

9th Berlin Workshop on Developmental Toxicology

13 – 14 September 2018, Berlin

Developmental toxicity assays with freshwater snails
(*Biomphalaria* sp., Planorbidae)

Francisco J.R. Paumgarten

National School of Public Health, Oswaldo Cruz Foundation,

Rio de Janeiro, RJ, Brazil.

(paum@ensp.fiocruz.br or fran CPAUM@hotmail.com)

- ➔ **Developmental toxicity** tests have been more and more conducted with mollusks (mostly freshwater snails) such as *Physa acuta*, *Lymnea stagnalis*, *L. luteola*, ***Biomphalaria glabrata***, *B. tenagophila*, *B. alexandrina*, *Helix pomatia*, *Cantareus aspersus*, and *Marisa cornuarietis*.
- ➔ Most of these studies were performed to evaluate the impact of molluscicides, plant extracts and environmental chemicals on the reproductive performance of mollusks/snails.
- ➔ Multigeneration **reproduction** and **dominant lethal studies** have also been performed with *Biomphalaria sp* snails.

Multigeneration snail reproduction **study** (*B. tenagophila*: Oliveira-Filho et al 2009_{ab})

Snail dominant lethal assay (*B. glabrata*: Nakano et al 2003)



Albino and wild-type *B.glabrata*

[Martins LS, 2010]

Snail germ cell mutation test: *exposed wild-type snails are crossed with unexposed albino snails and heterozygous embryos are evaluated for malformations*

In 2016, OECD issued an “Effect on Biotic system” (Section 2) guideline for a mollusk reproductive toxicity test with *Lymnea stagnalis*. The endpoint evaluated by this assay is the general reproductive performance of the snail. The test **guideline 243** is intended to assess the potential impact of chemicals on ecosystems; **not to screen chemicals of developmental toxicity.**

In this presentation we will show some preliminary data on the feasibility of using a snail (*B.glabrata*) test protocol to screen chemicals of potential developmental toxicity.

Biomphalaria glabrata

B. glabrata is a freshwater snail native to Brazil and one of the snails that are intermediate hosts for *Schistosoma mansoni*, a parasitic worm (trematode) that causes liver and intestinal schistosomiasis, a disease endemic in Brazil, the Caribbean, Middle East and Africa.

