Detection of latent heritable genetic damage in populations of aquatic snails, *Lymnaea stagnalis*, exposed *in situ* to genotoxic pollution.

by

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Abstract

We hypothesized that populations exposed to chronic levels of genotoxins for many generations *in situ* will accumulate latent heritable genetic damage leading to increases in mutational load and decreased population fitness. Common pulmonate snails (*Lymnaea stagnalis*) were collected from three sites that differed in pollution level [Manitoulin Island (reference), Beauharnois (moderate pollution), and Varennes (highly polluted)]. These organisms have a rapid generation time and are capable of self-fertilization. Fitness indicators were clutch size, survival (hatching to day 30) and growth (length from hatching to day 90). Recessive deleterious mutations that have accumulated through time are masked as snails preferentially outcrossed, but are expressed when snails self-fertilize. Results obtained by comparing fitness components of snails derived from selfing and outcrossing experiments showed that in sites with higher levels of pollution inbreeding depression increased for clutch size and survival. Results for growth rate were similar to those for clutch size and survival in the two least polluted sites, but not for the most polluted site, due possibly to biased mortality of smaller snails at this site. After correction for size biased mortality, the growth estimates showed patterns similar for the two polluted sites when compared to the reference site. This study suggests that long-term *in situ* exposure to genotoxic pollution may effect population fitness due to the accumulation of latent heritable genetic damage.

Résumé

Nous avons postulé que les populations exposées de façon chronique à des génotoxines *in situ* durant plusieurs générations accumuleront des dommages génétiques héréditaires latents qui augmenteront la charge en mutation et réduiront le fitness de la population. Des escargots communs (*Lymnaea stagnalis*) ont été récoltés à trois sites qui avaient des niveaux de pollution différents [Île Manitoulain (site témoin), Beauharnois (pollution intermédiaire), Varennes (pollution élevée)]. Ces organismes ont un court temps de génération et sont capables de s'auto-fécondés. Les indicateurs de fitness étaient la taille de la couvée, la survie (de l'éclosion jusqu'au 30^{è} jour), et la croissance (la longueur à l'éclosion jusqu'au 90^{è} jour). Les mutations récessives nuisibles qui se sont accumulées au cours du temps sont généralement masquées chez les escargots, car ils se reproduisent préférentiellement par croisement; cependant elles peuvent être exprimées lorsqu'ils s'auto-fécondent. Les résultats obtenus en comparant les composantes du fitness des escargots à partir d'dexpériences de croisements et d'autofécondation ont démontré que le coefficient de consanguinité augmentait en fonction du niveau de pollution pour la taille de la couvée et la survie des escargots. Des résultats similaires ont été obtenus pour la croissance des escargots, à l'exception du site le plus pollué, possiblement à cause d'une mortalité élevée pour les petits escargots de ce site. Lorsque la taille moyenne fut corrigée pour la mortalité sélective, la croissance des escargots des sites pollués était similaire. Les résultats de cette étude indiquent qu'une exposition à long terme *in situ* à des génotoxines peut avoir un effet sur le fitness

des populations due à une accumulation de dommages génétiques héréditaires latents.

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Preface

Authorship and Style

The Faculty of Graduate Studies and Research requires that the following text be reproduced in full in order to inform the reader of Faculty regulations:

Candidates have the option of including, as part of the thesis, the text of a paper submitted or to be submitted for publication, or the clearly-duplicated text of a published paper. These texts must be bound as an integral part of the thesis.

If this option is chosen, **connecting the texts that provide logical bridges between the different papers are mandatory**. The thesis must be written in such a way that it is more than a mere collection of manuscripts; in other words, results of a series of paper must be integrated.

The thesis must still conform to all other requirements of the "Guidelines for Thesis Preparation". **The thesis must include:** A Table of Contents, an abstract in English and French, and introduction which clearly states the rationale and objectives of the study, a comprehensive review of the literature, a final conclusion and summary, and a thorough bibliography of reference list.

Additional material must be provided where appropriate (e.g. in appendices) and in sufficient detail to allow a clear and precise judgement to be made of the importance and originality of the research reported in the thesis.

In the case of manuscripts co-authored by the candidate and others, **the candidate is required to make an explicit statement in the thesis as to who contributed to such work and to what extent.** Supervisors must attest to the accuracy of such statements at the doctoral oral defense. Since the task of the examiners is made more difficult in these cases, it is in the candidate's interest to make perfectly clear the responsibilities of all authors of the co-authored papers.

This thesis consists of one chapter written in accordance to the guidelines of The Faculty of Graduate Studies and Research. I designed and executed the entire research presented herein, under the supervision of Dr. Joseph Rasmussen (Dept. of Biology, McGill University) and Dr. Daniel Schoen (Dept. of Biology, McGill University). I collected and cultured the study organisms with the help of many technicians. I wrote the thesis in full. Dr. Rasmussen and Dr. Schoen contributed significantly to the development of the ideas presented in this thesis. Marc Trudel kindly translated the abstract.

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One final thought.....

"One of the penalties of an ecological education is that one lives alone in a world of wounds. Much of the damage inflicted on land is quite invisible to laymen. An ecologist must either harden his shell and make believe that the consequences of science are none of his business, or he must be the doctor who sees the marks of death in a community that believes itself well and does not want to be told otherwise."

Aldo Leopold
The Round River

Oh yeh, thanks A.....

"dosis sola facit venenum"....the dose alone makes a poison

Paracesus 16th century physician-alchemist

INTRODUCTION

With ongoing industrial, agricultural and domestic activities, watersheds continue to be exposed to contamination, often with unknown effects on the biota (or ecosystems). Commonly, aquatic systems receive industrial and/or domestic effluents that may be genotoxic. According to Brusick (1987), "Agents that produce alterations in the nucleic acids and associated components at subtoxic (below physiological toxicity) exposure levels resulting in modified hereditary characteristics or DNA inactivation" are classified as genotoxic. The study of genetic toxicology, or genotoxicology, is concerned with the effects of toxins on the genetic material of organisms. High levels of genotoxins have been measured in water systems near industrialized areas (Stahl, 1991 and Houk, 1992). Genotoxic chemicals can be detected in biota (Malin, 1988; Baumann and Whittle, 1988) and tissue extracts of biota from industrialized areas and have been shown to elicit a response in bacterial bioassays used to detect genotoxicity (White, 1998a).

Organisms in aquatic environments can experience both acute and chronic exposure to genotoxins. The effects of acute exposure (high potency and short duration) to cytotoxic or genotoxic pollution can act through different biochemical pathways on biota, but have similar end points such as irritation, necrosis, and death. The effects of chronic low level (below the acute effect threshold) exposure to genotoxins can be more subtle, and the end points are difficult to define. Possible end points include changes in life expectancy (growth

potential and fecundity), mutagenesis/carcinogenesis, or nonreversible tissue degeneration (Brusick, 1987).

Somatic and germ cell mutations

Genotoxic effluent waste can alter genetic material in both somatic and germ cells (Kurelec, 1993; Depledge, 1996; Bickham and Smolen, 1994). Somatic cell mutations (non-heritable genetic alterations) can cause disease such as teratogenesis if exposure is at the embryo stage, or it can result in life threatening neoplasia (cancers). The development of neoplasia would cause mortality in the organism (usually in the later stages of life), but if it occurs after recruitment the ecological impact could be minimal. On the other hand, if teratogenesis occurs early and embryos fail to survive, it could result in a recruitment impairment or failure. Brown *et al.* (1973) found a higher percentage of fish with tumors in a polluted watershed when compared to a reference (less polluted) site and more recently, Omair *et al.* (1999) detected tumors in a variety of zooplankton in Lake Michigan. Germ cell mutations accumulated over many generations of low-level exposure to genotoxins can cause latent heritable genetic damage (recessive mutations). It has been suggested that such effects can render a population vulnerable and ultimately lead a species to extinction. This was termed "Genotoxic Disease Syndrome" (Kurelec, 1993 and Depledge, 1996).

The most commonly used genotoxicity bioassays are short-term tests based on prokaryotic organisms (Houk, 1992). These bioassays provide

information on the potential genotoxic effects of pollution. However, fundamental differences in DNA replication and repair machinery between prokaryotes and eukaryotes (Aravind *et al.*, 1999) make extrapolation of genotoxic effects to eukaryotic organisms questionable. On the other hand, all eukaryotic organisms share highly conserved DNA replication and repair machinery (Aravind *et al.*, 1999). Therefore, it is desirable and preferable to have a method to assess long-term effects of genotoxins that is based on an eukaroyotic organism.

Consequences of heritable mutations

Continual exposure to genotoxic pollutants can cause populations to accumulate latent heritable genetic damage and thereby increase the genetic load--the burden of disease and death that is created by the effects of deleterious genes (Fraser and Nora, 1986). This is expected to lead to fitness decline in the population. The expression of lethal recessive mutations with large effects can cause death or failure to reproduce whereas sublethal and mildly deleterious recessive mutations will cause fitness declines of a lesser degree (Charlesworth and Charlesworth, 1987; Crow, 1993). Despite their enormously different homozygous effects, Crow (1993) estimated that lethal and mildly recessive deleterious mutations are maintained equally in populations, with a mean persistence of approximately 40 generations. Hedrick (1994) also suggested that the genetic load in *Drosophila* appear to be comprised equally of lethals and mildly deleterious mutations.

It has been hypothesized that the accumulation of mildly deleterious recessive mutations can lead to the extinction of asexual populations and small sexual populations. Lynch *et al.* (1995a) referred to this as the "mutational meltdown of sexual populations". Lynch *et al.* (1995b) also suggested that small populations (in the range of 100-1000 individuals) are especially susceptible to extinction due to the accumulation of recessive mutations.

A classic tool for detecting of recessive mutations is by inbreeding a normally outcrossing organism, such as *Drosophila* (Wallace, 1956). In a laboratory study, White *et al.* (1999) were the first to study heritable effects of a common environmental genotoxin, Benzo[a]pyrene. They were able to show a decrease in survivorship of Fathead minnows, *Pimephales promelas*, whose grand-parents had been exposed to subtoxic levels of the mutagen. This study found that a single generation exposure was sufficient to produce a marked increase in the genetic load. Such effects have never been examined on organisms exposed *in situ* to low-level genotoxic pollution over many generations and will be the main focus of this thesis.

OBJECTIVE

The objective of this thesis is to test the hypothesis that chronic exposure (over many generations) of biota to *in situ* low-level genotoxic pollution can cause populations to accumulate latent heritable genetic damage and thereby increase the genetic load. This would lead to fitness decline in the population. The genetic load can be assessed by examining fitness differences of inbred and

outbred progeny derived from the study population. This is also expressed as inbreeding depression. If the deleterious mutation rate of organisms from the polluted sites is elevated as a result of exposure to genotoxic contamination, inbreeding depression will be higher than in populations from the sites without genotoxic pollution. The coefficient of inbreeding depression is expressed as $\delta=1-w_i/w_o$, where w_i is the mean trait value from inbred progeny and w_o is the mean trait value from outcrossed progeny (Jarne & Delay, 1990; Lande and Schemske, 1985; and Charlesworth and Charlesworth, 1987).

Study organism

A freshwater species of snail, *Lymnaea stagnalis,* ubiquitous in the St. Lawrence ecosystem, including the Great Lakes (Clarke, 1973) was used to evaluate effects of long-term exposure to genotoxic pollution. This pulmonate snail is hermaphroditic and can reproduce through self-fertilization when isolated (Cain, 1956; and Van Duivendoben *et al.*, 1985). This makes it ideal for use in inbreeding experiments. *Lymnaea stagnalis* is a relatively hardy animal, easy to raise in laboratory conditions (Pip, 1986; and McMahon, 1983). Fitness indicators such as clutch size, post-hatchling survival, and growth rate are also easy to measure. As an eukaryote, it is a better genetic model for higher organisms than most prokaryotic bioassays, which are the most commonly used genotoxicity bioassays (Houk, 1992). Eukaryotic organisms have more DNA, are diploid, and their DNA are all organized similarly and contained in a nucleus (Darnell *et al.*, 1986). Eukaryotes have proteins complexed to DNA and a higher

order chromatin structure that is significantly more complicated than prokaryotes. DNA replication and repair machinery for eukaryotes are more elaborate and linked to cell cycle checkpoints thus further distinguish it from prokaryotes (Aravind *et al.*, 1999).

