INTERNAL REPORT

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ERISS Protocol for the creekside monitoring of Magela Creek waters
I. Freshwater snail, ***Amerianna cumingii***, Reproduction and survival test

Prepared by : CL Humphrey
BF Lewis
I Brown
JL Suggit

The Supervising Scientist for the Alligator Rivers Region has research, supervisory and co-ordination responsibilities related to effects on the environment and uranium mining in the Alligator Rivers Region and research and supervisory responsibilities related to effects on the environment of non-uranium mining in a conservation zone declared within the Region.

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ERISS PROTOCOL FOR THE CREEKSIDE MONITORING OF MAGELA CREEK WATERS

I. FRESHWATER SNAIL, *Amerianna cumingii*, REPRODUCTION AND SURVIVAL TEST

C.L. Humphrey, B.F. Lewis, I. Brown and J.L. Suggit

Environmental Research Institute of the Supervising Scientist

FORWARD

The following protocol is in DRAFT form. It is our intention that the written procedures for conducting the freshwater snail reproduction and survival test for creekside monitoring be handed over to RUM staff for practical implementation. After feedback from company staff, the protocols will be revised. Further to this, the protocols are incomplete in figures (photomicrographs) describing the embryonic stages (including abnormalities) of *Amerianna cumingii*. Whilst this will not affect immediate application of the protocol, the figures will greatly assist in this and will be prepared and incorporated in later versions.

Eventually, full application of the protocol and analysis of the data gathered under its procedures will require accompanying creekside monitoring information. In particular, there are two internal reports in preparation describing the creekside monitoring systems on Magela Creek (including routine maintenance) and the data gathered so far upon *A. cumingii*. The paper by Humphrey *et al.* (1995) outlines the rationale for the snail test and the design (BACIP) employed.

Reference

Humphrey, C.L., Faith, D.P. & P.L. Dostine (1995). Baseline requirements for assessment of mining impact using biological monitoring. *Aust. J. Ecol.* (in press).

DRAFT ONLY

ERISS PROTOCOL FOR THE CREEKSIDE MONITORING OF MAGELA CREEK WATERS

I. FRESHWATER SNAIL, *Amerianna cumingii*, REPRODUCTION AND SURVIVAL TEST

1. Objective

Detection in the field, of adverse effects of dispersed mine waste-waters upon freshwater snail reproduction, embryonic development and the survival of the juvenile snails over a 15-day test period.

2. Principle of the Test

Pairs of *Amerianna cumingii* (Mollusca, Gastropoda), all of similar shell size (length 10-12.9 mm), are exposed simultaneously for four days, in containers in tanks, to a continuous flow of creek waters drawn from one of two sources, i.e. upstream of the mine site (control water) and downstream of the mine site (test water). During this time the snails are free to lay 'egg masses' upon the test containers. (An 'egg mass' refers to a discrete batch of eggs, each egg in its own capsule and each batch of eggs being surrounded by a gelatinous coat.) The adults are then removed and a selected number of egg masses retained for testing over the next 10-12 days in the respective treatment-waters. Juvenile snails escape from the egg mass after 6-8 days and at the end of the test, four days later, the number of eggs produced and the number of juvenile snails surviving in each treatment are compared. Where significant differences are found to occur between a time-series of results from the two sites, this is taken as evidence of the presence of an unacceptable concentration of mine waste-waters in the creek water.

3. Test Organism

The test species, *Amerianna cumingii* (Mollusca, Gastropoda), occurs in lentic floodplain habitats of the Alligator Rivers Region. Important aspects of the biology of the snail are summarised in Appendix 1. All specimens required for testing are taken from laboratory stocks (Appendix 2) which have been cultured from a sample of animals collected from Magela Creek in 1986. Adult snails of between 10-12.9 mm shell length are used for testing, this being the optimal size for egg laying. (Larger animals [>13 mm] in particular may be senescent.)

All test animals must be free of overt signs of disease or shell damage. Snails can be weighed prior to testing and their weights compared with those of similar-sized individuals if there is any doubt of the health of the snails. For the size range of snails tested (10-12.9 mm shell length) corresponding weights should be between the range 250 - 500 mg. Snail pairs which fail to lay egg-cases after a four-day exposure to natural (control) creek waters are regarded as abnormal [see section 7(i)]. Shell or internal damage to one or both of the snails, arising from mis-handling, is often found to be the cause. Snails must therefore be handled carefully during all stages of testing; shell damage in particular will lead quickly to the death of animals. Further details on the handling of animals are provided in Appendix 2. More extensive failure of snails to freely produce healthy egg cases may indicate presence of disease (e.g. trematode parasitism). Should this be observed, new stocks from the field should be obtained if required (Appendix 2).

Snails used in a creekside test must not be used in subsequent tests. Snails exposed to control creek waters in previous tests may be used only as stock for further snail production at the laboratory. Snails exposed in tests to creek waters downstream of Ranger are destroyed at the completion of the test.

4. Equipment

(a) Egg-laying chambers

Sixteen egg-laying chambers are required per treatment (= different water type). Each of these consists of an open-ended cylinder of transparent, colourless Perspex or polycarbonate, 70 mm long, 51 mm external diameter and 1.75 mm material thickness. Nylon mesh screens are inserted approximately 5 mm into each end of the chamber, using internal circlips as stoppers. Circlips are constructed from small lengths (5 mm) of PVC pressure pipe (class 12, 50 mm internal diameter). A sufficiently small section is cut from the PVC 'ring' so that upon insertion of nylon screen and resulting circlip into the chamber, the ends of the circlip meet exactly. The mesh serves to prevent escape of snails while allowing a flow-through of water. Screens of two mesh sizes are required. Screen of mesh-size ranging between 1.0 and 2.5 mm is used initially to retain the adult snails. Later in the testing procedure, (immediately prior to juveniles escaping from egg masses,) a smaller size mesh (e.g. 600 x 250 μm) is fitted in order to retain the young snails. (Newly-hatched snails measure 600-700 μm shell length x 400 μm width.) At one end of each chamber and outside of the area in which egg masses might be laid, an identification code is either etched, or marked on the chamber with indelible ink, using a felt-tipped pen.