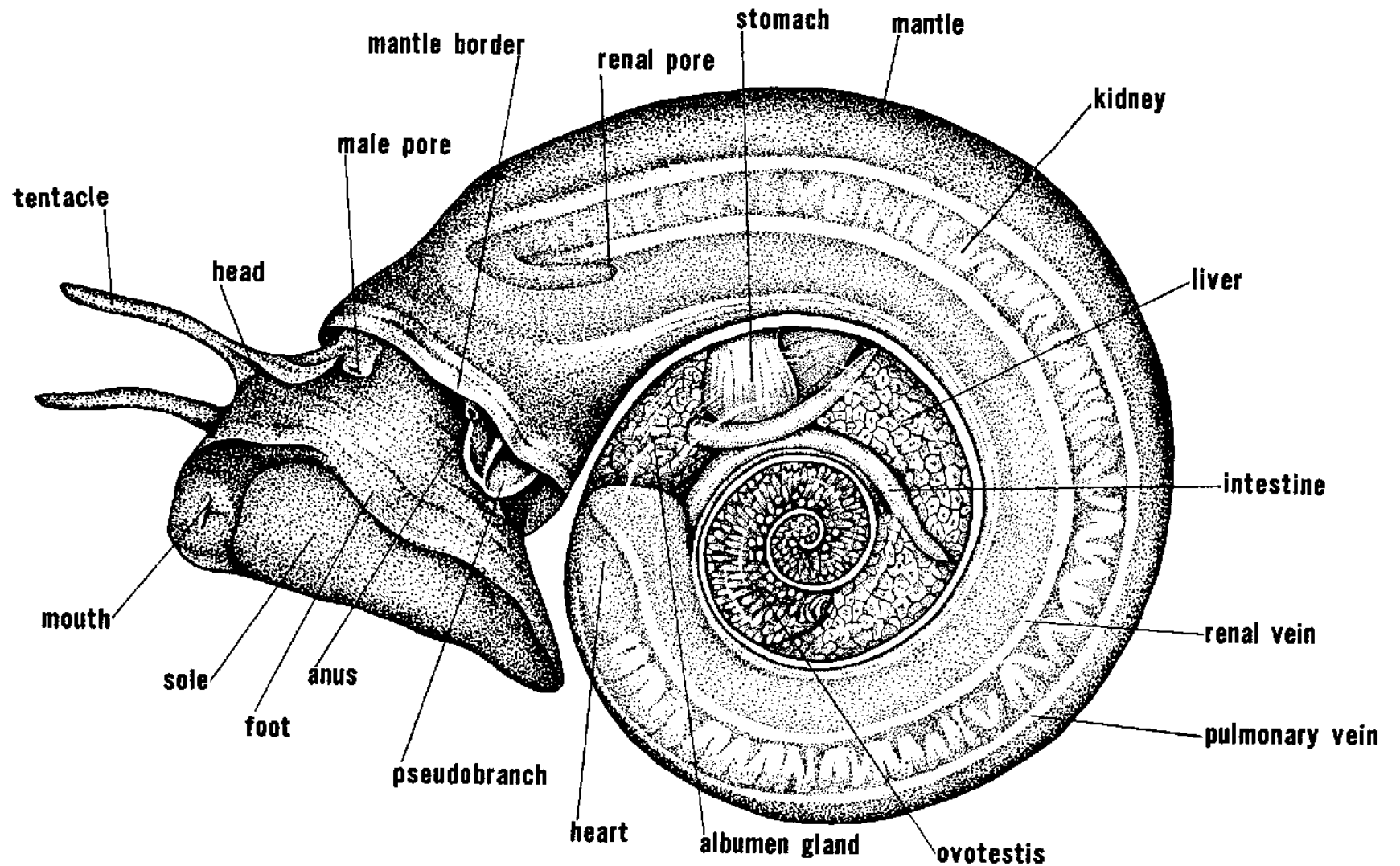
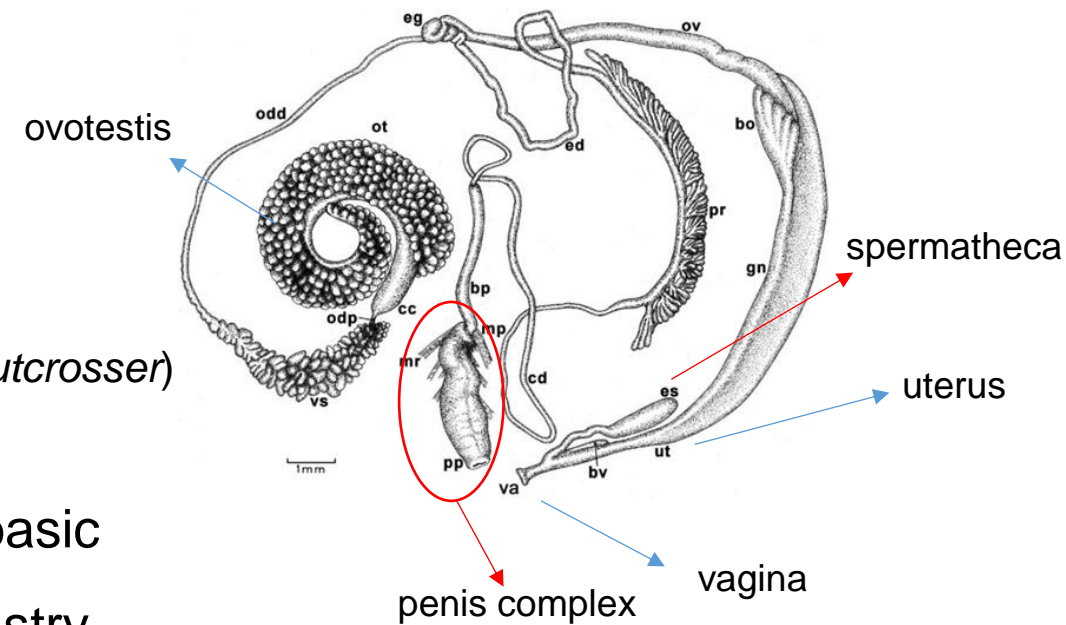


Fig. 8. A *Biomphalaria* with the shell removed and seen from the left, enlarged (after Demian)

Simultaneous hermaphrodite

self-fertilization
cross-fertilization (*preferential outcrosser*)

Since it is a snail of medical interest, there are many basic research studies on ***B. glabrata*** physiology, biochemistry, immunology, behavior, and genetics.