The final criteria was that only sampling sites where the test animals were very abundant (several snails per m^2) were used in this study, since it is known that these snails will only self-fertilize when no other conspecifics are encountered (Jarne and Charlesworth, 1993). This minimized the likelihood that the population would purge any genetic load that was accumulated through time.

Study site

The St. Lawrence River is one of the world's largest rivers and Canada's main water artery. There are approximately 1300 vascular plant species, 185 fish species, 115 bird species, 16 amphibian species, 14 reptile species, and 20 mammal species associated with the St. Lawrence ecosystem (SLC, 1996). Commercial and sport fisheries are important economic activities on the St. Lawrence River; the landed value of freshwater species was estimated at $3.2 million in 1992 (MENVIQ, 1993).

There are over 300 municipalities and 2300 industries located along the shoreline of the St. Lawrence River that impact the water quality of the river (Statistics Canada, 1991 and SLC, 1996). Every year, approximately 265 000 t of liquid waste containing oil, grease, sludge, solvents, acids, PCBs, heavy metals, cyanide and other highly toxic substances are released into the St.

Lawrence River directly as effluent waste (SLC, 1990). An estimated 30 000 chemicals, 800 of which are considered dangerous, are used in the Great Lakes / St. Lawrence drainage area (Government of Canada, 1991).

The largest number of industrial plants and mills are between the Valleyfield and Trois-Rivières stretch of the St. Lawrence River (SLC, 1996). A majority (75%) of the dangerous waste comes from the Montreal area (SLC, 1996). Wastewater or organic extracts of water, sediment, and tissue of biota from this area are genotoxic when measured with the SOS Chromotest, a bacterial bioassay (White *et al.*, 1996a; White *et al.*, 1996b; Langevin *et al.*, 1992 and Costan *et al.*, 1993).

Three sites were chosen to represent a gradient of exposure of the study organism to genotoxic contamination (Figure 1). The reference site (low pollution) was located at Manitoulin Island in the Georgian Bay of Lake Huron. Rowan and Rasmussen (1992) showed that fish from the Georgian Bay had lower levels of PCBs and other organochlorines than sites downstream in the St. Lawrence watershed. This area receives no direct or identifiable industrial inputs and is not downwind of any urban or industrial sites. Langevin *et al.*, (1992) were able to show that surface water extracts from areas with minimal industrialization were not genotoxic when tested with the SOS Chromotest.

The two polluted sites are close to the Montreal area. Beauharnois is located at the west end of Montreal Island; water at this site has not yet passed through the industrial area of Montreal. Varennes, on the other hand, is situated to the east of the island (downstream from the island's industrial area), and has an order of

magnitude higher genotoxic loading than Beauharnois (Table 1). White and Rasmussen (1998b) were able to show that organic extracts of industrial and domestic waste waters from the Montreal area were genotoxic when tested with the SOS Chromotest. Furthermore, a mass-balance analysis by the same authors showed genotoxic loading from the municipal wastewater treatment facility located at the east end of the Island is greater than the majority of industrial facilities. The two polluted sites are also representative of the types of industries and genotoxic pollution that can be found along the St. Lawrence River (White *et al.*, 1998c). Tissue extracts from fish and macroinvertebrates at the study sites also test positive for genotoxicity (White *et al.*, 1998a). Thus, based on the loading estimates from (White *et al.*, 1998c), Beauharnois was considered the moderately polluted study site and Varennes was the highly polluted study site.

Figure 1. Map of study sites.

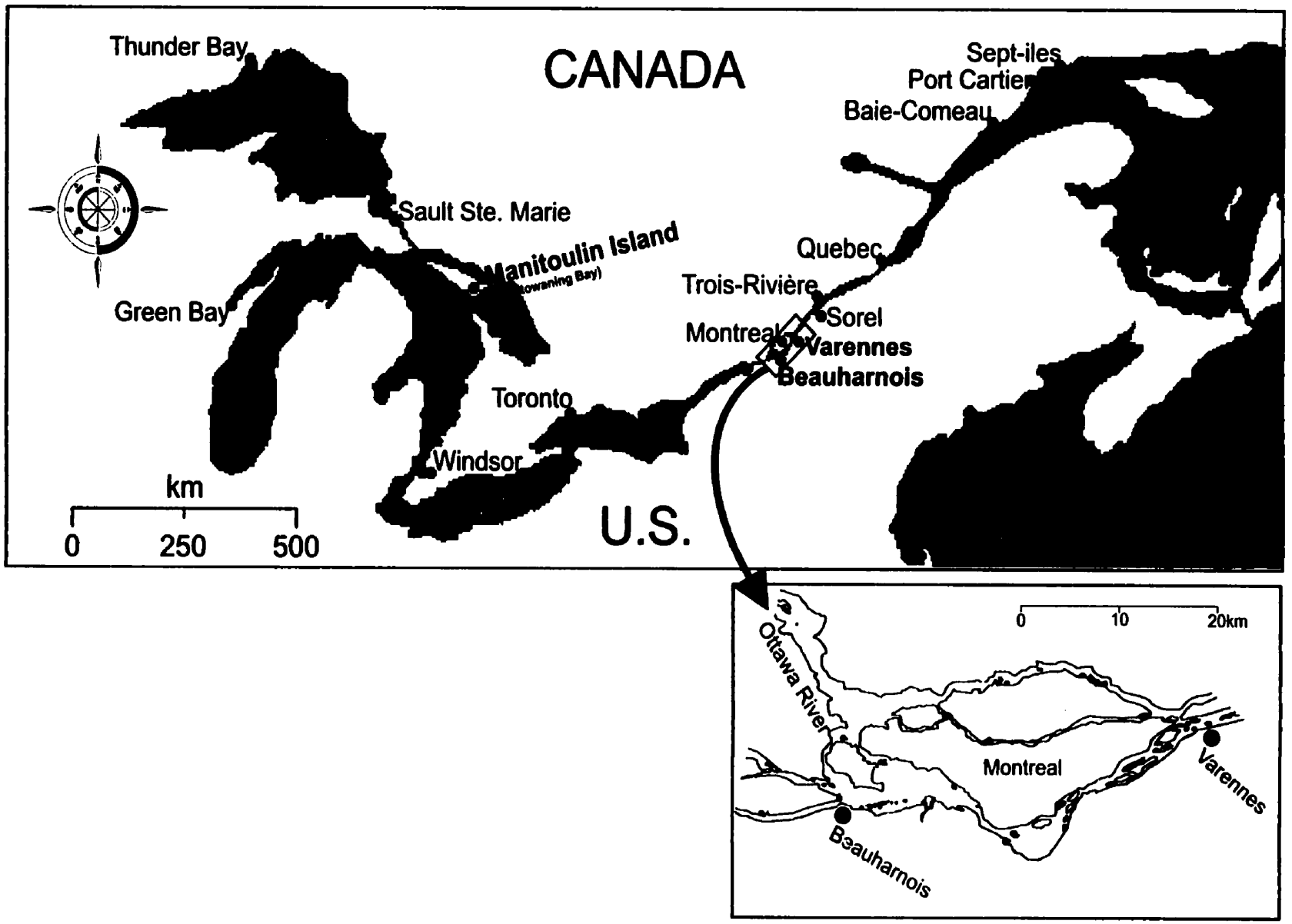

CANADA
Thunder Bay
Sept-iles
Port Cartier
Baie-Comeau
Sault Ste. Marie
Manitoulin Island
Quebec
Trois-Rivière
Green Bay
Sorel
Montreal
Varennes
Beauharnois
Toronto
Windsor
km
0
250
500
U.S.
0
10
20km
Ottawa River
Montreal
Varennes
Beauharnois

Table 1. Sampling site characteristics.

Site	Discharge (dry mg L^{-1})	Associated Industries	Genotoxic loading (g BaP Equiv. day^{-1})
*Beauharnois	7780	PP, Al, In	372.6
*Varennes	9500	Org (2), In (2), Pe	1349.5
**Manitoulin Island	------	No direct inputs	No direct inputs

Sources: *White, 1998b; **Rowan and Rasmussen, 1992 and Langevin *et al.*,1992

PP pulp and paper
Al=aluminum founding
In=inorganic chemical production
Org=organic chemical production
Pe=petroleum refinery

MATERIALS and METHODS

In the fall of 1996 and 1997, fifty snails were collected from each of the three study sites where snails were abundant. The snails were transported back to the lab in their native water in a temperature-controlled 30 L container. Snails were acclimated for 48 hours to 20^{0}C (Boag and Pearlstone, 1979; and Noland and Carriker, 1946) and a 16 hrs light / 8 hrs dark cycle (Jarne and Delay, 1990) in the original container, and then transferred to a 30 L glass aquarium. They were fed *ad libitum* with romaine lettuce every two days (Noland and Carriker, 1946). The water supply for all the experiments was tap water stored in a 1000 L Nalgene container that was continuously aerated and filtered to remove chlorine and other possible water impurities.

Snails from the wild populations were allowed to mate randomly for one generation in the aquarium, and egg capsules were collected every two days. Experiments were conducted with the randomly mated offspring of the snails collected at the field sites. This ensured that tissue-borne contaminants present in the snails from the field would not be present in the experimental animals, and that only heritable effects of the *in situ* exposure would be detected. Furthermore, it ensured that all experimental snails were virgins before the breeding experiment (Chen, 1993; Jarne *et al.*, 1991; Jarne and Charlesworth, 1993). Species of *Lymnaea stagnalis* have ovotestis and are thus able to produce both oocytes and spermatocytes. They are also capable of storing sperm, which may be used later to fertilize the oocytes. Selfing occurs unless stored allosperm (foreign sperm) obtained through copulation are present, in which case the eggs are outcrossed as they mature. In this way, pulmonates

avoid selfing by active copulatory behavior (Jarne and Charlesworth, 1993). Eggs are produced before sperm and are released into the hermaphroditic duct and will be fertilized if sperm are present. If no allosperm are present, after 2 or 3 weeks, the snails' own sperm (autosperm) will enter the hermaphroditic duct bringing about self-fertilization. Therefore, the only way to be certain about the breeding history of a particular snail is to place the snail in its respective mating container as a virgin to eliminate the chance for cross-contamination with autosperm or allosperm from previous reproductive bouts.

Breeding regime

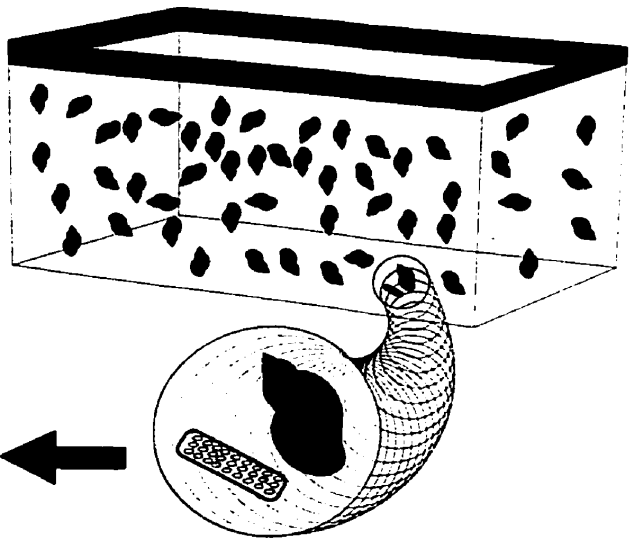
For each site, a stock of 50 wild snails (P_o) were randomly mated for one generation and twenty egg capsules collected. Capsules were collected by scraping the container wall with a razor blade; care being taken not to rupture the ootheca of the capsule. Each capsule was then hatched and raised individually (F_1) in a 100ml container. Thirty days after hatching, the offspring (F_1) from the 20 capsules were randomly chosen for the breeding experiment (Figure 2a). These capsules were divided into 2 groups of 10 capsules, designated for replicates 1 and 2. Replicate 1 consisted of two 1000ml outcrossing containers (each outcrossing container was composed of 10 different snails each from the 10 different capsules). Replicate 1 also consisted of 2 sets of ten 100ml selfing containers each with 1 snail from the same capsules as the outcrossing snails. The remaining 10 capsules were treated similarly to make up replicate 2 (Figure 2b). Fitness indicators were measured on the F_2 generation.