(b) Test containers

These consist of transparent and colourless Perspex vessels, rectangular in plan and cross-section, each capable of holding at least 8 egg-laying chambers. (Where organic compounds are of concern, glass containers should be used.) Each test container must be capable of holding a

volume of test solution equivalent to at least 3 L/g of snail, while leaving a freeboard of at least 5 cm. By way of example, the preceding specifications can be met in a test container that is slightly flared in cross-section (for stacking) with maximum surface dimensions 440 mm in length x 290 mm in breadth, and height of 310 mm. In this container, a volume of 17 L of test-water is sufficient to hold 8 egg-laying chambers containing snails of the required size.

Tubing distributing incoming water is fixed onto an aquarium clamp fitted to the inside of one end of the container. The tubing should reach a depth of two-thirds of the water level in the container. The outflow is located at the opposite end of the container. A convenient design for the outflow is one consisting of two plastic 90°-bend connectors. One connector is located inside the container and, through a hole in the container wall, adjoins the other located on the outside. The outside connector faces downwards and the inside connector upwards to serve as a stand-pipe. Thus, the angle of the inside connector may be adjusted (or plastic tubing added to the connector) in order to achieve a desired water volume. If attack and predation by birds and other animals upon snails is likely to a problem, each container should be provided with a Perspex (or other clear plastic) cover for protection.

(c) Ancillary

- Vernier calipers (used to measure length of adult snails);

- binocular dissecting microscope with 6 Volt capability for field use;

- glass or clear plastic vessels in which to hold egg-laying chambers for microscopic viewing. The vessel must hold a sufficient depth of water that the chamber, lying on its side, is completely immersed. Glass histological staining dishes are ideal for this purpose;

- containers to transport the egg-laying chambers containing snails to and from the field;

- lettuce cutter (a short length of 20 mm (i.d.) PVC pipe, one end of which is chamfered in order to make a clean incision);

- two thermometers (maximum and minimum) per water 'treatment';

- portable dissolved-oxygen meter.

5. Test Procedure

(a) A relevant aspect of test design

As stated above, the treatment waters to which snails are exposed are drawn from one of two sources, namely upstream of the mine site (control water) and downstream of the mine site (test water). Two test waters may be drawn: fully-mixed receiving waters or waters from a 'mixing zone'. Duplicate waters are drawn independently for each treatment. For this, separate pumps should be located on each side of the creek channel for the cases in which control and receiving waters are drawn or 100 m apart on the side of the creek channel for the case in which mixing-zone waters are drawn. The duplicate waters of each treatment are pumped to separate header

tanks at the respective creekside stations. Each duplicate water then feeds a duplicate test container holding replicate egg-laying chambers.

(b) Cleaning of test containers

Prior to initial use in the Wet season, all test containers, egg chambers, nylon mesh and circlips must be cleaned, using successively, detergent, 10% nitric acid and demineralised water. Prior to use in all tests, containers should be filled with the appropriate waters at the creekside monitoring station and flushed through for a minimum of one hour.

After each test, containers are washed and rinsed with creek water at the creekside station (with no detergent). Any remaining sediment and detritus adhering to containers are wiped off with a towel. This process is much easier if carried out immediately after use while the containers are still wet. After cleaning, the test containers are dipped in 10% nitric acid. The acid wash may be conducted after a test or prior to a new test commencing. If conducted after a test and following the wash, containers are thoroughly rinsed with control creek water that, if necessary, has been transported to the creekside station for this purpose. If conducted prior to a new test commencing (within 2 hours), containers may be rinsed with treatment water. For either option, the acid-wash solution must be returned to the main laboratory for disposal. Containers left empty between tests are stored upside down on test benches until required. After each test, egg chambers are cleaned at the laboratory using successively, detergent, 10% nitric acid and demineralised water.

(c) Test responses

Egg production

At the laboratory, snails in the range 10-12.9 mm shell length (see Fig. 1) are taken and sorted into three 1.0 mm size classes (10-10.9, 11-11.9 and 12-12.9 mm). Shell length is taken as the distance between the apical tip and the shoulder (Fig. 1). Thirty-two snails are required per treatment. Each treatment duplicate is allocated 8 pairs of snails from the stock. (Thus a total of 32 snails per water type are exposed when duplicates are pooled.) Size classes *must* be distributed evenly amongst, and between the duplicates of, the different treatments. To ensure this, the following procedures are carried out for each group of 8 egg-laying chambers that will be assigned to each treatment duplicate:

(i) cover one end of each chamber with nylon mesh (using circlip inserts);

(ii) rest each chamber on the end bearing the nylon mesh, in a container holding laboratory snail stock-tank water at a level that half-immerses the chambers;

(iii) randomly select 5, 6 and 5 snails each of size class 10, 11 and 12 mm respectively;

(iv) distribute to successive egg-laying chambers the five 10 mm size snails;

(v) continue to distribute to chambers in turn the six 11 mm size snails, the last 3 snails of this size class being added to the first 3 chambers holding single 10 mm size snails;

(vi) add the five 12 mm snails to the remaining chambers so that a pair of snails is present in each chamber.

In this way, the following size pairs are present: 10 and 11 [x 3 pairs], 10 and 12 [x 2], 11 and 12 mm [x 3].