Reproductive organs of ***B. glabrata***

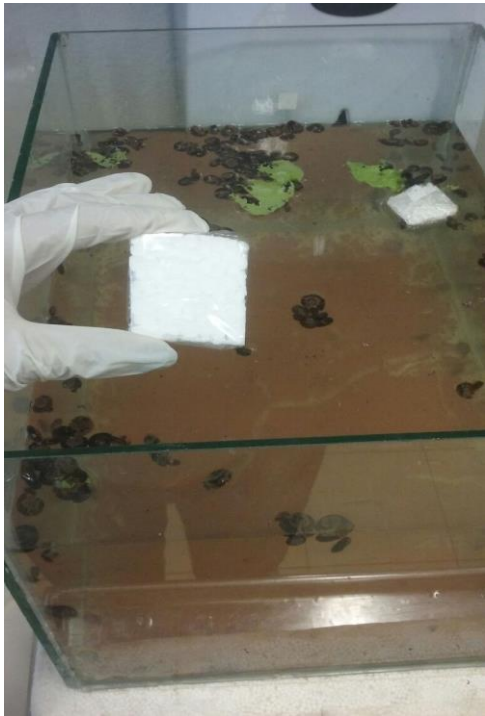
[Paraense 1975]

***B. glabrata* genome was sequenced and analyzed** (Adema *et al* 2017).

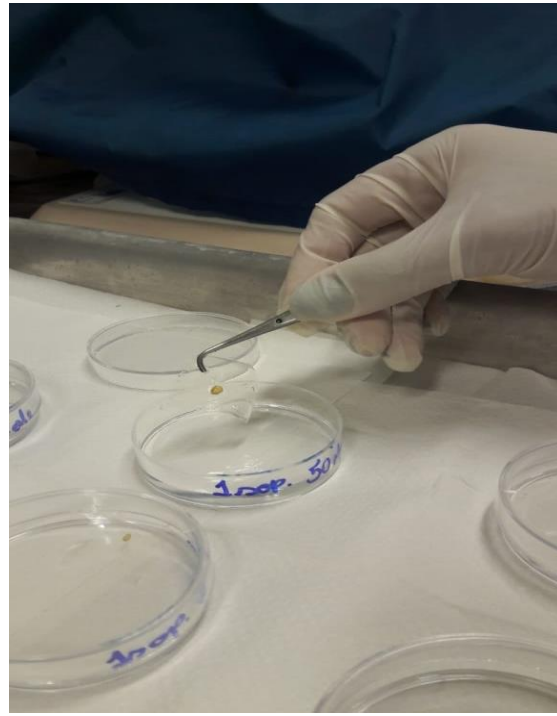
Its length is estimated as about 916 Mbp and comprises 18 chromosomes. It includes xenobiotic biotransformation enzyme genes, such as cytochrome P450 enzymes (99 genes for CYP superfamily), glutathione S-transferases (GST), and drug transporters, notably multi-drug resistance protein (efflux transporter) and solute linked carrier (influx transporter) (Zhang et al 2015).

The Snail DevTox Assay

Snail DevTox Assay



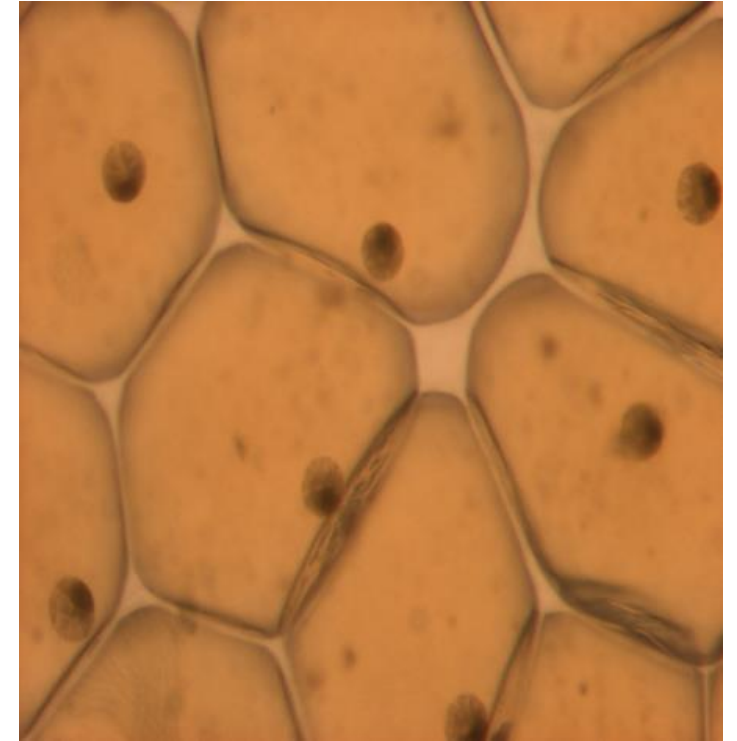
Egg masses (EM) laid on floating pieces of styrofoam wrapped with cellophane sheet.



EMs are placed into plastic Petri dishes with the assay softwater.