Figure 2a. Procedure for preparing organisms for breeding experiments.

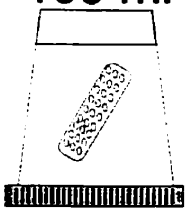
For each site, a stock of 50 wild snails (P_o) were randomly mated and 20 egg capsules collected. Each capsule was hatched and raised individually (F_1) in a 100ml container. Containers were placed in a 50L aquarium. Thirty day post-hatch, the 20 containers each with hatchling from a single capsule were used for outcrossing and inbreeding experiments.

Breeding regime

P_o Wild population n=50 snails randomly mating



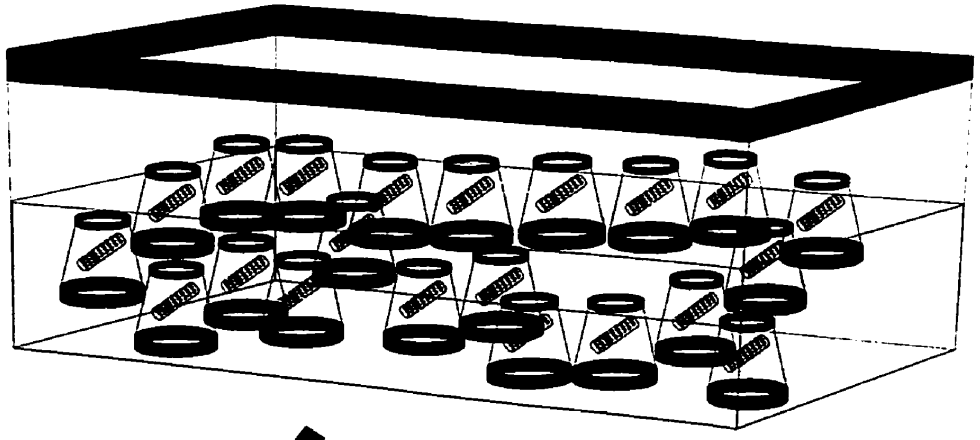
100 ml



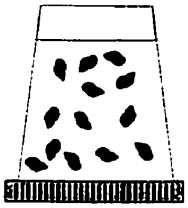
X 20 containers each with a capsule

The hatchlings from 20 capsules were used for the breeding experiments

F_1



100 ml



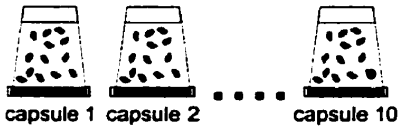
30 days post-hatch

X 20 containers each with hatchlings from a single capsule

Figure 2b. Breeding procedure for outcrossing and selfing experimental snails. Collected capsules were divided into 2 groups of 10, designated replicates 1 and 2. Replicate 1 consisted of two outcrossing containers (each outcrossing container was composed of 10 different snails each from the 10 different capsules). Replicate 1 also consisted of 2 sets of ten 100ml selfing containers each with 1 snail from the same capsules as the outcrossing snails. The remaining 10 capsules were treated similarly to make up replicate 2.

replicate 1

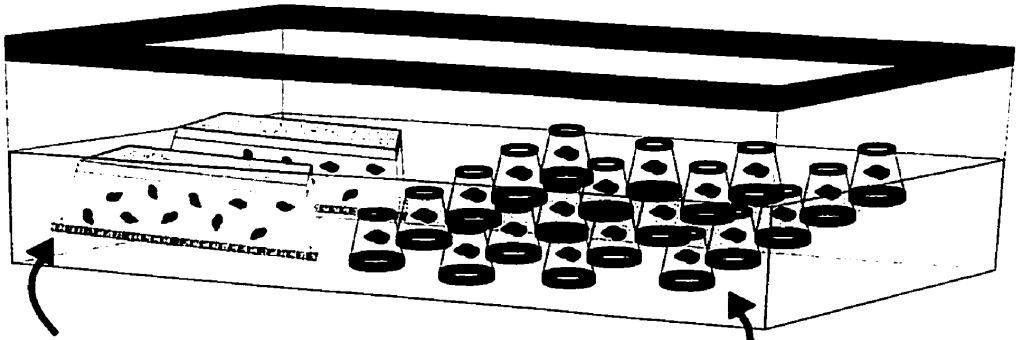
Group 1
capsules 1 to 10



replicate 2

Group 2
capsules 11-20

20 capsules from the wild population were divided into 2 groups.



two 1000 ml container each with 10 outcrossing snails

two sets of ten 100 ml container for individual selfing snail

F_2 offspring

measure clutch size, survival, and growth

replicate 2 was threated the same as replicate 1 but contained hatchlings from Group 2.

Since density has been reported to affect both growth and fecundity of the genus *Lymnaea* (Noland and Carriker, 1946), this set-up maintained a density of 1 snail per 100ml of water. Fitness indicators were measured on the F_2 generation by collecting capsules from both outcrossing and selfing containers.

A non-toxic method was used to clean all containers by soaking them in a salt water solution and then thoroughly rinsing with water. The tops of all the containers were cut off and replaced with a 200 μm polyester mesh, and the containers were then inverted. Small pieces of styrofoam were then glued onto the containers. These containers were designed to allow them to float and facilitate water circulation in an aquarium. To ensure a common water supply, each set of selfing and outcrossing containers were placed in a 50L aquarium. Every two days the snails were fed and each container was lifted out of the water and allowed to drain. This increased water exchange and removed any possible animal waste build-up. The containers were also visually inspected to ensure that there was no build-up of food.

EXPERIMENTAL DESIGN

To demonstrate possible latent heritable genetic damage due to genotoxic pollution, the clutch size, survivorship and growth rate of outcrossed and inbred progeny (F_2) from polluted sites were compared to those from a reference site (Jarne et al., 1991; Jarne and Delay, 1990; Chen, 1993). Larger differences in the values of the fitness indicators between outcrossed and inbred snails at the polluted sites when compared to the reference site would indicate greater

inbreeding depression and thus lower fitness for the population due to the effects of genotoxic pollution. The coefficient of inbreeding depression ($\delta = 1 - w_i/w_o$) was calculated for each of the measures of the following life history traits:

1. Clutch size:

Egg capsules were collected from F_2 snails in both outcrossed and selfed treatments for a period of one month. Capsules were removed from the containers within 24 hours of being laid. The clutch size was determined for the two treatments by counting the number of eggs per capsule under a dissecting microscope.

2. Survival:

Two capsules were collected from each selfing snail (20 selfing capsules in total) and 20 capsules were collected from each outcrossing container. Each capsule was raised individually in a 100ml container. The number of live hatchlings (F_2) per capsule was determined at hatch (day 0), day 10, day 20 and day 30.

3. Growth (length):

The size measurement used was the length from the apex to the tip of the aperture of the snail. For all treatments, snail lengths were measured every 10 days from hatch to day 90. A video camera was mounted on a dissecting microscope and each snail was filmed on a VHS recorder along with a calibration ruler. The images on the videotape were captured with a TV tuner/frame

grabber, which allowed for still image capture. The images were analyzed with "Imagetool" software (Uthscsa, copyright 95-97). To minimize interobserver error one person did all the measurements.

Statistical analyses

Two-way ANOVAs were used to detect differences among sites for pollution level and breeding system (outcross and inbred) for clutch size and hatchling survival. Since inbreeding depression (i.e., the difference between outcross and inbred snails) is expected to be positively correlated with the level of pollution at the site, the interaction between site X breeding system should be statistically significant. A pairwised t-test with Bonferroni correction was used to compare the coefficient of inbreeding depression (obtained from growth results) of the reference site with each of the polluted sites. All percentage data were arcsin(sqrt)-transformed to normalize the distributions before statistical analysis. However, to simplify visual presentation all means are presented as percentage survivals.

RESULTS

Clutch size

Mean clutch size tended to be larger in outcrossed snails than in inbred snails. The difference between outcrossed and inbred snails tended to be greater in polluted sites when compared to the reference site; furthermore, this difference tended to increase with the level of pollution at each site (figure 3).

The interaction for pollution level at each site and breeding system (outcross/inbred) for clutch size was statistically significant with $p<0.0001$. The analysis shows that the trend of increasing difference in mean clutch size between outcrossed and inbred snails with increasing level of pollution at the site was statistically significant (Table 2). The estimate of inbreeding depression for clutch size was 0.66 for Varennes, 0.48 for Beauharnois, and 0.29 for Manitoulin Island (Table 3). Thus, the level of inbreeding depression increased with the level of pollution at the site.

Fecundity

We attempted to determine the fecundity of snails (#eggs/snail/day) from clutch measurements. Fecundity showed similar trends with the level of pollution as clutch size (Figure 4). However, the ANOVA (interaction for pollution level at each site and breeding system (outcross/inbred) for fecundity) was not significant probably because sample size, and hence statistical power, was low (Table 4). Therefore, clutch size was used instead of fecundity in this study.

Figure 3. Mean clutch size (error bars are $\pm$ 1 SE) for outcrossed and inbred snails from the three study sites.

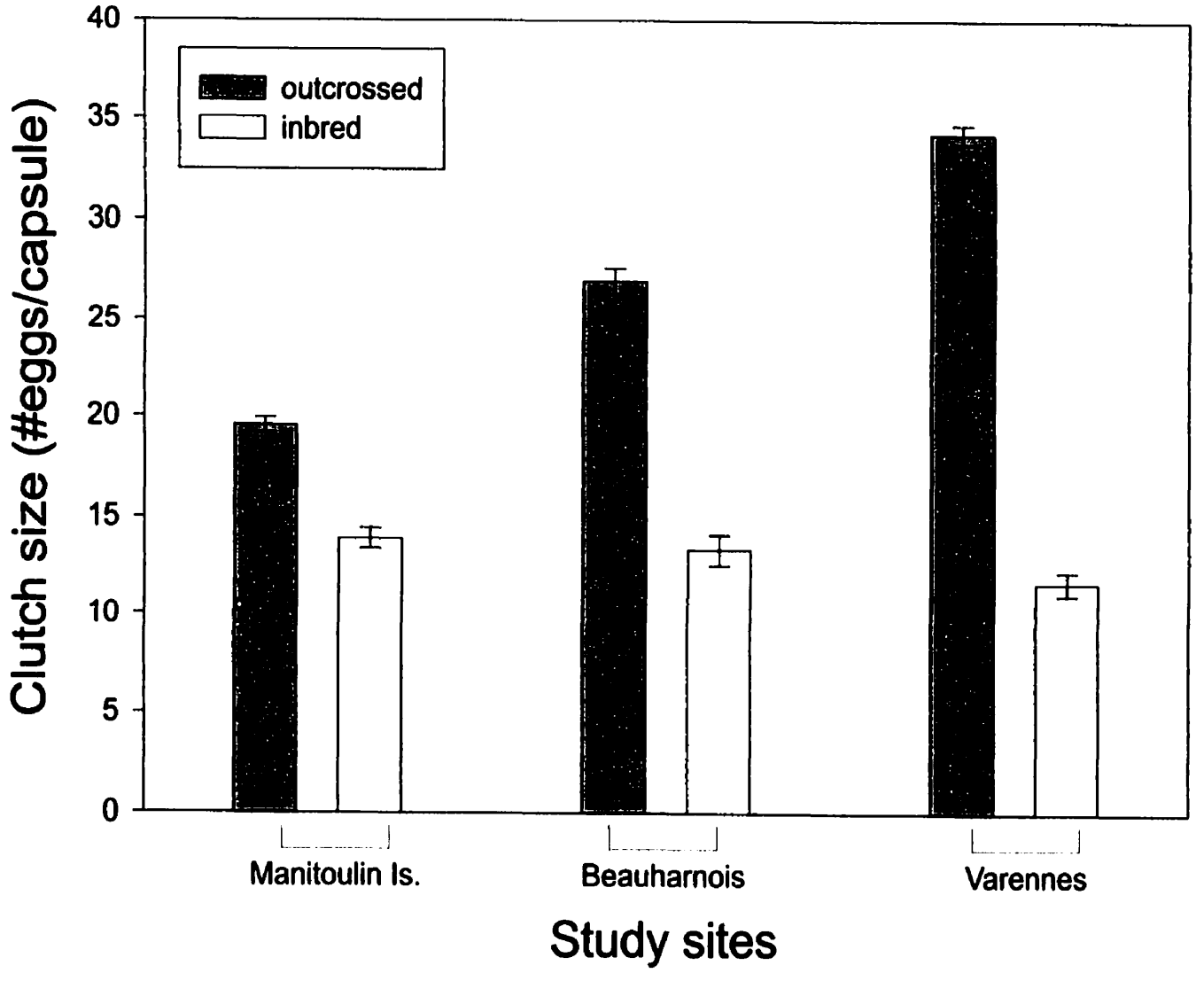

outcrossed
inbred
Clutch size (#eggs/capsule)
0
5
10
15
20
25
30
35
40
Manitoulin Is.
Beauharnois
Varennes
Study sites

TABLE 2. ANOVA for mean clutch size as a function of pollution level at the site and breeding system (outcross/inbred).