All effort must be made in a creekside test to use this allocation of size classes of snails for treatment duplicates. Should a situation arise, however, where there are insufficent numbers of snails of a particular size class, additional snails from one of the other two size classes may be used, providing the overall mean size of snails tested lies in the range 11-11.9 mm. As before, size classes must be distributed evenly amongst, and between the duplicates of, the different treatments. This may be achieved using an appropriate modification of the procedure of allocation described previously. No snails outside of the size range 10-12.9 mm may be used in creekside tests.

Two 20-mm diameter discs of freshly-cut, outer lettuce leaf, washed in demineralised water are added to each egg-laying chamber containing the snail pair. The open end of the chamber is then covered by nylon mesh to retain the snails. The sizes of the snails in each chamber are recorded. The groups 8 egg-laying chambers with their enclosed snails are carefully transported to the field in containers holding laboratory snail stock-tank water.

At the creekside monitoring station, the 8 egg-laying chambers and enclosed snail pairs of each treatment duplicate are successively placed in the test containers. (This follows cleaning and flushing of the test containers as outlined above.) The test containers are positioned on the benches at the creekside station in such a way as to avoid bias in experimental design. (See Humphrey (in prep.) for details of the container distribution. This covers the two cases (a) different treatment waters from adjacent sites drawn to a common creekside station and (b) different treatment waters drawn from sites remote from one another and drawn to separate creekside stations.) The test is deemed to have commenced when all egg-laying chambers have been placed in the test containers, the test containers have been positioned appropriately and the containers are receiving treatment waters at the appropriate flow rate (section 6).

During the period of exposure of adult snails, pairs of snails are inspected daily and dead or injured animals removed and replaced. This information is recorded. At the time of this inspection, circlips and mesh from the laying chambers are removed, cleaned and rinsed in clean treatment water and faeces and uneaten lettuce removed from the chambers. Each snail pair is fed by addition to the chamber of two 20 mm diameter discs of fresh outer lettuce leaf previously washed in demineralised water. The mesh covers are then re-inserted and the chambers returned to their respective test containers. The entire procedure of inspection, cleaning, feeding and reassembly of chambers should be carried out in a separate container of treatment water such that the time the snails are out of water (in transferring between the test containers) is no longer than a few seconds. Finally, the containers themselves should be inspected and cleaned of any fouling organisms, uneaten lettuce and faeces.

After 4 days, the circlips, mesh and snails are removed from the laying-chambers. Considerable care must be taken in dislodging snails from the inner walls of the chambers to prevent damage of the egg masses. The egg masses attached to the inner wall of each chamber are then examined under a binocular dissecting microscope in order to count the number of embryos. The numbers of egg masses and of embryos per egg mass in each chamber for the 4-day period are recorded. Chambers may be examined either in the field or at the laboratory. For either location, it is important that snails from all treatments are removed from chambers within one hour of each other. For laboratory examination, each chamber must be transported carefully

from the field in a container holding a sufficient volume of its respective treatment water to cover the chamber. For microscopic examination, either in the field or laboratory, each chamber is placed on its side in a clear plastic or glass dish. The dish must hold a sufficient volume of its respective treatment water to cover the chamber.

Juvenile survival

Two or three egg masses are then selected from and retained in six of the eight chambers in each treatment duplicate so as to give (ideally,) between 50 and 70 (but not less than 30) embryos per chamber, for further exposure to the waters being investigated. Selection is made on the basis of similar numbers of embryos and relative synchrony in the developmental stage of the embryos (see Appendix 1) both within and amongst water types. To meet this requirement, egg masses most recently laid (preferably in the previous 24 hours) are retained. Egg masses not required for further testing are removed from the chambers (e.g. scratched free with the clean fingertip). If this procedure has been carried out at the laboratory then the chambers must be transported carefully back to the field site, again covered in appropriate water. The six chambers containing egg masses are then placed back into the respective duplicate test container (= 12 chambers per treatment). No mesh is required on the chambers at this stage.

The entire process of egg mass examination and selection, and replacement of the chambers back into the treatment waters must be completed within 12 hours. Chambers that are examined in the laboratory must be held in treatment waters corresponding in temperature to that occurring in the field. (Holding of the chambers at a different temperature, e.g. in air-conditioned rooms, must be avoided as far as possible.)

Over the ensuing 6-8 day period (depending on water temperature, see Appendix 1), embryonic development proceeds, the eggs hatch (= eclosion), and a little later the juveniles escape from the egg masses. Two or three days prior to eclosion, each chamber should be placed daily under a dissecting microscope in the field (as described above) for examination. In this examination, a visual assessment is made of the state of development of the embryos (see Appendix 1). This enables the number of viable embryos in each chamber to be counted just prior to eclosion. (These 'pre-eclosion' numbers are used as the denominators of the 'juvenile survival' ratio (section 7) i.e. final number of live snails/pre-eclosion number of live embryos.) However, even though egg masses were selected initially on the basis of their similarity in developmental state, nevertheless, the time from eclosion to escape from the egg mass in replicate chambers takes place over a period of approximately three days. Thus, the time between which the pre-eclosion count is made and that of count of juveniles, will vary for each replicate chamber - and, even at times, for each egg mass within a single chamber. It is therefore essential to identify and follow closely the development of individual egg masses by making observations with a sufficient frequency.

The pre-eclosion count must be made during the embryonic stage, i.e. while each animal is still within its own egg capsule. (It is difficult to make accurate counts during the period of about two days from the time of eclosion of the young to their escape from the egg mass because of the movement and 'milling' of the juveniles within the egg mass.) After the counts have been made, the fine nylon mesh (e.g. 600 x 250 μm) screen is inserted into the open ends of each chamber. This mesh remains in place until the test is terminated. During the period over which

the chambers with their fine-mesh covers are retained in the test containers, it is essential to ensure that there are no air bubbles present in the chambers. (These can be removed by gentle suction using 5 mm plastic tubing in the mouth.)

