meet the assumptions of normality and attempts to normalize the data had the reverse effect on the data from juvenile coots, non-parametric statistics were used. Results from the naive adult group were compared to that of juvenile coots exposed to metacercariae from the same snail species, *G. parvus*.

Prior exposure to *C. mutabile* did not influence the susceptibility of adult coots to infection by the parasite. No significant differences were found between the percentage of flukes parasitizing previously infected or naive adults coots (Mann-Whitney *U* test, $Z = 1.066$; $P = 0.286$).

Figure 6.1. Viability (=infectivity success) of *Cyclocoelum mutabile* metacercariae from 7-8 week old infections in three species of snails (G.p., *Gyraulus parvus*; S.e., *Stagnicola elodes*; P.e., *P. exacuous*) in juvenile and adult coots. Coots were either naive (O), or had previous exposure to *C. mutabile* (●).


60
50
40
30
20
10
0
Percent viable (± S.E.)
d.p.
s.e.
P.e.
d.p.
d.p.
Juvenile
Adult

6.4. Discussion

No significant difference in the infectivity of metacercariae from the three species of snails was found. Therefore, all three species would be effective in transmitting *C. mutabile* to coots. These findings differ from those of Scott (1980) who found significant differences in the infectivity of *T. cucumerinum* subsp. from three species of snails to ducks.

However, the greater host response to and the longer average excystment time required by metacercariae derived from *S. elodes* reported by Goring (1993) was reflected in this study. The lowest infectivity in juvenile coots was associated with metacercariae from this species. The high passage rate of food through the digestive tract of birds (Duke, 1986) coupled with the longer excystment time of metacercariae from *S. elodes* could account for the lower numbers that established. A smaller proportion may excyst in time or in the right region of the gut to establish in the birds. Alternatively, a stronger host response, resulting in the complete encapsulation of the metacercariae, could lower the infectivity of the metacercariae by killing the metacercariae directly. Faliex (1988) has shown that the immune response of the grey mullet, *Liza ramada*, prevents the uptake of nutrients by the metacercariae of *Labratrema minimus*. If a comparable mechanism occurs in snails infected by *C. mutabile*, this too could limit the infectivity of the metacercariae. The metacercariae used in this study were

from 7-8 week-old infections. Since host response is greater in older infections (Goring, 1993), differences due to host response may have been observed if older infections had been used. However, as the transmission window for this parasite is quite narrow (Chater 5) the snail host response would not have had a chance to develop to a point where the metacercariae are affected by the time the snail is eaten by a coot and would not greatly influence the transmission of this fluke.

Scott *et al.* (1982) have reported similar findings for two other species of cyclocoelids (*T. c. cucumerinum* and *T. c. sisowi*) from *S. elodes*. They found metacercariae of both these species to have the lowest infectivity when obtained from this snail. This lower infectivity occurred without a noticeable host response in the snails.

Although both juvenile and adult coots are susceptible to infection, juveniles were far more susceptible than adults. Fewer adults became infected and a lower portion of the metacercariae fed to coots developed to adult flukes. These differences could well account for the reported differences in *C. mutabile* prevalence in these two age classes each fall (Colbo, 1965; McLaughlin, 1986). However, the results reported by McLaughlin (1986) differ markedly with those found here. Indeed, in examining the infectivity of metacercariae from *S. elodes* to 3-4-week-old, 1, and 2 year old coots, McLaughlin (1986) found the proportion of metacercariae establishing remained fairly constant between coots of different ages (10-21%) and no

differences in the proportion of coots of different ages that become infected. The coots used by McLaughlin (1986) were much younger than those used in this study (3-4-week-old *vs* 2-month-old). Thus, the size of the coot livers would differ significantly. As *C. mutabile* undergoes a 15-18 day period of development in the liver prior to migrating into the airsacs (McLaughlin, 1977), it is possible that the smaller liver in the younger coots used by McLaughlin (1986) may limit the number of flukes that established in the younger birds. Such a density-dependent limit on the number of infections establishing has also been suggested for *Fasciola hepatica* in cattle (Cheng, 1976).

Differences in the food habits of juvenile and adults could also partially account for the difference in prevalence of *C. mutabile* observed in coots in the wild. Adult coots feed predominantly on vegetation but Jones (1940) found nearly half of the food of juveniles consisted of animal matter. The high percentage of animal matter declines as the coot's digestive system becomes more adept at breaking down fibrous material. Driver (1988) found the animal portion of the diet of juvenile coots to be 84% when less than 29 days old, dropping to 21 % by the time the birds were greater than 60 days old. However, the total percentage of snails remained constant at 6-10% of all food consumed in all age classes (Driver, 1988). In contrast, Jones (1940) found less than 2% of the adult coots diet to consist of snails.