	F-ratio	df	p
(s) pollution level at the site	55.6	2,2228	<0.0001
(b) breeding system	838.8	1,2228	<0.0001
s X b	105.0	2,2228	<0.0001

TABLE 3. Estimate of inbreeding depression (δ) for clutch size at each site.

Pollution level Site	Clean Manitoulin Is.	Intermediate Beauharnois	High Varennes
δ	0.29	0.48	0.66

Figure 4. Mean fecundity (error bars are $\pm$ 1 SE) for outcrossed and inbred snails from the three study sites.

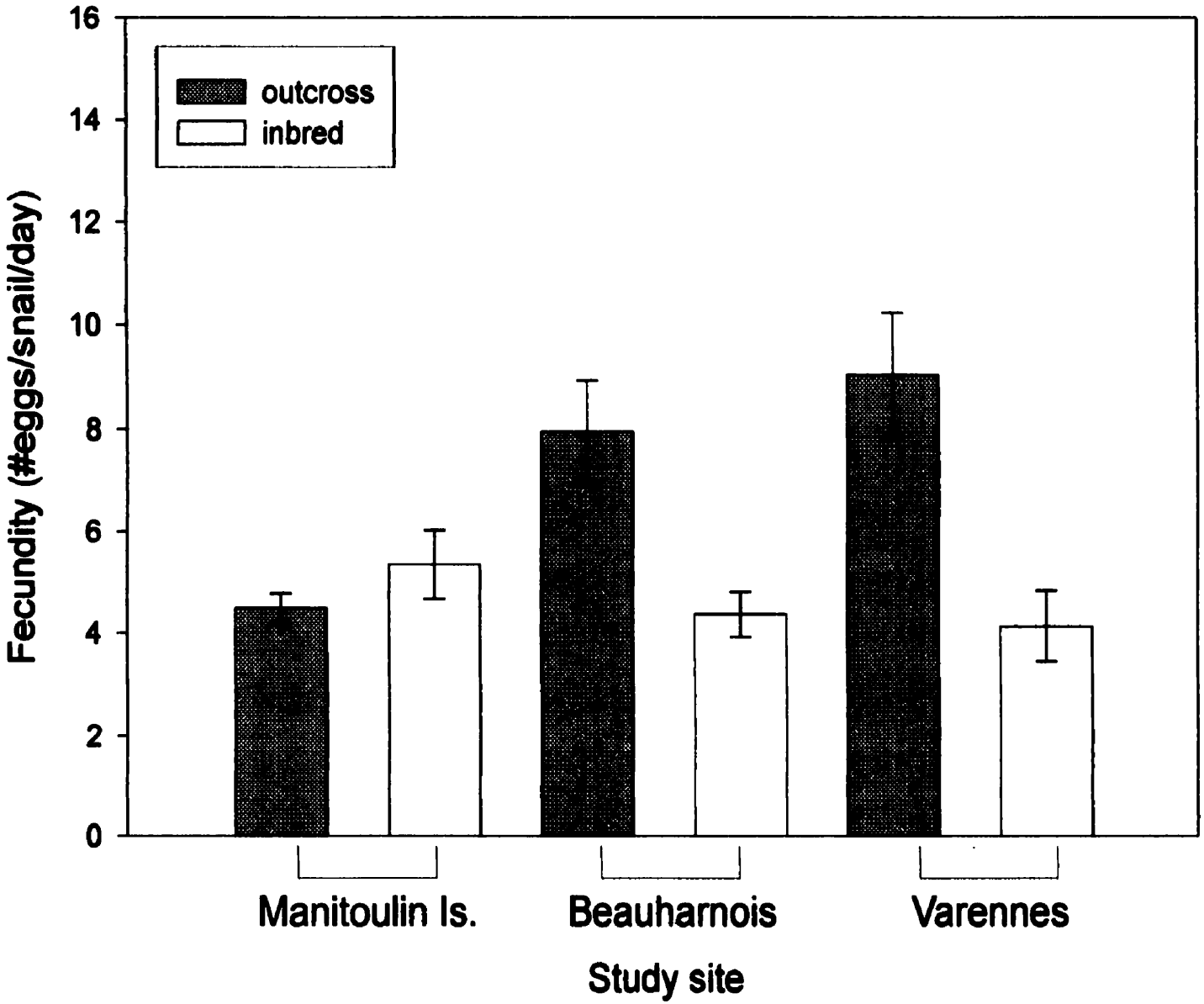

outcross
inbred
Fecundity (#eggs/snail/day)
0
2
4
6
8
10
12
14
16
Manitoulin Is.
Beauharnois
Varennes
Study site

TABLE 4. ANOVA for mean fecundity as a function of pollution level at the site and breeding system (outcross/inbred).

	F-ratio	df	P
(s) pollution level at the site	0.901	2,109	0.409
(b) breeding system	5.712	1,109	0.019
s X b	2.708	2,109	0.071

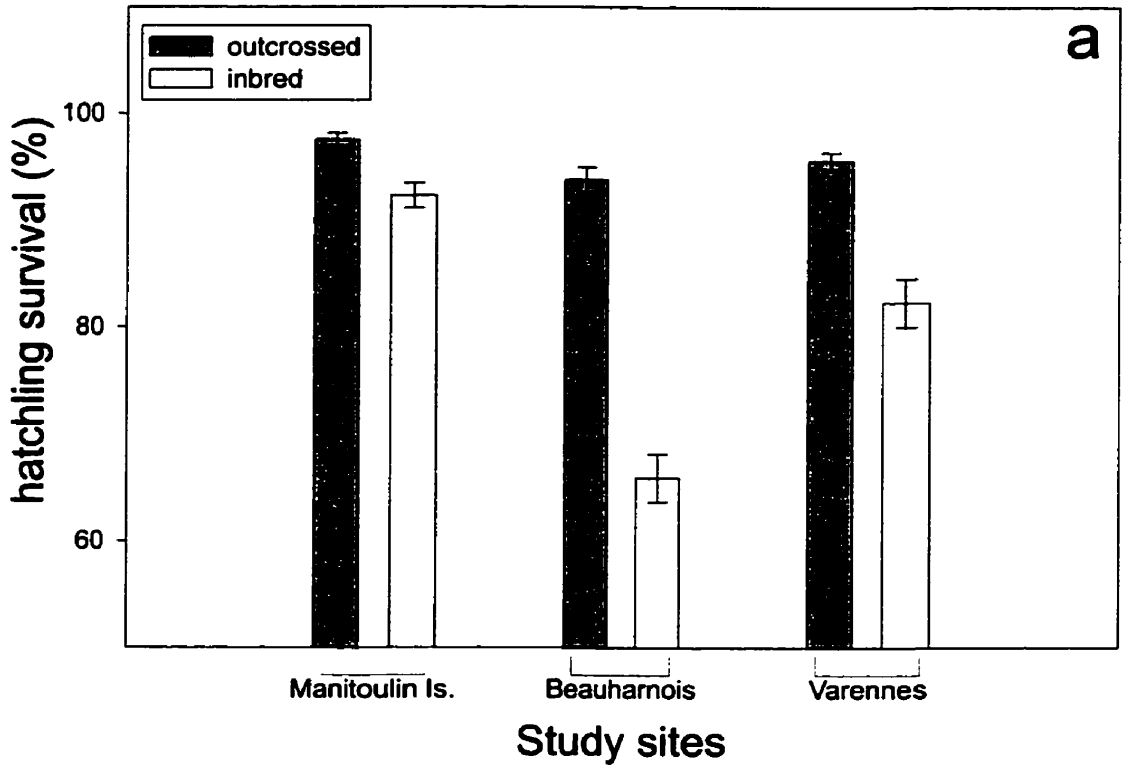
Survival

Survival for inbred snails tended to be lower in polluted sites when compared to the reference site, while survival for outcrossed snails tended to be similar across all sites. (Figure 5a-5d). As a consequence, the differences between outcrossed and inbred survival of snails tended to be higher in polluted sites when compared to the reference site, and tended to increase along the pollution gradient by day 30 (Figure 5d). Statistical analysis showed that the interaction between pollution level at the site X breeding system was significant at all time points (Table 5).

Estimates of inbreeding depression were consistently larger in both polluted sites when compared to the reference site for all time points. For example, at hatch, δ is 0.03 at the reference site as compared to 0.28 at Beauharnois and 0.13 at Varennes. This trend is also seen at day 10 and 20. However, it was not until day 30 that the estimate of inbreeding depression became significantly higher for the Varennes (δ=0.40) than for the Beauharnois (δ=0.30) (Table 6).

Figure 5a. Percentage hatch survival (error bars are $\pm$ 1 SE) for outcrossed and inbred snails from the three study sites.

Figure 5b. Percentage survival (error bars are $\pm$ 1SE) for outcrossed and inbred snails at day 10 from the three study sites.


a
outcrossed
inbred
hatchling survival (%)
100
80
60
Manitoulin Is.
Beauharnois
Varennes
Study sites