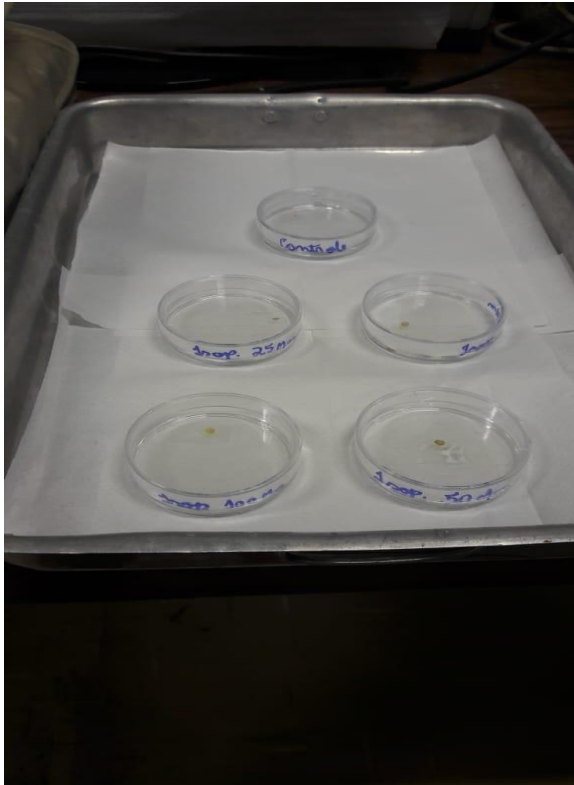


EMs are then examined under a stereomicroscope to score the embryo development stage.



B. glabrata blastula stage (10-23h)
Only EMs with embryos in the blastula stage are further tested.

Snail DevTox Assay



pH 7.0 ± 0.2 synthetic softwater
(40-48 mg/L as CaCO_3)

EMs in the blastula stage are immersed in the assay softwater solution and transferred to a climatic photoperiod controlled chamber.



Climatic photoperiod chamber:
 $25 \pm 1^\circ \text{C}$; 12-h photoperiod;

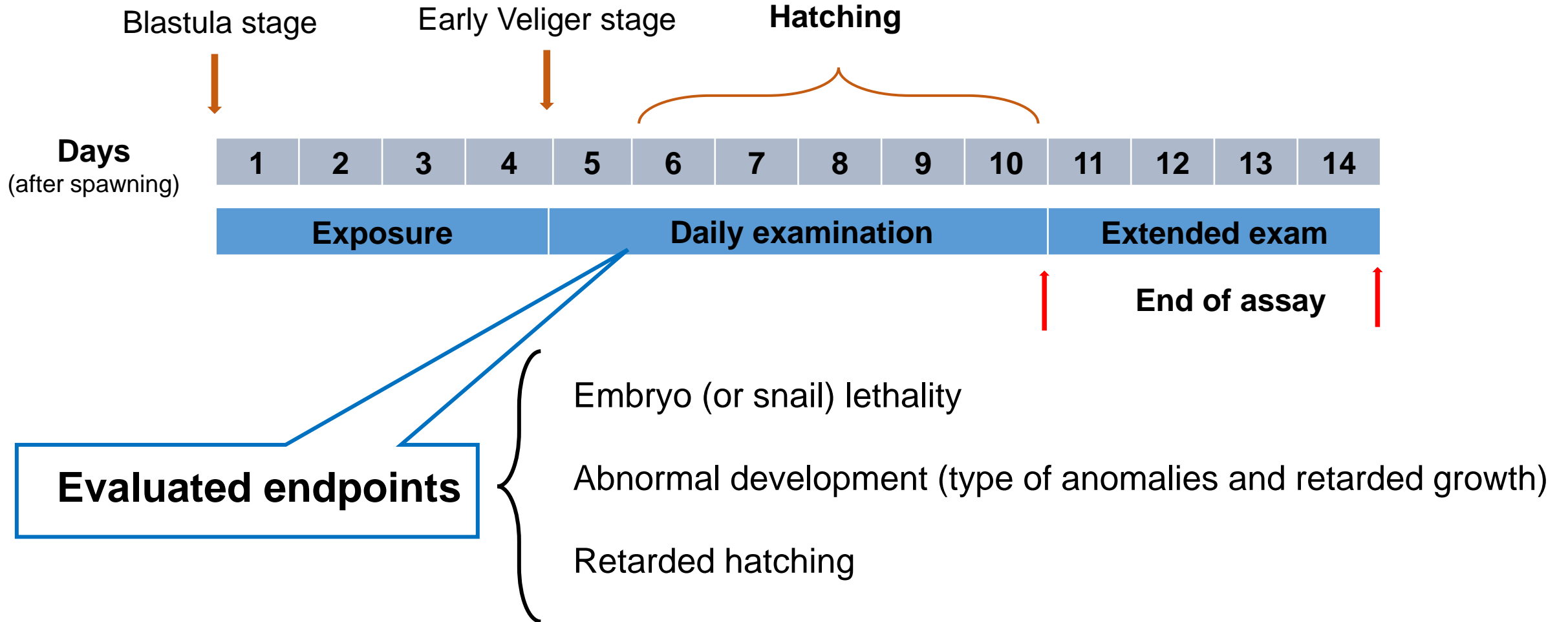
EMs are exposed to test chemicals dissolved in the assay water.