As noted above, snails may escape from the different egg masses present in a particular chamber at different times (- although escape within a single egg mass is usually synchronous). Therefore it is essential that daily microscope examination of the embryos and juveniles continues until the young escape from the egg mass. For an individual egg mass, the 'time of escape' is identified as being that of the escape of the median animal. Where snails from the different egg masses present within a particular chamber escape at different times, then the 'time of escape' is identified as that of the median animal to escape from the last egg mass in which this occurs. Where the number of juvenile snails present in an egg mass is low, the egg mass appears to have greater strength and integrity, and hatching may be prolonged (over several days). In these cases (and providing juveniles are present in the 'milling' phase), the egg mass may be ruptured by gently breaking it open with a sharp pointed instrument.

Following identification of the 'time of escape' for snails within a replicate chamber, the juvenile snails are exposed to the treatment waters for a further four-day period. Chambers are left undisturbed in the test containers for this time. At the end of the four-day exposure period, counts of juvenile snails are made under a dissecting microscope in the field or at the laboratory. (Again, for laboratory counts, chambers are transported carefully from the field in containers holding the respective treatment waters.) At the time of examination, each chamber is removed from the test or holding container, water allowed to drain from the chamber and the mesh enclosures carefully removed. Under dissecting microscope, counts are carefully made of live juveniles adhering to the mesh and inner surface of each chamber. Counts should be completed on the last day of the four-day exposure period.

6. Test Environment and Conditions

Test environment. The culturing of snails to be used in the tests (Appendix 2), and all manipulation and testing should be carried out in premises or areas free from harmful vapours and dusts, and undue disturbance. The environment under the creekside shelters should be as uniform as possible, to avoid differences in test conditions amongst the test containers (Brown *et al.*, in prep.). Otherwise, the test containers from within and amongst different water types must be arranged under each of the shelters such that there is no bias in test conditions (Humphrey, in prep.).

Flow rate. Gravity feed, from a single header tank for each treatment duplicate, provides the required continuous flow to each test container. The flow rate to the containers is dictated by both the hydraulic head from the header tank and the numbers of hose lines concurrently in use (Brown *et al.*, in prep.). Within these limits, when flow is free and unrestricted (i.e. hose lines are not fouled by detritus and algal build-up), the header tanks should deliver water to each test container at a rate of ~300-500 mLs/ min. Where this flow condition cannot be met then the flow to each test container should be such as to ensure a replacement not less than 90% over a 2-hour period.

It is essential that in any particular test (within and amongst different water treatments) each test container receives the same flow rate, that this rate is as close as possible to optimal (as defined above) and that, as far as possible, amongst different tests a similar flow rate is observed. Flow rates to each test container must be checked daily at least. Any deviations from the optimal rate should be rectified, e.g. by cleaning or replacing hose lines, fittings etc. or by flushing the entire system (Brown *et al.*, in prep.) and the variation recorded (time, date, degree of flow variation, test container involved and estimated duration).

The rate of egg production and oviposition of *A. cumingii* is dependent upon water temperature (Appendix 1). Lengthy interruptions to water flow through test containers will result in differences in water temperatures between and amongst treatment duplicates. In particular, a stoppage of a pump occurring in the early morning (0-0800 hours), if of prolonged duration, will lead to a reduction in water temperature and rate of oviposition of snails in the particular duplicate test container relative to other treatment duplicates. Consequently, data from a treatment duplicate are rejected wherever a pump stoppage: (i) was estimated to have occurred for longer than 6 hours, or (ii) resulted in a water temperature differential of more than 2°C between the duplicate containers of a particular treatment (ascertained from daily maximum and minimum temperature records held for waters in each duplicate container - see section below).

Recording of test conditions. At the commencement of the test and at daily intervals thereafter, water samples are collected from the header tanks containing each treatment duplicate and brought back to the laboratory promptly (within 1 hour) for measurement of pH, conductivity and turbidity. Apart from water samples taken at the commencement of the test, all other samples are collected between 0700 and 0900 hours. Minimum and maximum water temperatures are also read at the time of collecting the water samples, from a thermometer sited in each test container. The minimum and maximum water temperature readings taken each morning relate to the current and previous 24 hour periods respectively. The concentration of dissolved oxygen (DO, mg/L) in the water in each test container is measured *in situ* on the second and fourth days of exposure of adult snails, and at 4-day intervals thereafter (egg mass exposure). (Oxygen concentrations in Magela Creek waters are normally at or above their air-saturation value.) Measurements of DO are made between 0700 and 0900 hours.

It is essential that for proper interpretation of creekside monitoring data, accompanying hydrological and physico-chemical data are available. Apart from measurement of the water quality variables described above, data pertaining to selected metals and major ions will also be required. For creekside monitoring conducted in Magela Creek, collection and analysis of waters for U, Mn, SO_4, Cl and dissolved and total organic carbon of treatment waters should be undertaken at least once during each of the following three components of the test: adult exposure, exposure of egg masses and exposure of post-escape juveniles.