Behavioral differences could further explain the

differential prevalence of *C. mutabile*. Males and nonbreeders congregate on larger water bodies in late summer, the juveniles remaining closer to the nesting site (Ryder, 1963; P. Ward, personal communications). As rates of infection in snail populations are often highest near bird roosts and nests (Sousa & Grosholz, 1991; and references therein), the juveniles would be expected to carry a heavier burden of the parasite.

Prior exposure to the parasite seems to have little effect on susceptibility to further infections. This result is in accordance with those of McLaughlin (1986) who found current *C. mutabile* infections to have no effect on either the proportion of coots becoming infected by challenge doses or the intensity of new infections. However, the low infectivity seen in adult coots in this study would not have allowed statistical differences to have been observed. Other studies examining acquired resistance to flukes in birds have yielded variable results. Fried (1963) and Nollen (1971) found previous or concurrent infections with *Philophthalmus hegeneri* and *P. megalurus*, respectively, to have no effect on the establishment of subsequent infections. On the other hand, Huffman & Roscoe (1986) have found previous infection with *Sphaeridiotrema globulus* to significantly reduce the number of infections that may establish when reexposed to the parasite.

While admitting to the low number of snail species utilized, this study suggests that infectivity of *C. mutabile* metacercariae is not influenced by the snail host. I have also pointed out how differential susceptibility, diet and behaviour are all factors

that may contribute to the differences in the late summer prevalence of *C. mutabile* in adult and juvenile coots. Thus, for the three snail species examined, the susceptibility and seasonal density of the different species in the environment remain the primary components underlying the transmission dynamics of the parasite.

Chapter 7.

Overview

The purpose of this study was to obtain a better understanding of the seasonal transmission of *Cyclocoelum mutabile* to snails and through this, a better understanding of the factors that underlie the seasonal occurrence of this fluke in the American coot, *Fulica americana*. Infections of *C. mutabile* in coots show a distinct seasonal pattern. Coots on the wintering grounds remain infected throughout the year (Kinsella, 1973; Eley, 1976). Resident coots and other species such as gallinules (*Gallinula chloropus* and *Porphyrula martinica*) and sora rails (*Porzana carolina*) which also harbour this parasite (Kinsella & Hon, 1973; McDonald, 1969) continually reseed the environment in the wintering areas.

As the flukes live for only 10-12 weeks, infections within migrant coots arriving on the breeding grounds are lost by mid to late June. Recruitment of new infections does not occur until mid July (Colbo, 1965; McLaughlin, 1986). The delayed onset of recruitment of new infections and results from this study suggest that infections do not overwinter in the snail host. Thus, infections acquired each summer must be the result of the reseeding of the environment with eggs produced by flukes returning with the coots that same spring.

It was found that a temperature threshold exists at 14°C that must be reached before the eggs hatch. Eggs maintained at

lower temperatures remain viable for at least four weeks and show no decline in hatching success once this threshold is reached. Miracidia hatching from eggs stored for up to 7 weeks at low temperatures showed no decline in infectivity. Thus eggs deposited in the field before water temperatures warm to 14 °C should not hatch. However, once water temperatures rise to this level, any eggs that may have accumulated probably hatch normally and *en masse*. Miracidia hatching from eggs of all ages have equivalent infectivity.

This delayed hatching of eggs may have an important effect on the transmission of the parasite to the snails. Snail populations in the spring are transitional with a good many of those present dying as new cohorts start to emerge. The delay in hatching has two consequences. First, most of the older, senescent snails will have died and therefore not act as sinks for miracidia. Secondly, and more importantly, populations of young snails will have appeared by this time.

The high transmission efficiency and longevity of miracidia at 14 °C would enable miracidia to locate the widely dispersed snails at that time. Miracidia hatching later in the spring would enter an environment with an increasing density of snails. The lower transmission efficiencies reported at higher temperatures would probably not be as important to the transmission of the parasite as the relative values indicate.

Young (smaller) snails are more susceptible to infection than larger snails. Thus, most of those snails encountered by

miracidia hatching in the late spring would be of the most susceptible sizes. The effect of temperature on the susceptibility of snails, although significant, showed no consistent pattern.