Exposure: semi-static renewal system (e.g. renewal every 24-h) for 96-h.

After 96 h, test-substance solutions are replaced (every 24 h) with assay softwater solution.

Whenever possible, Petri dishes with assay water only (untreated) and those with tested concentrations of a chemical are concomitantly evaluated.

Snail DevTox Assay



Snail DevTox Assay

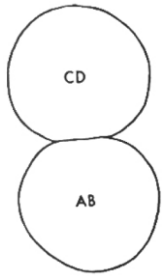
TABLE 1. Main stages of the embryonic development of *B. glabrata* at 25°C.

Embryonic stage	Time interval between observations	Number of stage in figures
Beginning of the 1st cleavage	0	1B
2nd cleavage	80 min.	10
3rd cleavage	160 min.	16
4th cleavage	230 min.	20
blastula	15 hrs.	21
gastrula	26 hrs.	23
early trochophore	43 hrs.	25
late trochophore	66 hrs.	27
early veliger	96 hrs.	28
late veliger	120 hrs.	29
hippo stage	144 hrs.	30

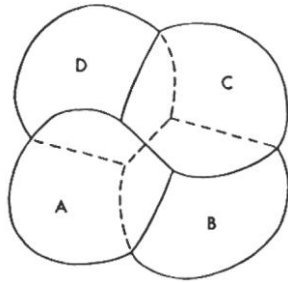
Exposure Period { blastula, gastrula, early trochophore, late trochophore, early veliger, late veliger }

[Kawano et al, 1995]

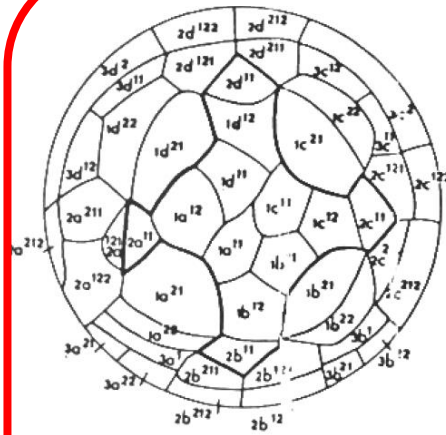
Snail DevTox Assay: *B.glabrata* embryo stages



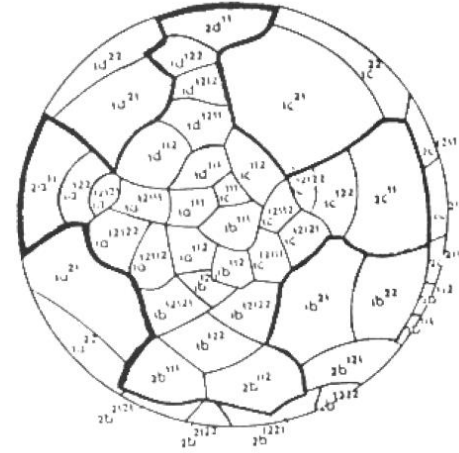
0 h: 1st cleavage



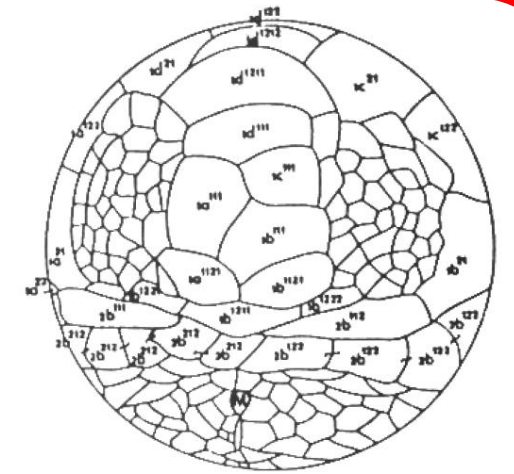
80 min:
2nd cleavage.