7. Statistical Analysis of Test Data

Statistical tests for the detection of intermittent (e.g. pipeline releases) or sustained and continuous effects arising from dispersion of mine waste waters to creek systems may validly be applied using the BACIP (Before, After Control, Impact, Paired differences) design (Stewart-Oaten *et al.*, 1986, 1992). Egg production and survival-rate data from a time series of such

tests are required for this purpose. The statistical procedures to be used are detailed in a separate protocol (Humphrey, in prep.). For (BACIP) analysis of data derived from trials using freshwater snails, the following information is required: (i) for the egg production response, the mean number of eggs laid per snail pair for the 16 replicate pairs of snails exposed to each treatment; and (ii) for the juvenile survival response, the proportion of surviving juveniles pooled over the 12 replicates from each treatment. Analyses of the test data for (i) and (ii) are conducted as follows:

(i) Egg production

Step 1: Rejection of zero values

Tabulate the total number of eggs laid by each of the 16 replicate pairs of snails over the four-day exposure period. Reject any values for which a zero is recorded. Failure of snails to lay eggs after a four-day exposure to natural waters is an uncommon and abnormal response indicating unhealthy test organisms (see section 3). Otherwise, absence of any egg production may be more frequently observed after snails have been exposed to high concentrations (only) of Ranger mine waters (e.g. >30% RP2 water) (Lewis, 1992). (Thus inclusion of these data would not be relevant to most field monitoring applications.)

Step 2: Pooling of replicates

Before calculating the arithmetic mean number of eggs laid by each pair of snails for the 16 replicate pairs of snails exposed to each water type, it is necessary first of all to establish that the sample means of the treatment duplicates (each comprising 8 replicate snail pairs) are not significantly different from one another. A two-sample Student *t*-test (for equal or unequal sample size) is performed using the replicate data from each of the treatment duplicates. (MINITAB software is convenient for this operation.) Data are analysed at the 1% level. If the analysis is significant ($P<0.01$) it may indicate a simple offending replicate. One replicate (from the 16, excluding zero values) may be deleted to ensure that further data analysis can proceed (- see analysis of 'Juvenile survival' data below for discussion of statistical outliers). After the duplicate data have been shown to be 'compatible' (i.e. no significant difference between duplicate means), the mean number of eggs laid per snail pair (for all replicate data) is calculated.

A statistically significant difference in the sample means of the treatment duplicates could indicate improper experimental procedures used during the test, in particular, interruptions to continuous flow through a duplicate test container. Data from the offending duplicate are discarded from further statistical analysis.

(ii) Juvenile survival

Homogeneity of replicates

Tabulate numbers of both pre-eclosion embryos and post-escape (surviving) juveniles for each of the 12 replicates (= 6 x 2 duplicates) from each water type. The homogeneity of the 12 replicates is checked using the standard chi-square test for the homogeneity of proportions (e.g. Zar, 1984: section 5.8). MINITAB software is convenient for this operation. Data are entered in **two columns** - *'alive'* = post-escape, surviving count, and *'dead'* = [pre-eclosion count] - [post-escape, surviving count] - of ***n* replicate rows**. A worked example of the chi-square test, as applied to fish-embryo survival data (OFR 51), is provided in Brown (1992). Data are analysed at the 1% level.

If the chi-square analysis is significant ($P<0.01$) it may indicate presence of one or two 'offending' replicates. Up to two replicates (from the 12) may be deleted to ensure that further data analysis can proceed. This arbitrary rule (allowing removal of two outliers) is based on practical grounds since the test data are costly to obtain and should not be totally discarded. Brown (1992) advocated removal of one outlier from 3 replicates in the ERISS fish-embryo gudgeon test on the same grounds. Removal of 2 outliers from 12 replicates, therefore, does not appear unreasonable. If there are 3 or more aberrant replicates, the test is terminated and the experimental procedure reviewed.

Using only those replicate data for which homogeneity has been demonstrated (i.e. a non-significant ($P > 0.01$) result in the chi-square analysis), a pooled count of r post-escape, surviving juveniles from n initial (pre-eclosion) embryos is calculated.

(iii) Incompatibility of data between treatment duplicates

Should there be no ready explanation for a significant difference observed between duplicate data from either egg production or juvenile survival responses, all treatment data are rejected. Should such a significant difference be readily explained by inappropriate experimental procedures applied to one of the duplicates (e.g. pump failure), data from the 'unaffected' duplicate may be used in graphical portrayal of trends in a time series of data but should not be used in formal hypothesis testing.

8. References

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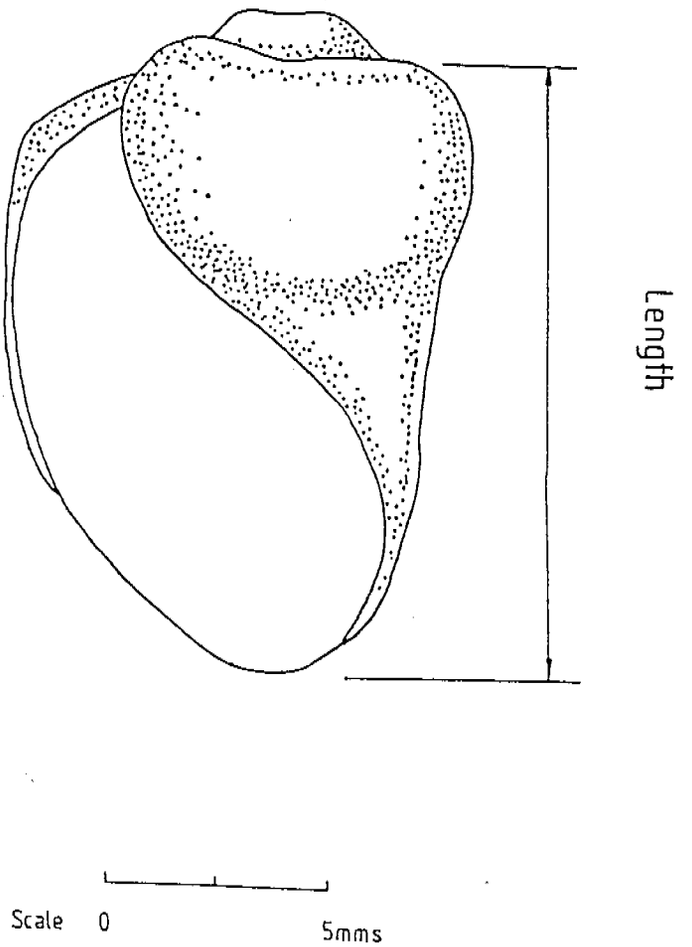

Figure 1. Ventral view of the shell of Amerianna cumingii. (After Jones (1992).)