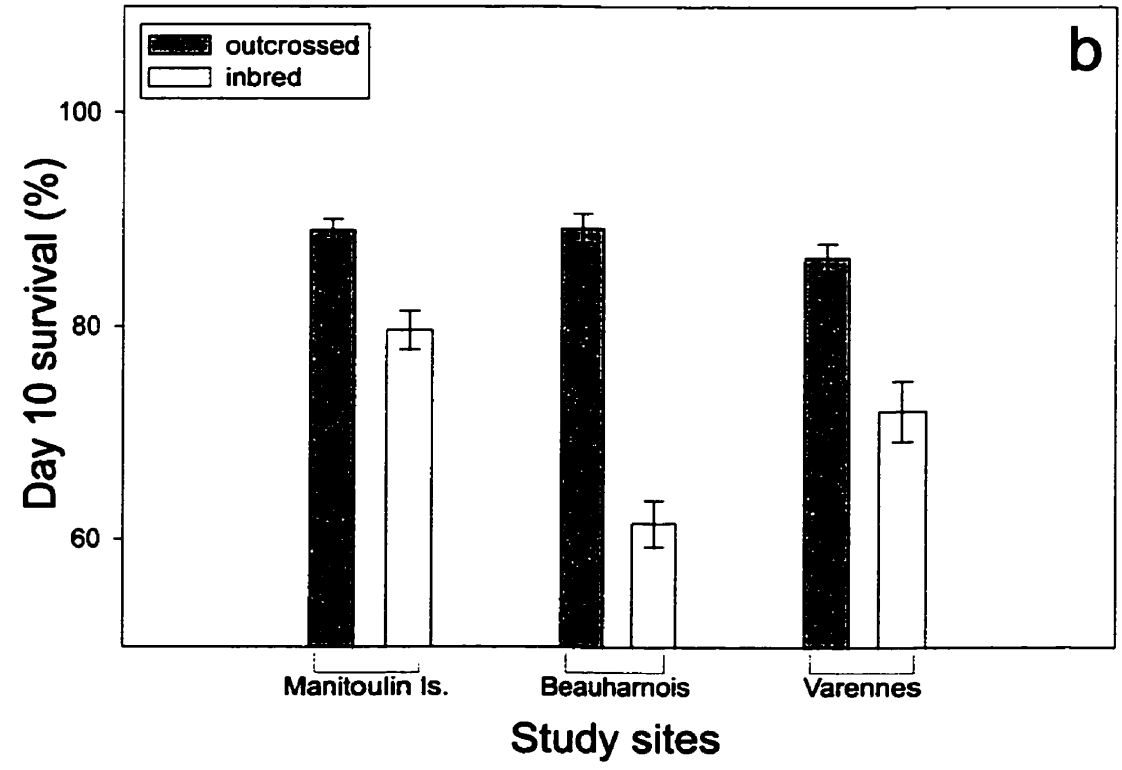
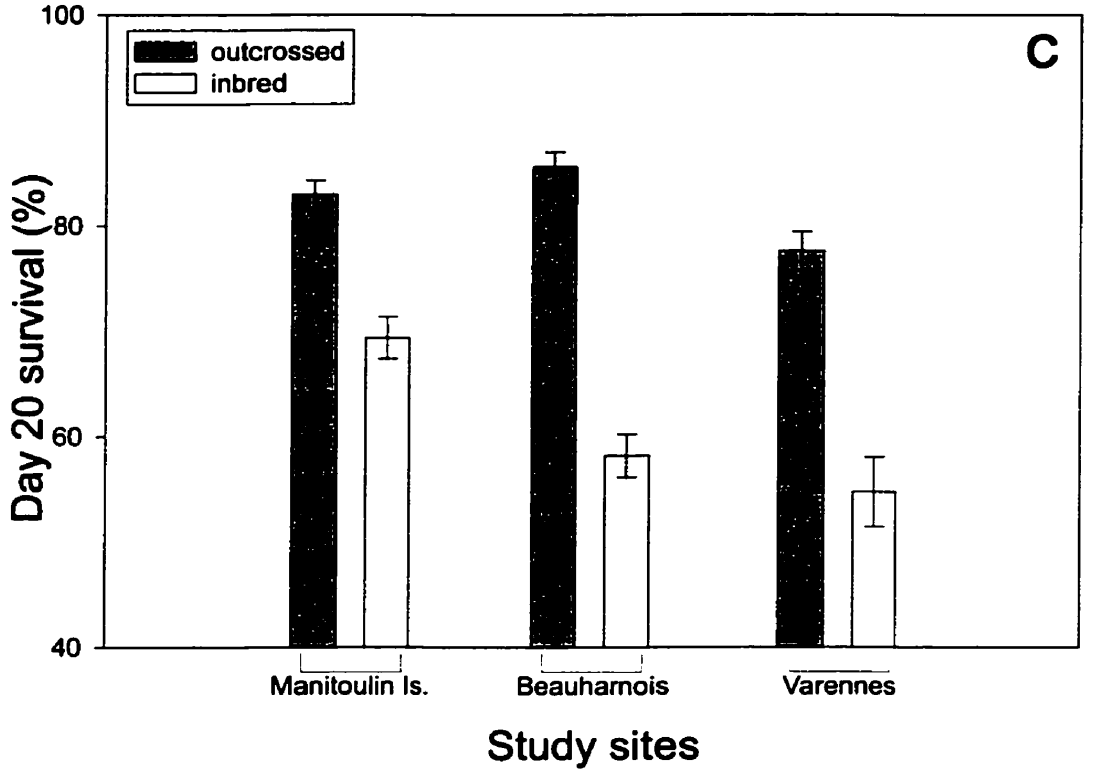

b
outcrossed
inbred
Day 10 survival (%)
100
80
60
Manitoulin Is.
Beauharnois
Varennes
Study sites

Figure 5c. Percentage survival (error bars are ± 1 SE) for outcrossed and inbred snails at day 20 from the three study sites.

Figure 5d. Percentage survival (error bars are ± 1 SE) for outcrossed and inbred snails at day 30 from the three study sites.


100
80
60
40
outcrossed
inbred
c
Day 20 survival (%)
Manitoulin Is.
Beauharnois
Varennes
Study sites

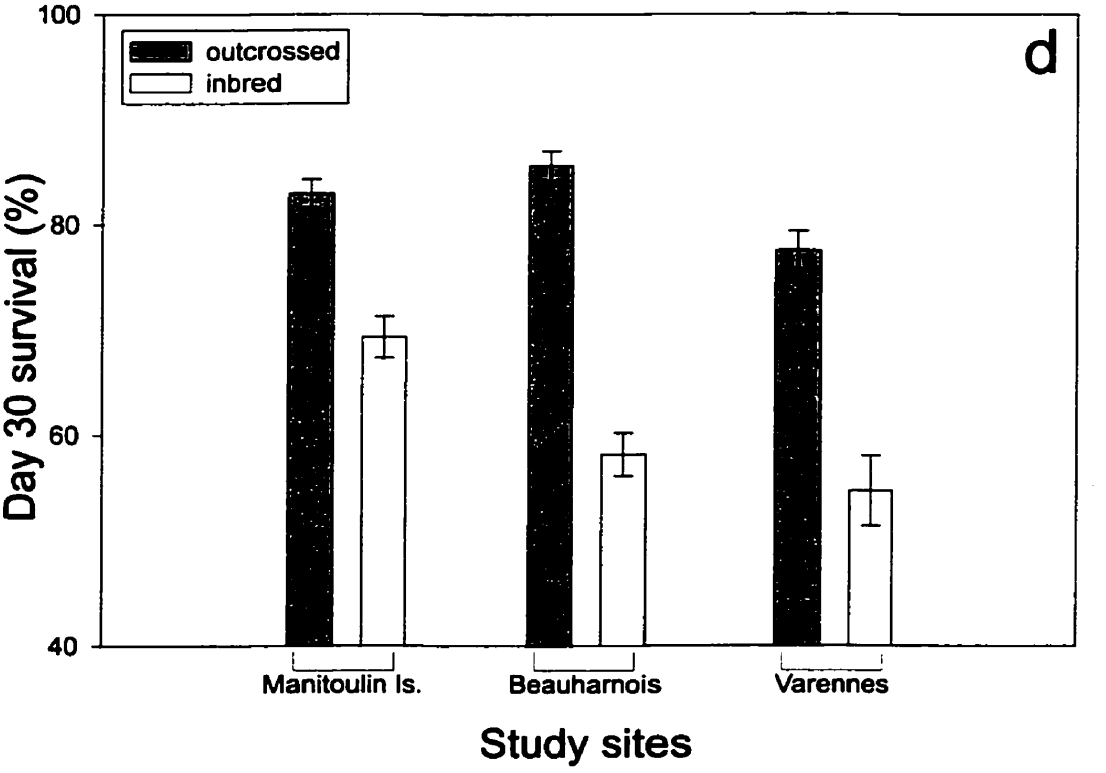

100
80
60
40
outcrossed
inbred
d
Day 30 survival (%)
Manitoulin Is.
Beauharnois
Varennes
Study sites

TABLE 5. ANOVA for post-hatch survival at four time points as a function of pollution level at the site and breeding system (outcross/inbred).

		F	df	*p*
HATCH	(s) pollution level at the site	50.6	2,631	<0.0001
	(b) breeding system	111.2	1,631	<0.0001
	s X b	29.3	2,631	<0.0001
Day 10	(s) pollution level at the site	15.1	2,631	<0.0001
	(b) breeding system	104.1	1,631	<0.0001
	s X b	16.4	2,631	<0.0001
Day 20	(s) pollution level at the site	14.1	2,634	<0.0001
	(b) breeding system	143.2	1,634	<0.0001
	s X b	7.7	2,634	=0.0005
Day 30	(s) pollution level at the site	29.5	2,629	<0.0001
	(b) breeding system	186.4	1,629	<0.0001
	s X b	6.0	2,629	=0.0025

TABLE 6. Estimate of inbreeding depression (δ) for survival at four time points at the three study sites.

Pollution level Site	Clean Manitoulin Is.	Intermediate Beauharnois	High Varennes
	δ	δ	δ
Hatch	0.03	0.28	0.13
Day 10	0.08	0.30	0.16
Day 20	0.14	0.30	0.30
Day 30	0.18	0.31	0.40

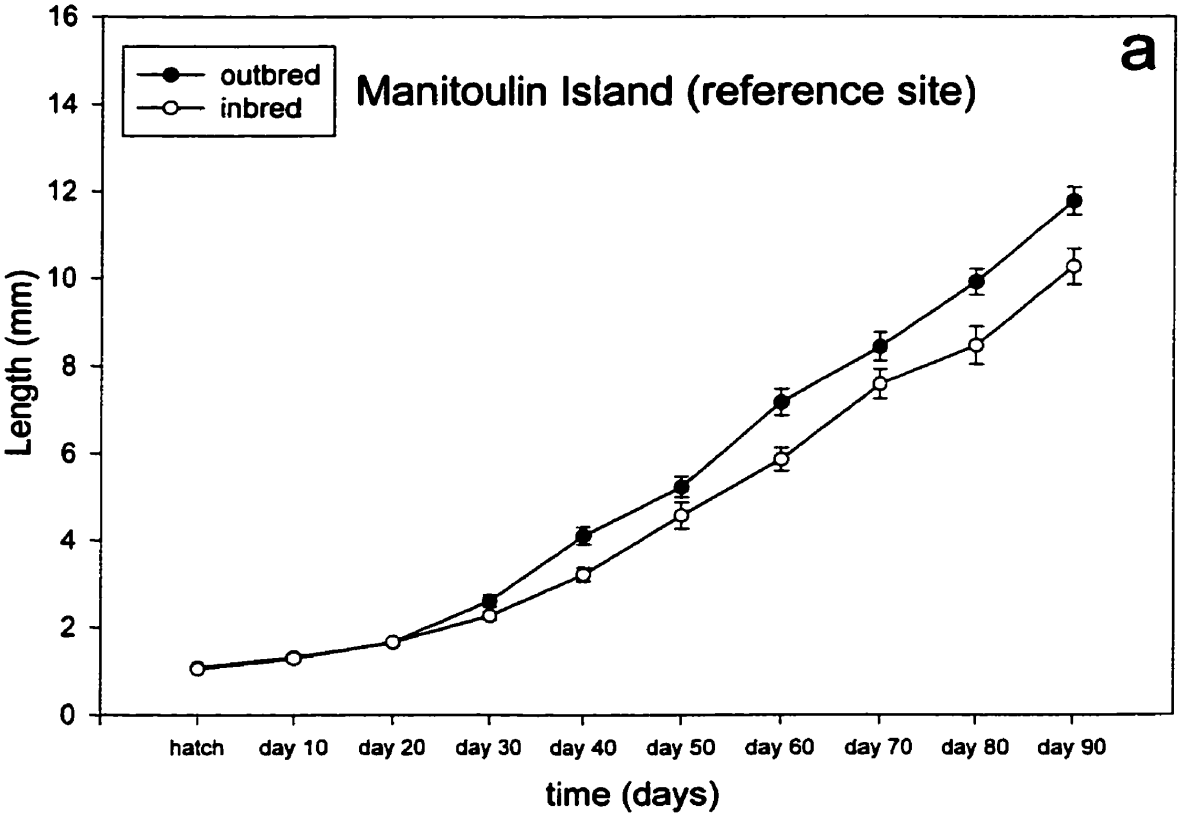
Growth (length)

Length of inbred snails from the reference site and from the mildly polluted site tended to be smaller than outcrossed snails from day 40 to the end of the experiment (Figure 6a, 6b). In snails from the most highly genotoxic site, the difference was only apparent on day 80 and day 90 (Figure 6c). The estimates of inbreeding depression were calculated for all time points at the three study sites and ranged from 0.0278 to 0.2171 at the reference site, 0 to 0.2877 at Beauharnois, and 0 to 0.1232 at Varennes (Table 7). To determine if there were significant differences between the reference site and the polluted sites, pairwised t-tests with Bonferroni correction were performed. Inbreeding depression was significantly greater in Beauharnois than in Manitoulin Island with $p=0.0038$ (Table 8). It was expected that the inbreeding depression would be greater in Varennes than in the reference site, but δ was greater in Manitoulin Island than in Varennes ($p=0.0392$; Table 8). This result is likely due to size biased mortality of smaller snails from the inbred progeny at the Varennes site resulting in a bimodal frequency distribution of the lengths from day 40 onward to day 90 (Figure 7b, 7c, and 7d respectively). The proportion of snails from the smaller mode decreased from 32.9% to 14.8% during that period (Figure 8). The frequency distributions from hatch to day 30 were all normally distributed (Figure 7a and 7b respectively). This suggests that there was size biased mortality that favored the larger snails. Therefore, the growth rate of inbred snails from Varennes was overestimated. Bimodal distribution was not observed in neither outcrossed nor inbred snails for the other two sites. To correct for the effect of

size biased mortality from the analysis, the growth trajectory was recalculated for the inbred snails from the Varennes site from day 40 to day 90 assuming that small snails represented 32.9% of the population and then calculating a weighted mean size of the snails from the large and small cohort. The adjusted size of inbred snails tended to be lower than the outcrossed snails from day 50 until the end of the experiment (Figure 6c, corrected inbred curve). The corrected inbreeding depression coefficients ranged from 0.12 to 0.22, and tend to be higher than the values of the reference site from day 70 to day 90, but remained smaller than the values for snails from the intermediately polluted site (Table 7).