15 h: Blastula
(64 blastomeres)

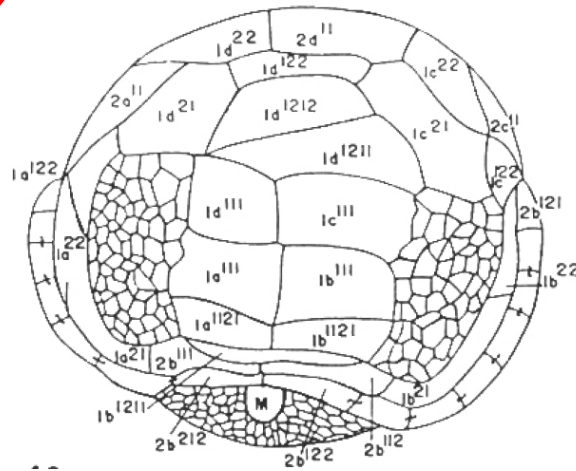


26 h: Gastrula

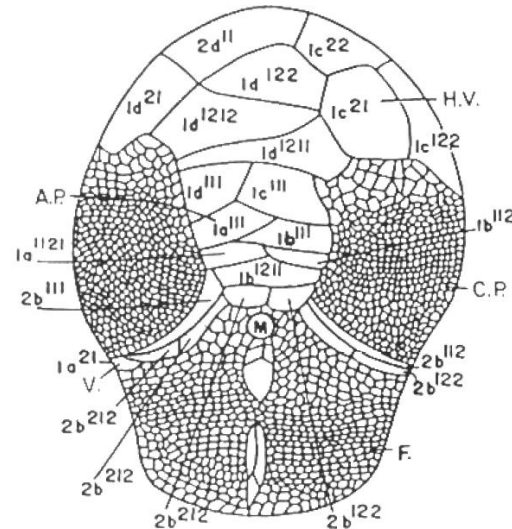


34 h: Early trochophore

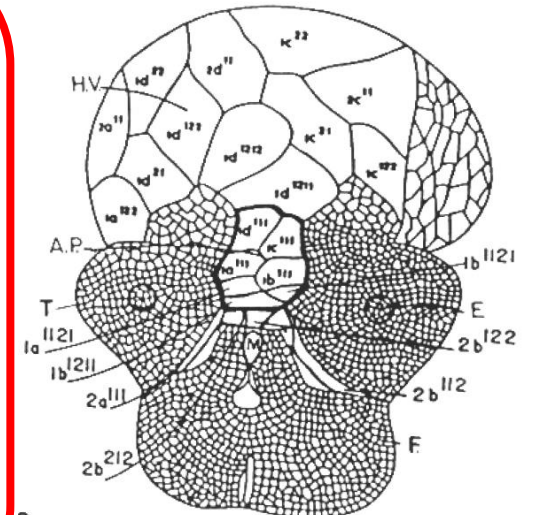
Exposed embryonic
development stages



40 h: Trochophore



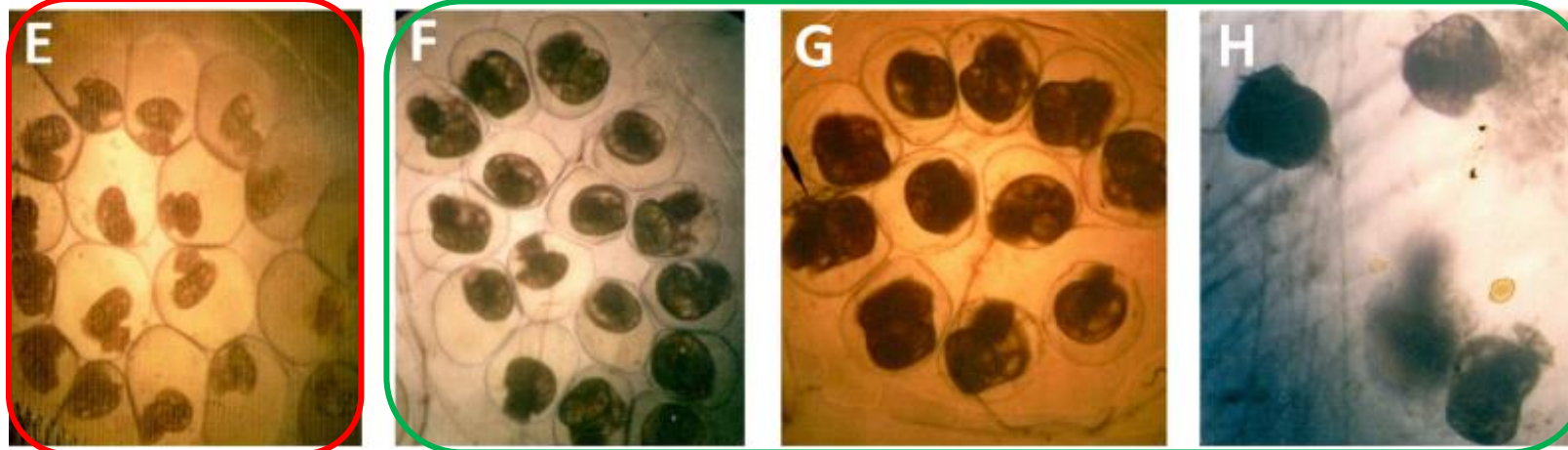
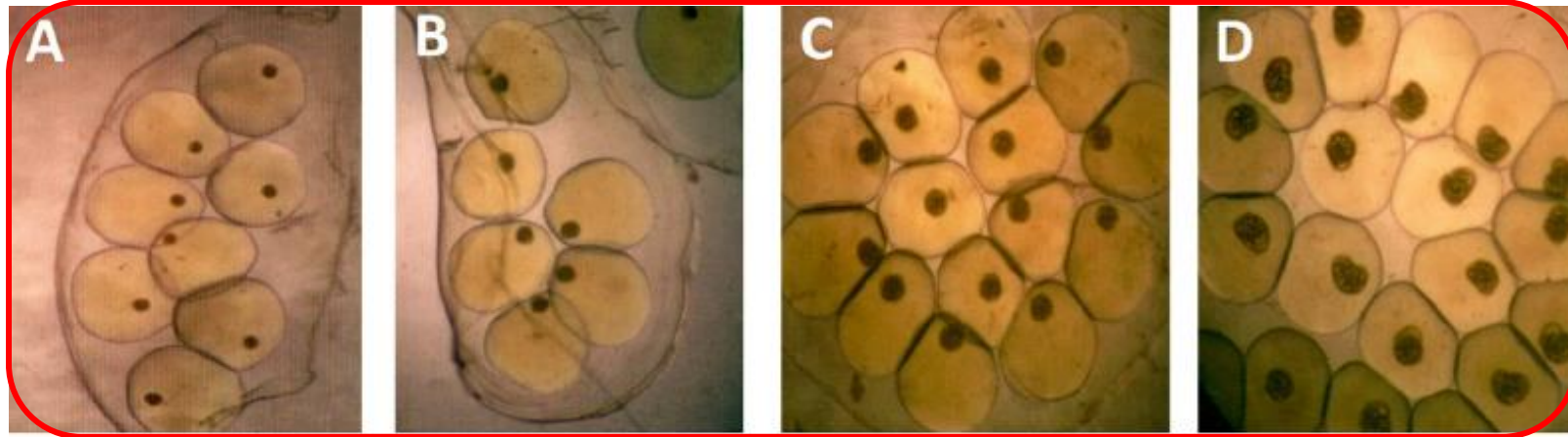
96 h: Early Veliger



120 h: Veliger

Stages of embryonic development: *B.glabrata* snail

Exposure



Follow up

A: Blastula; B: Gastrula; C: Trochophore (early); D-trochophore; E- Veliger (early);

F: Veliger; G; Hipo stage; D- Newly hatched snail

Veliger means veil, sail (from Latin “*velum*”) bearer.