Appendix 1

Aspects of the biology of the freshwater snail, *Amerianna cumingii*, under culture

Jones (1992) studied aspects of the biology of *Amerianna cumingii* under culture whilst Lewis (1992) provided additional embryological information. Minor investigations upon reproduction have subsequently been pursued at ERISS. Important findings from these collective studies are summarised below.

Reproduction

1. As with pulmonates generally, *A. cumingii* is hermaphroditic, the gonad being referred to as the ovotestes. Both cross- and self-fertilisation may occur. Cross-fertilisation is the preferred mode of reproduction, resulting in genetic diversity in the species. (It was this rationale that led originally to the *pairing* of snails for ERISS toxicological and monitoring studies using snail reproduction as the test response.) Over the Wet season months, October-May, egg production and oviposition are continuous.

2. 'Egg masses' are oviposited most commonly between 0200-0800 hours upon any smooth-surfaced, fixed substrate in the water. An egg mass is a discrete batch of eggs, each egg in its own capsule and each batch of eggs being surrounded by a gelatinous coat. The number of eggs contained in an egg mass may range between 1 and 50, more commonly between 10 and 25.

3. Snails commence egg-laying at a size of 6 mm shell length, corresponding to an age (after escape from the egg mass) of about 5-6 weeks. The rate of oviposition is dependent upon a number of factors, including snail size and age, density of snails in a container, diet, water temperature and other physico-chemical factors.

- Fecundity is positively correlated with size of snails. However, the fecundity-size relationship may be unimodal should the larger snails (>13.0 mm shell length) be close to the maximum age (~ 7 months); then, egg production peaks in snails of size range between 10.0 and 12.9 mm with larger/older snails producing fewer egg masses (= senescence).
- With increasing density of snails (1-4) in an egg-laying chamber, the number of egg masses produced per snail decreases. Jones (1992) found, as a consequence, that similar numbers of eggs were laid over a one-week period in chambers, irrespective of snail density.
- The number of egg masses produced by snails is enhanced when an epiphytic substrate is provided. However, of all diets studied - including two palatable native aquatic macrophytes - only the provision of lettuce has so far resulted in sustained (up to three weeks) production of egg masses.
- Over a range of water temperatures studied in the laboratory (25, 30, 33°C), rate of egg mass production increased with increasing water temperature over a 7-day period. The influence of other physico-chemical factors upon reproduction has not been investigated, though there is some anecdotal evidence to suggest that low dissolved oxygen concentrations (< 4.0 mg/L) and elevated suspended solids (compared to background Wet season creek values) may suppress production of egg masses.

Early development

4. The development of the early life stages of *A. cumingii* is typical of freshwater pulmonates generally, and thus follows that for *Lymnaea palustris*, as described by Morrill (1982). The sequence and main stages of embryonic development of *A. cumingii* at 30°C are:

(i) *Cleavage period* (up to 49-cell stage). Completed within 24 hours.

(ii) *Gastrula period* (placode-shaped embryo the result of invagination of the cell-ball). Occurs between 24-48 hours.

(iii) *Trochophore larva* (the most conspicuous feature of which are larval liver cells forming a cuplike layer around the endodermal lumen; other larval organs and adult primordia appear). Developed by about 48 hours.

(iv) *Veliger larva* (exhibits the first signs of asymmetry; bilobed foot and liver, rudimentary shell). Developed by about the third day.

(v) *"Hippo" stage* (embryo resembles a miniature hippopotamus; shell covers the visceral mass). Develops between the third and fourth days.

(vi) *Adult-like stage until hatching* (further differentiation and growth of the adult organs, culminating in hatching of juvenile snails). Develops between the fifth and seventh days.

(vi) *"Milling" phase of juvenile snails* (i.e. following hatching, milling of juveniles within the egg mass until escape). Occurs between about the seventh and eighth days, after which juvenile snails escape from the egg mass; escape may be protracted and extend into the tenth day.

5. The rate of embryonic development is dependent upon water temperature. At 25°C, development of the veliger and 'hippo' stages may be slower than at 30°C (as described above) such that escape of juveniles commences on about the ninth day. As stated in 4., development up to the commencement of egg-mass escape takes about 8 days at 30°C, and 7 days at 33°C.

Growth and longevity

6. Growth of post-escape, juvenile snails was followed for a 5 week period during the 1990-91 Wet season. From an initial shell length of 700 μm, juvenile snails approximately doubled in size after the first week, thereafter increasing in size by about 1 mm each week.

7. Over an eight-week period during the 1990-91 Wet season, Jones (1992) measured the weekly growth rate of adult snails (size range 4-14 mm shell length) at the laboratory and at a field creekside station. The mean weekly growth rate over all size classes was 0.57 mm at the laboratory and 0.69 mm in the field. The highest weekly growth rate was 1.47 mm in the field, corresponding with the highest mean daily water temperature measured over the eight week period (32.1°C). At both laboratory and field, growth generally declined over the 8 weeks in association with a decline in water temperature.

8. Commonly the maximum age of snails under laboratory culture is about 7 months although occasionally individuals have reached an age of 35 weeks.

Diet

9. Freshwater pulmonate snails may be termed 'grazing herbivores' with a diet consisting of living and decaying plant material. Epiphytes and/or detritus are undoubtedly important food sources for *A. cumingii* in the wild. Laboratory studies provided some support to this claim. Feeding trials also showed that out of about 12 native aquatic macrophytes tested, only two species, *Caldesia oligoeocca* and *Persicaria attanuata* were palatable to snails. As noted above (3.), only the provision of lettuce has so far resulted in sustained growth and reproduction of snails under mass laboratory culture.