Figure 6a. Mean length (error bars are $\pm$ 1 SE) of outcrossed and inbred snails from Manitoulin Island (reference site).

Figure 6b. Mean length (error bars are $\pm$ 1 SE) of outcrossed and inbred snails from Beauharnois (intermediate level of pollution).


a
outbred
inbred
Manitoulin Island (reference site)
Length (mm)
0
2
4
6
8
10
12
14
16
hatch
day 10
day 20
day 30
day 40
day 50
day 60
day 70
day 80
day 90
time (days)

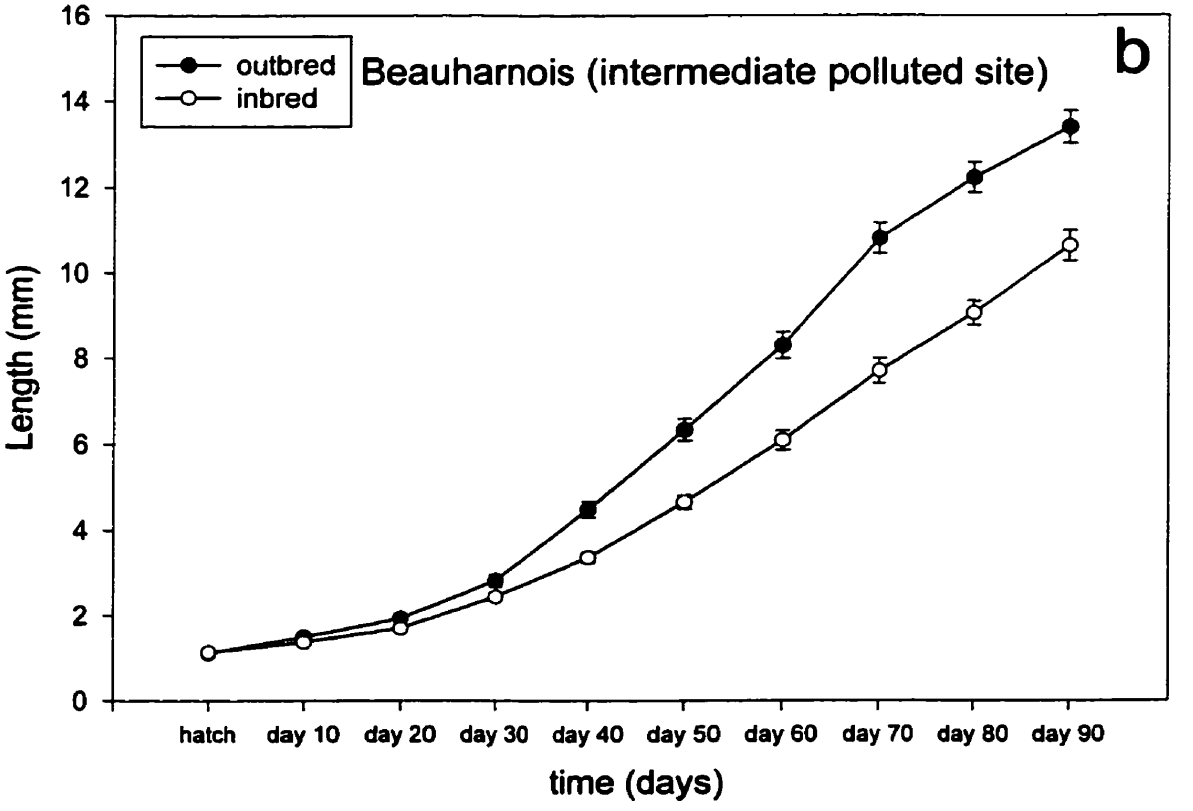

b
outbred
inbred
Beauharnois (intermediate polluted site)
Length (mm)
0
2
4
6
8
10
12
14
16
hatch
day 10
day 20
day 30
day 40
day 50
day 60
day 70
day 80
day 90
time (days)

Figure 6c. Mean length (error bars are $\pm$ 1 SE) of outcrossed and inbred snails from Varennes (most polluted site).

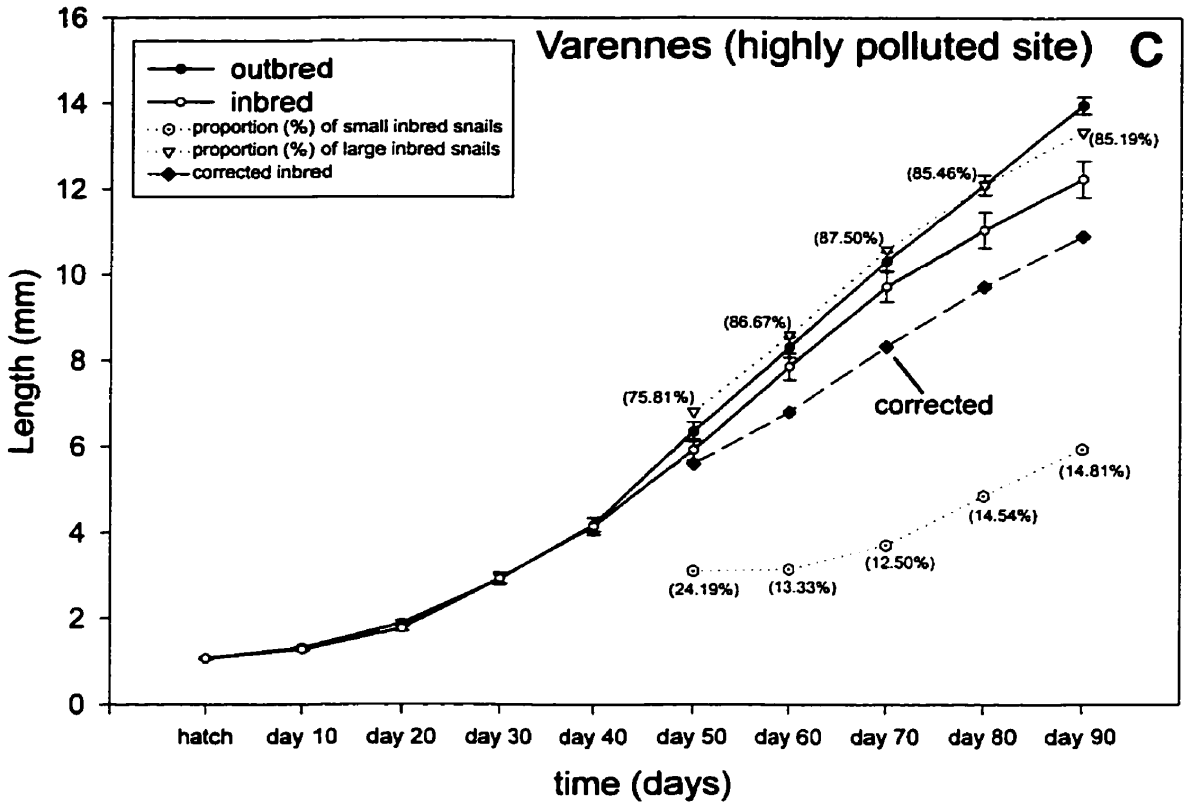

Varennes (highly polluted site)
C
outbred
inbred
proportion (%) of small inbred snails
proportion (%) of large inbred snails
corrected inbred
Length (mm)
time (days)
hatch
day 10
day 20
day 30
day 40
day 50
day 60
day 70
day 80
day 90
(75.81%)
(86.67%)
(87.50%)
(85.46%)
(85.19%)
(24.19%)
(13.33%)
(12.50%)
(14.54%)
(14.81%)
corrected

Table 7. Coefficient of inbreeding depression (δ) between outcrossed and inbred snails for growth (length) at each time point for all three study sites.

Pollution level Site	Clean Manitoulin Island	Intermediate Beauharnois	High Varennes	Corrected Varennes site
	δ	δ	δ	δ
HATCH	0.0278	0	0	–
Day 10	0.0153	0.0766	0.0229	–
Day 20	0	0.1189	0.0585	–
Day 30	0.1308	0.1336	0	–
Day 40	0.2171	0.2507	0.0072	–
Day 50	0.1262	0.2646	0.0677	0.12
Day 60	0.1827	0.2661	0.0541	0.18
Day 70	0.1008	0.2877	0.0581	0.19
Day 80	0.1463	0.2593	0.0867	0.20
Day 90	0.1283	0.2059	0.1232	0.22

0= negative coefficient of inbreeding depression

Table 8. Results of pairwised t-test with Bonferroni correction comparing coefficient of inbreeding depression

Paired t test	t	df	p
Manitoulin Is. vs Beauharnois	-3.8595	9	0.0038
Manitoulin Is. vs Varennes	2.4112	9	0.0392

Figure 7a. Frequency distribution of outcrossed and inbred snail lengths at hatch to day 20 at the Varennes site.