The wide host specificity of the miracidia reported here and previously has obvious implications on the transmission of the parasite. Most snails encountered are potential hosts and the lack of a free-swimming cercarial stage does not limit the distribution of the parasite within the snail community. However, the apparently undiscriminating action of the miracidia could be a liability when a snail community is comprised of a large number of insusceptible or refractive species. This could be why so few infections were found in the field aspect of these studies.

The temperature regime recorded in one of the ponds manipulated in this study suggests that snails first become infected in late June or early July. Once infected, the reduced life cycle of *C. mutabile* allows for the production of new metacercarial generations within snails within one month. This corresponds with the initiation of recruitment of the parasite by coots each year.

Recruitment of new infections involves mostly young of the year coots (Colbo, 1965; McLaughlin, 1986). The results reported in this study suggest that differences in the susceptibility of young and old birds could account for this fact. Behavioral differences may enhance the observed differences in prevalence of

the fluke between the two groups of birds.

The contribution of the infections acquired by coots in mid to late summer to the recruitment of further infections by the coots remains unknown. The prepatent time in *C. mutabile* is about 4 weeks (McLaughlin, 1983). Thus infections acquired by coots in late July would only become ovigerous by late August. The hatching threshold detected and temperature regime recorded under natural conditions suggests that most of the eggs of these parasites would not hatch due to declining temperatures. Even if these eggs do result in infections in snails, the declining temperatures would slow the development of the infections within them. Also, many of the coots, shifting habitats prior to migration, would not be in areas where infected snails would be present. These unhatched eggs may possibly overwinter and hatch the following spring but this aspect of the life cycle remains unexplored. The literature, while showing many examples of overwintering of unembryonated eggs, yields no examples of overwintering of embryonated eggs. Further, many of the smaller potholes in the prairies freeze to the bottom, the snails surviving by digging into the mud.

The large population of flukes present in coots returning to the wintering grounds would result in a surge of eggs being deposited in wetlands throughout the migration. The broad specificity of the miracidia for the snail host would allow an undefined proportion of snails in these populations to become infected. Unfortunately, studies on this aspect of the ecology

of *C. mutabile* have not been done.

Given the apparent low infectivity of metacercariae to adult coots, perhaps this large influx of eggs deposited by juvenile birds on the wintering grounds is necessary to allow sufficient numbers of snails to become infected to account for the infection of adult coots returning to the breeding grounds the following spring.

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Appendix

Mean infectivity (% ± S.E.) of single *Cyclocoelum mutabile* miracidia to four size classes of eight species of snails. Dashes represent size class-species combinations that were not tested. Size classes and sample sizes are given in Table 4.1.

Snail species	Size class	Temperature 14°C	16°C	20°C
S. elodes	1	40.2 (3.1)	63.0 (3.0)	45.6 (3.2)
	2	22.5 (2.4)	34.6 (2.6)	28.1 (2.3)
	3	16.7 (2.3)	14.9 (2.0)	24.1 (2.6)
	4	13.4 (2.2)	15.7 (2.1)	17.2 (2.1)
L. stagnalis	1	66.2 (2.7)	59.4 (2.9)	50.1 (3.2)
	2	49.7 (2.9)	63.7 (2.8)	50.7 (2.9)
	3	40.5 (3.1)	54.3 (2.7)	59.6 (2.7)
	4	32.2 (3.6)	45.5 (3.3)	39.4 (3.4)
P. exacuous	1	70.5 (2.6)	59.9 (2.7)	86.5 (1.8)
	2	51.3 (3.6)	46.2 (3.1)	69.0 (3.0)
A. crista	1	81.6 (2.3)	62.6 (3.0)	81.8 (2.1)
G. circumstriatus	2	56.9 (4.0)	31.2 (3.4)	34.1 (3.2)
G. parvus	1	72.0 (2.9)	55.4 (2.7)	59.9 (3.0)
H. trivolvis	2	3.5 (2.0)	4.8 (2.7)	9.2 (2.5)
	3	2.8 (1.6)	3.5 (1.7)	1.8 (1.3)
P. jennessi	1	5.6 (1.4)	4.9 (1.2)	4.5 (1.3)
	2	3.9 (1.1)	3.4 (1.0)	3.2 (1.0)
	3	3.3 (1.2)	3.0 (1.0)	3.4 (1.5)
	4	5.4 (1.7)	-	-
P. gyrina	1	0	0	0
	2	0	0	0
	3	0	0	0
	4	0	0	0