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Appendix 2

Culture and handling of *Amerianna cumingii*

Culture

General maintenance of adult snails. As noted in section 3, the present ERISS culture of *Amerianna cumingii* was derived from a sample of animals collected from (unspecified) floodplain habitats of Magela Creek in 1986. Should new stocks be required at any time, snails may readily be obtained from many of the surface waters occurring on the floodplain edge; snails are common, for example, at the floodplain edge adjacent to the Water Resources (PAWA) gauging station 821-0021, located northwest of Jabiluka Billabong (ref. 12°26'S 132° 50'40"E). For establishing a new culture of snails, a sample size of not less than 10 snails collected from the field should be used.

Stocks of snails at the ERISS of the size used for testing are normally cultured and maintained in large fibreglass or plastic tanks of holding capacity 150-700 L. Holding tanks are located oudoors and may be exposed to partial sunlight and rainfall. However, care should be taken to avoid runoff from galvanised rooves or other sources of potential contamination. Tanks should not be exposed for prolonged periods of time to direct sunlight so that water temperature at any time exceeds 35°C. Other than direct stress to snails, such conditions also promote rapid build-up of unwanted phytoplanktonic algae. Cultures do not appear to be favoured by any particular size dimension of the holding tanks (e.g. water depth) other than the observation that larger volume tanks will support greater numbers of snails. It is important at all times that water in the tanks is aerated. New tanks or tanks held in storage for appreciable periods that are to be used for culture should be cleaned prior to use, using successively, detergent and 10% nitric acid followed by several thorough rinses with 'stock waters' (see below).

Stock waters used in the holding tanks are collected from Magela Creek: upstream of the Ranger release pipe and away from the influence of Georgetown Billabong during the period of flow in the creek (Wet - early Dry season), and from Buffalo Billabong, located 13 km downstream of Ranger at other times of the year (Dry season). Compared with other relatively accessible sites in the creek catchment, waters from Buffalo Billabong most closely combine desirable attributes of close resemblance to Wet season water quality in Magela Creek and least influence from human (mining and non-mining related) activities in the catchment.

Adult snails are cultured on a diet consisting of the outer leaves of fresh lettuce. (Limp or wilted leaves should not be used.) Supplies of lettuce can normally be obtained from large grocery stores or supermarkets which have a practice of discarding outer lettuce leaves prior to shelf display. At ERISS, a suitable supply of lettuce is obtained twice-weekly and refrigerated thereafter at 4°C. Prior to introduction to the tanks, lettuce is washed thoroughly in a supply of the stock waters. In this manner, soil and possible surface pesticide residues (if present) are removed. Snails are normally fed daily, although if a supply added to the tanks *ad libitum* remains fresh in the stock waters for a period of time greater than 24 hours (e.g during the Dry season when water temperatures are lowest), then snails can be fed at a less frequent interval. Otherwise, no more lettuce should be added to cultures than can be eaten by snails before it begins to decay. Should limp or decaying lettuce be found at any time in the tanks it should be removed. While snails will find, and feed upon, lettuce floating on the water surface, it is

preferable to anchor the leaves to the bottom of the holding tanks, using small and clean stones as weights, or by enclosing the lettuce in a coarse plastic mesh that sinks to bottom of the tank.

Water in the holding tanks will need to be changed more frequently during the Wet season than during the Dry season. During the Wet season (November-April), water should be changed whenever water clarity declines appreciably, or monthly, whichever occurs first. A decline in water clarity is usually the result of fouling from breakdown of uneaten food left in the tanks. During the Dry season, deterioration in water quality arising from waste food is less of a problem (presumably because of reduced water temperatures at this time). Over this period, water should be changed whenever water clarity declines appreciably, or monthly, whichever occurs first. Further notes on methods to promote good quality of the stock waters are contained below. At each water change, old water is drained from the tanks except for the bottom ~10 cm harbouring snails. Waters are drained at a sufficiently slow rate that snails adhering to the tank walls have time to retreat to the bottom with the falling water level. Should snails become exposed on the tank walls during this procedure, they are gently pushed free with the forefinger - see notes on 'handling' below. Tanks are then promptly refilled. Walls of the tanks are not normally cleaned at each water change unless either contamination is suspected or there is dense growth and build-up of filamentous algae.

For the greater part of the Dry season, there is no effort devoted to intensively culturing snails (i.e. producing new stock). In any case, low water temperatures at this time of year will suppress breeding activity to a large extent (Appendix 1). A number (up to 300) of snails of the range optimal for breeding - derived from the breeding program of the previous Wet season - is maintained over this period (i.e. 'passive' culture) prior to an active breeding program commencing in September. The progeny of this (September onward) breeding program only, is used for testing in Magela Creek during the ensuing Wet season (i.e. the adult Dry season stock is not used for this purpose). Thus, only one or two holding tanks are required to sustain and maintain the breeding stock over the greater part of the Dry season.

Intensive culture of snails. The advantages in using animals for Wet season testing derived from an intense breeding program conducted during the previous late Dry season, are (i) potentially reduced variation in egg production arising from use of a single and discrete cohort of snails, and (ii) greater 'vigour' of the test animals, the oldest snails not having reached maximum age (> 7 months).