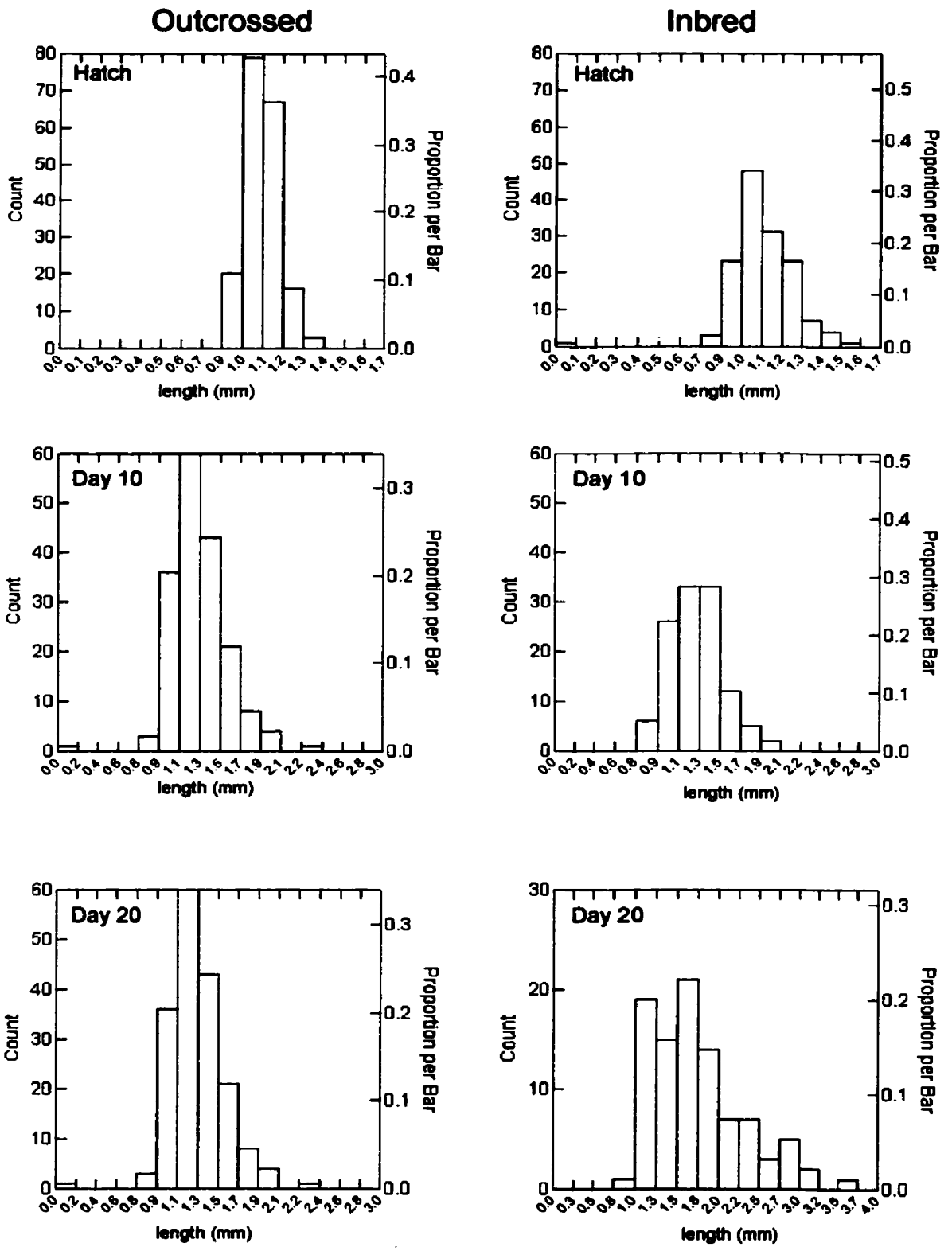

Outcrossed
Inbred
Hatch
Day 10
Day 20
Count
Proportion per Bar
length (mm)

Figure 7b. Frequency distribution of outcrossed and inbred snail lengths at day 30 to day 50 at the Varennes site. The division between small and large snails in the bimodal distributions at day 40 and 50 are marked with a black vertical line.

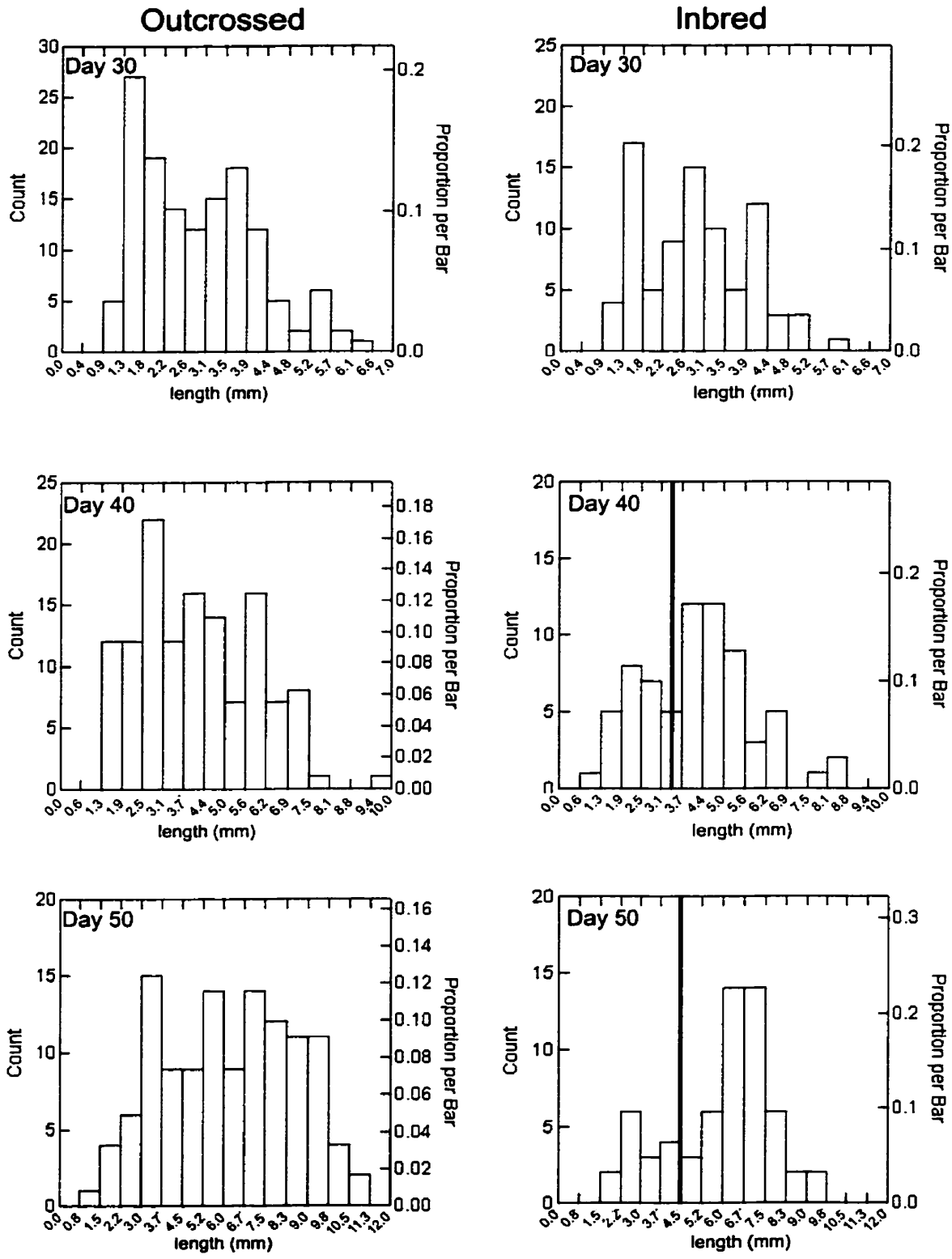
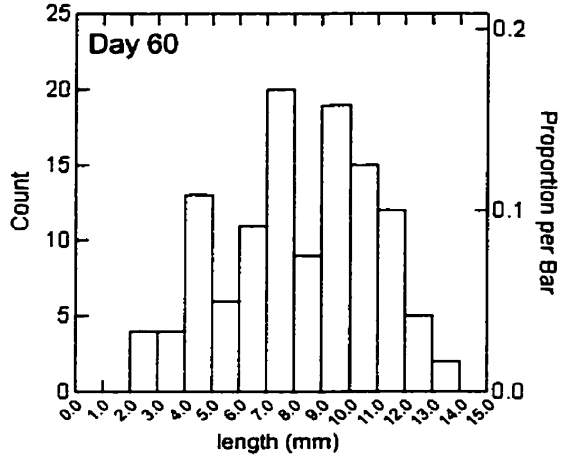
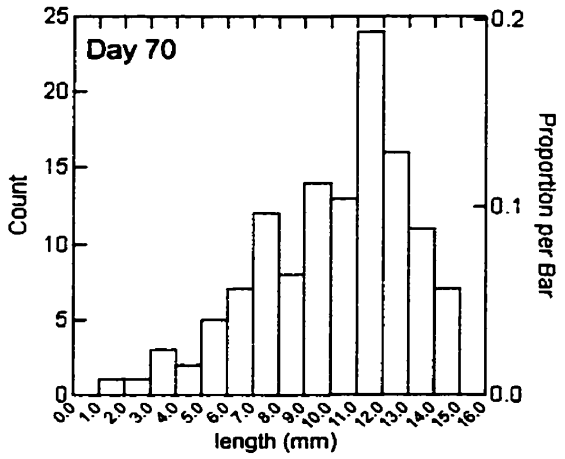
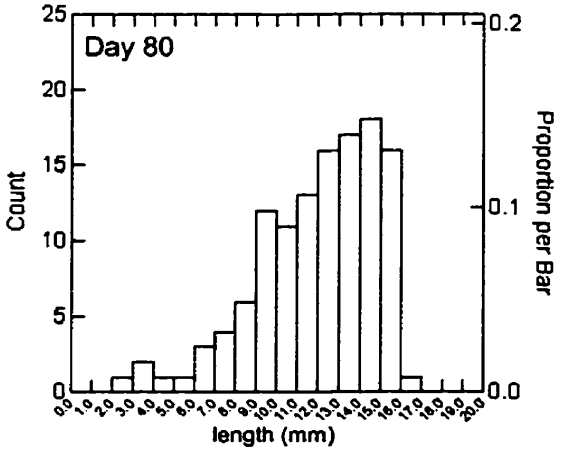

Outcrossed
Inbred
Day 30
Day 40
Day 50
Count
Proportion per Bar
length (mm)

Figure 7c. Frequency distribution of outcrossed and inbred snail lengths at day 60 to day 80 at the Varennes site. The division between small and large snails in the bimodal distributions at day 60 and 80 are marked with a black vertical line.

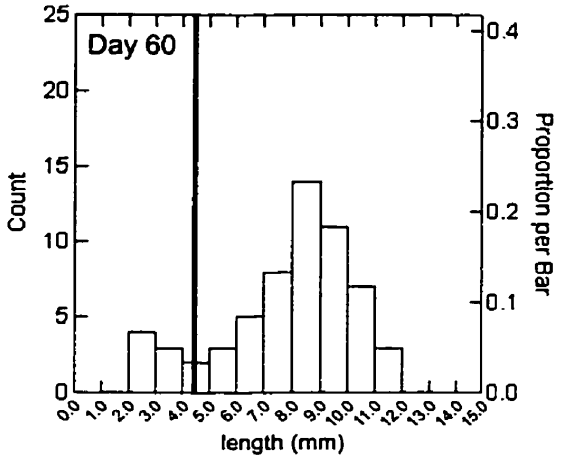
Outcrossed

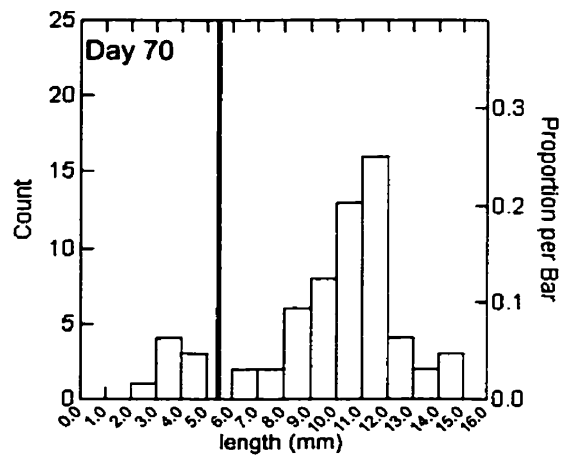






Inbred





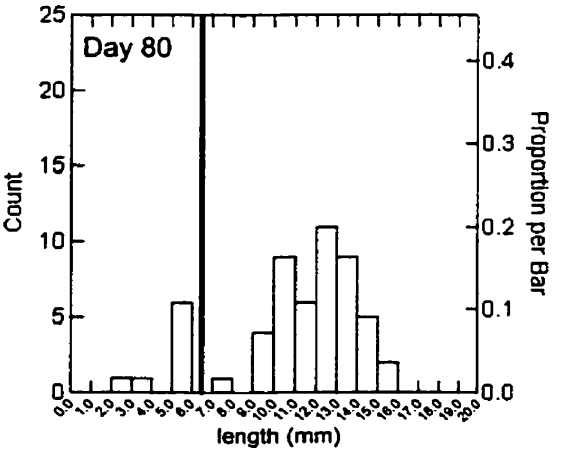
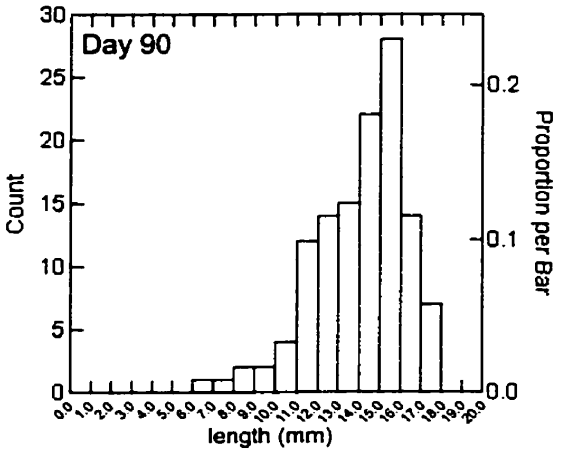


Figure 7d. Frequency distribution of outcrossed and inbred snail lengths at day 90 at the Varennes site. The division between small and large snails in the bimodal distribution at day 90 is marked with a black vertical line.