In September of each year, additional holding tanks are prepared for the breeding program. It is in these tanks that the new snail stocks are reared and, as described above, containers of any size (>100 L capacity) will suffice for this purpose. Six breeding tanks (3 x 150 L and 3 x 300 L) usually suffice for the ERISS's Wet season needs. Tanks are filled with tap water and leaf litter added so that as this is wetted and sinks, it covers the bottom of the tank to a depth of ~10-15 cm. Whilst a comprehensive appraisal of the type (parent source) of leaf litter that most suits the needs of juvenile snails has not been undertaken, the litter found at the base of commonly-occurring native *Acacia* and *Eucalyptus* shrubs and trees is adequate for this purpose. The vegetation and ground cover from which the litter is collected must be free of contamination, e.g. previous spraying of pesticides, dumping of waste waters or other materials etc.

The water is changed regularly over the ensuing 10 days (tap water) as the leaf litter ferments and plant tannins and saponins are leached into the water. After the fermentation period has

ceased and tank waters remain clear for longer than 48 hours, the water is drained and billabong water introduced. Experience has shown that survival of juvenile snails is enhanced significantly when the snails are reared with an accompanying substrate of leaf litter. Presumably, the litter and organic detritus are important in nutrition, either being eaten directly by snails, and/or in providing the growth medium for important microbial food items.

Large numbers of egg cases are then obtained from the adult snail stock, using in general, the procedures outlined in the protocol above (section 5). Thus, adult snails are placed into clear plastic laying chambers (2-4 snails per chamber) and held in the holding tanks for a sufficient period of time (up to four days) so that ~10 egg cases or more have been oviposited upon the chamber walls. Lettuce is supplied to the chambers (as a food supply), when required. After sufficient egg cases have been laid, adult snails are removed and the chambers placed in the rearing tanks, containing the leaf litter, for incubation of the egg masses. A stocking ratio of about 5 laying chambers per 150 L of rearing tank volume is generally adequate.

Other than constant aeration of the rearing tanks, no other maintenance is required until the largest of the juveniles that have hatched from the egg masses are 2-3 mm in length - a period of about 4 weeks from commencement of egg case incubation. At this point, additions of small amounts of lettuce to the rearing tanks are made; lettuce is allowed to float free on the water surface and no attempt is made to remove decaying material. Indeed, the decline in water quality in the tanks that would otherwise appear from breakdown of uneaten food left in the tanks (see above), is rarely evident in rearing tanks. It is possible that microbial communities flourishing in the leaf litter and detritus rapidly assimilate waste food products. Leaf litter added to the tanks holding adult snails (described above), will similarly result in better water quality than when absent, and therefore will lead to an extension of the period between successive water changes.

Snails >5 mm may be removed from the rearing tanks and placed in new holding tanks. Culturing of these small adult snails to a size suitable for testing can then proceed more intensively using a diet of lettuce. Because of the fragile nature of the shell, young snails ~5-6 mm in length should not be removed from the walls of the culture tanks by direct handling. It is preferable to 'bait' these snails with pieces of lettuce so that the latter, with attached snails, may then be removed and tranferred directly to the holding tanks.

Further subdivision of snails into holding tanks (each containing a different size class) for eventual testing, may be carried out as deemed appropriate.

Unwanted, co-habiting macrofauna. Snails should be cultured in isolation of other organisms. Nevertheless, unless regular and thorough cleaning of tanks is conducted, it is inevitable that a small assemblage of aquatic organisms will colonise tanks occupied by snails. Some of the larger-growing organisms may present a problem for snail culture. Apart from tadpoles, the only other conspicuous macrofauna appearing in tanks are odonate larvae. Whilst these invertebrates are predatory, it is unclear as to whether or not they prey upon juvenile snails. Rather, because of their benthic habit and therefore the need to disturb tank substrate considerably for removal, the control of these animals is deemed unwarranted. In any case, densities of odonates colonising the tanks are relatively low.

The adverse effects of tadpole infestations on warmwater fish pond production at least, are well documented; these include reductions in primary production, competition for space and artificial

feeds, and serving as vectors for fish disease and parasites (Kane *et al.*, 1992). Of these cited adverse effects, the most obvious as it affects ERISS snail stocks, is competition for the food supply - larger tadpoles in particular will ravenously consume lettuce placed in the tanks. An additional, potential problem is the active grazing of large tadpoles upon the tank walls and possible ingestion (even if inadvertent) of juvenile snails with this feeding activity. (There has been no effort made to verify this latter suggestion.)

Colonisation of tanks by tadpoles should be avoided. The most effective technique for control is to cover tanks with a sheet of coarse plastic mesh to prevent frogs entering and spawning at the water surface. It is important that the mesh does not come into contact with the water surface. Otherwise, should either frog spawn or tadpoles be observed in the tanks, they should be removed with appropriate dipnets, taking care in the process not to disturb or injure snails.

Handling

Snails should only be handled and removed from water when necessary. The longest period of time that snails will need to be held out of water is for measurement of shell length. This procedure must be performed promptly so that prolonged exposure to air is avoided. It is preferable that shell measurement is carried out in a humid environment; the procedure must not be routinely carried out under direct sunlight.

As stressed throughout the protocol, whenever handling snails considerable care is required in order that the fragile shell and internal organs are not damaged. The adhesion of snails onto test or holding container walls presents problems when their removal and handling are required. Snails may be particularly difficult to extricate from the corners of containers - a problem accentuated with decreasing volume of holding container. Snails must *never* be picked up from container walls directly by thumb and forefinger; forcing snails from this position of adhesive attachment can easily lead to damage of shell and internal organs. Rather, snails are encouraged to relax and 'retract' from this position by gentle and repeated 'flicking' of, or pressure upon, the base of the foot of the animal with index finger. It is essential that this procedure is not hastily performed. Should handling be required, it is far easier to gather snails from lettuce, a substrate on which attachment is relatively weak. As noted above (culturing of snails), lettuce may be used to bait snails for this purpose.

It is essential that no insect repellants are used by workers at any time when handling snails.

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