Outcrossed



Inbred

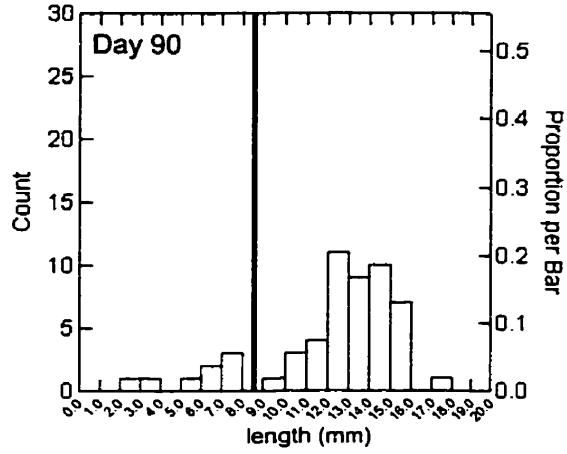
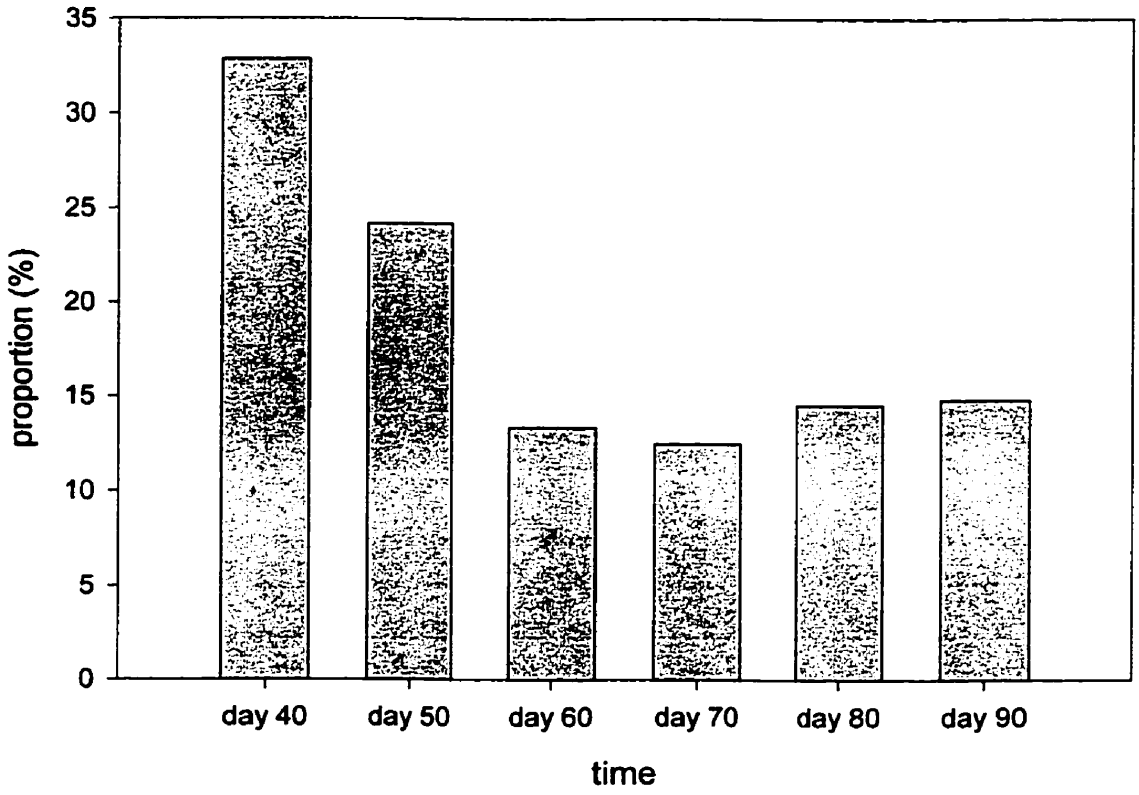


Figure 8. Proportion (%) of small snails in the bimodal distributions calculated for inbred snails from the Varennes site at day 40 to day 90.


35
30
25
20
15
10
5
0
proportion (%)
day 40
day 50
day 60
day 70
day 80
day 90
time

DISCUSSION

The analysis performed in this study showed that *Lymnaea stagnalis* populations from the three sites differed significantly in their estimates of inbreeding depression and that the levels of inbreeding depression for three different fitness indicators tended to increase along the upstream to downstream pollution gradient. These observations are consistent with the hypothesis that organisms accumulate latent heritable genetic damage in relation to the level of *in situ* pollution. A population that is continually accumulating latent heritable genetic damage (genetic load) could eventually become non-viable. Every deleterious mutation must eventually be eliminated from the population by premature death or reduced relative reproductive success (Crow, 1999). This phenomenon was termed "Genotoxic Disease Syndrome" by Kurelec (1993) and Depledge (1996).

In studies by Jarne *et al.* (1991), Chen (1993), Doums *et al.* (1994), Doums *et al.* (1996), and Jarne and Delay (1990), similar methods and a combination of three fitness indicators (used in this study) were used to determine inbreeding depression in hermaphroditic (land and freshwater) snails but in the context of evolution, not toxicology. The novelty of this study is that inbreeding depression appears to be related to increased exposure to genotoxic pollution, increasing the heritable genetic damage (recessive mutations) in the population.

Conditions that will obligate or increase selfing, such as low population density can place a population at risk if they cannot carry a large genetic load.

Non-genetic toxic effects of environmental contamination, population (abundance) fluctuations resulting from other ecological pressures and/or stochastic events might reduce population levels below the threshold where most snails could easily find a mate. In these situations, self-fertiization could occur. However, populations in an environment where they experience a high genetic load (burden) due to genotoxic contamination would compromise this mechanism (which provides the population a means to rebuild from low densities) and dramatically reduce the fitness of the population. This loss of fitness might lead to the extinction of local population. The cumulative effects of such events could imperil the species on a much larger scale.

There are several unique features in this study. First, populations obtained were already exposed *in situ*, allowing for the estimation of realistic effects of duration, composition and levels of genotoxins. Second, toxicologists generally define chronic exposure as 12 months or the life time of the animal (Brusick, 1987); this study goes beyond an organism's lifetime and incorporates the cumulative effects of many generations of exposure to all polluting activities at the study sites integrating spatial, temporal, and, environmental changes. In contrast, studies by Bluzat and Seugé (1983) and Woin and Bronmark (1992) exposed *Lymnaea stagnalis L.* to a herbicide for 45 days and for the life-time of the organism respectively. Third, polluted sites were defined in terms of low to high levels of potential genotoxicity but in fact all sites were well below any physiological toxicity level. Fourth, inbreeding depression measured in populations at these sites is the result of multi-generation exposure and is in

essence a cumulative historical marker of all past pollution events. Finally, using a hermaphroditic organism that is able to self increased the sensitivity of this study to detect latent heritable genetic damage because the chance of genes being identical by descent in the progeny of a selfed parent is higher than in full-sib mating of non-hermaphroditic and non-selfing organisms such as the Fathead minnow used in the study by White *et al.* (1999).

Matby and Calows' (1989) review of applications of bioassays (between 1979 and 1987) to environmental problems showed that more than 90% of the studies were classified as single-species laboratory tests with acute exposure of short duration. Most genotoxicological studies on water samples are usually confined to short-term acute toxicity tests using a multitude of bioassays such as sister chromatid exchange, the Ames test, SOS chromotest, or Microscreen phage-induction assay (Le Curieux *et al.*, 1993; McCann *et al.*, 1975; Meier *et al.*, 1987; Huang *et al.*, 1995; Helma *et al.*, 1996). Furthermore, previous studies on genetic damage focused on somatic cells such as neoplasia in fish from contaminated sites (Malin *et al.*, 1988 and Brown *et al.*, 1973). Also, somatic cell damage as revealed in studies of DNA and protein adducts, sister chromatid exchange (SCE), micronuclei in humans exposed to mutagenic compounds (Perera, 1987) and abnormal DNA profiles (aneuploidy and mosaicism) in frogs exposed to pesticides (Lowcock *et al.*, 1997) are strong evidence of genetic damage but not evidence of heritability.

Another method used to evaluate toxicity is to expose an organism to a single compound in a controlled environment and measure cytotoxic affects. In

studies by Khangarot *et al.* (1988); Woin and Bronmark (1992); and Bluzat and Seugé (1983), pulmonate snails were exposed to heavy metals, an organic pesticide or an organic herbicide, respectively. Cellular bioassays, single chemical exposure studies, and field studies each provide different levels of information concerning genotoxic contamination. However, they are not able to provide information on the effects of multi-generation chronic exposure of a population to complex mixtures of genotoxic contaminates that are found in the natural environment (i.e., effects on population and/or at the ecosystem level). Tests to detect the effects of long-term (multi-generation) exposure are much less common. Furthermore, few studies have been designed to test for possible latent heritable genetic damage from *in situ* exposure to genotoxins. This study is not only able to make statements on population effects by genotoxins but also able to quantify the effects of a nondescript complex mixture of genotoxins that is unique to each polluted site. Thus the approach used here has the potential to address population-level affects of genotoxic mixtures.

Due to the labor intensive activity of culturing aquatic snails, only three sites were analyzed. Further research using more reference and polluted sites would allow for stronger estimates of baseline and elevated levels of inbreeding depression in the study organism.

Closer examination of the data illustrates a limitation in using growth as a fitness measure. Estimates for inbreeding depression for growth were higher in the moderately polluted site (Beauharnois) when compared to the reference site (Manitoulin Island). It was expected that the inbreeding depression for growth

would be highest in Varennes but in fact it was lower than values at Manitoulin Island. Since survival is correlated with growth (i.e., an organism must be alive to grow) it is not surprising to see size biased mortality of smaller snails resulting in a bimodal distribution of lengths for inbred snails at the Varennes site. Figure 7c shows, a hypothetical growth curve for inbred snails from day 40 onward corrected for size biased mortality of smaller snails. Growth, however, remains a problematical fitness parameter since it is not clear that it can be estimated in an unbiased manner, even when no apparent bimodality was present. It is quite possible that smaller snails might be more likely to die in all of the treatments.

Additional inbred generations (and backcrossing them) would allow one to determine whether the genetic load in a population of snails from a polluted site could be purged resulting in the recovery of the population fitness. This would be additional evidence that inbreeding depression is present (Roff, 1997) and an interesting and revealing experiment to be performed in the future.

Finally, estimates of *in situ* selfing rate would help confirm the assumption that the test organisms are preferentially outcrossing in nature (Jarne and Charlesworth, 1993; Schoen and Clegg, 1984) and thereby help support the idea that inbreeding depression is due to the accumulation of mutations.

We know little about the consequences of long-term exposure to low levels of genotoxins on aquatic biota and human health. At this point, studies have been able to detect genotoxins in surface water, sediments, and the tissue of biota from polluted aquatic environments. Additionally, laboratory experiments have shown that a potent environmental genotoxin can cause latent heritable

genetic damage in biota. This information has laid the foundation for this study. In this study we have shown that snails from different sites differ significantly in the genetic load they appear to be carrying, and that these differences are consistent with the hypothesis that genetic load is increased through exposure to genotoxic pollutants. With refinements that will shorten and simplify the procedures used in this study, this method can be employed as a simple bioassay for risk assessment.

Clearly, further studies investigating genotoxic pollution on other organisms, populations and communities are needed to determine ecologically relevant impacts of prolonged exposure. Using this method of studying the fitness effects of chronic exposure to genotoxins on organisms in conjunction with measurements obtained from common bioassays used to measure environmental genotoxicity will allow for a better assessment of the impact of anthropogenic pollutants on aquatic organisms.

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