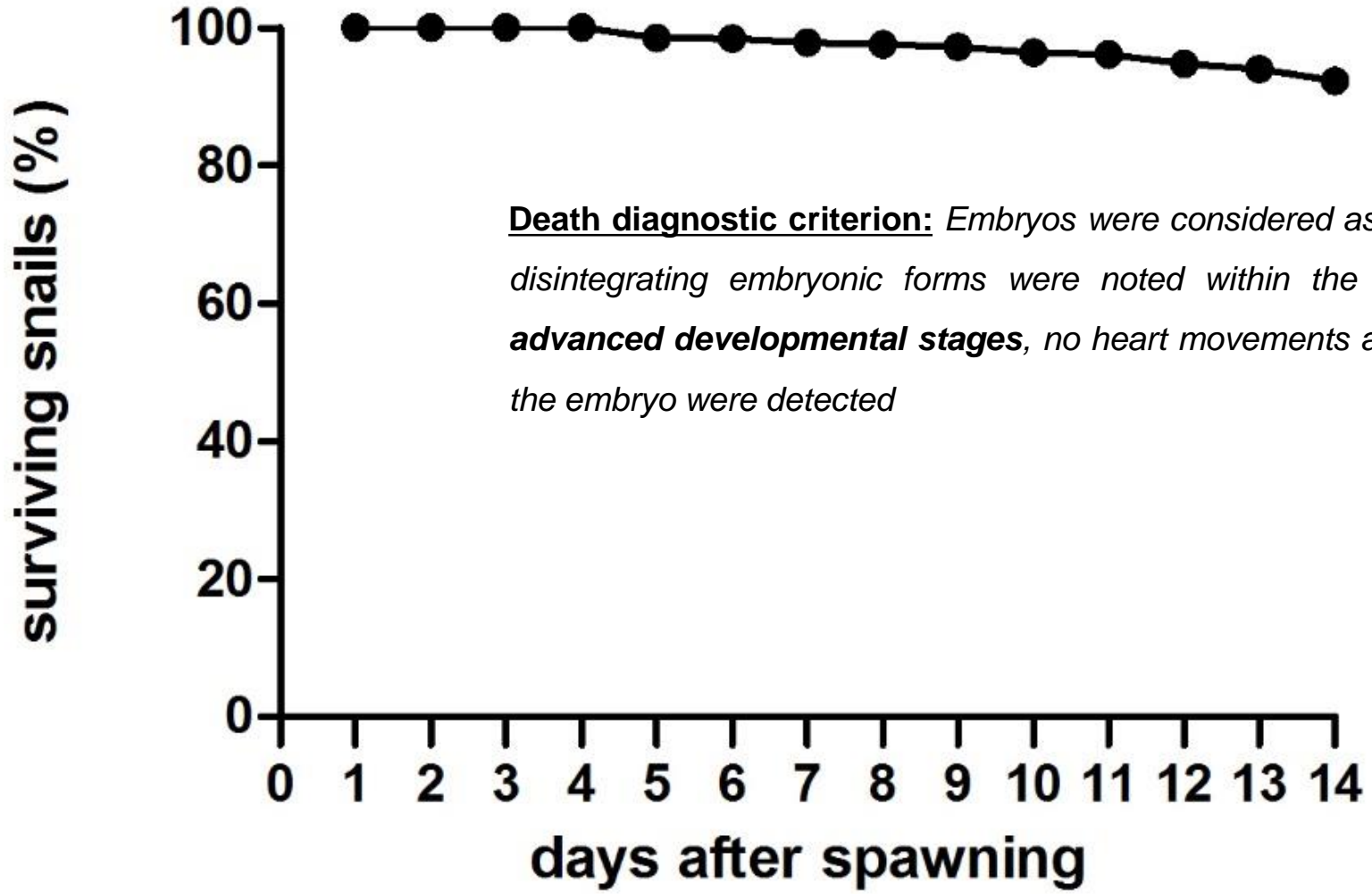
The historical control data

B. glabrata DevTox assay

B. glabrata historical control data

Embryos (eggs) N= 1038
Egg masses N= 69

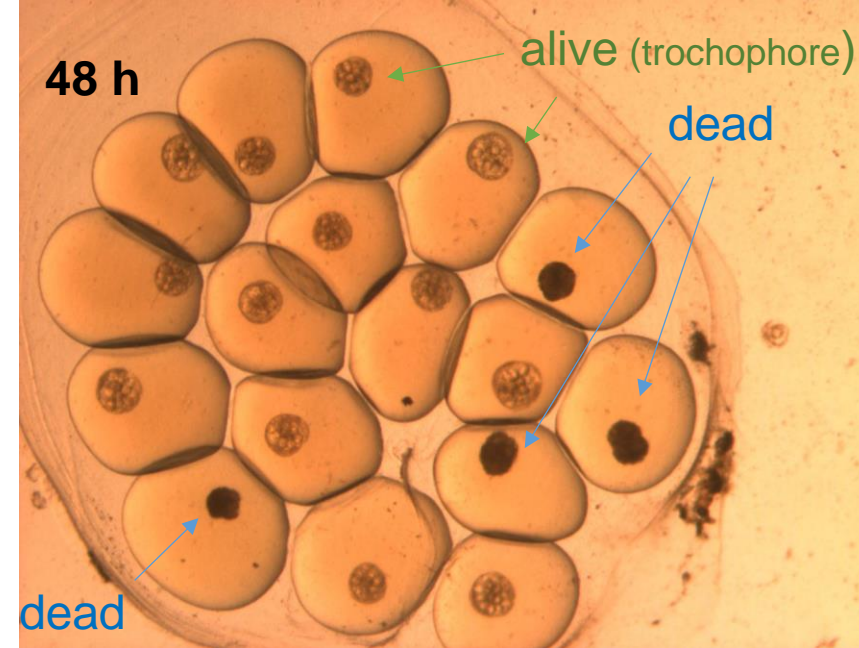
overall survival



Death diagnostic criterion: Embryos were considered as dead whenever disintegrating embryonic forms were noted within the egg and or, **at advanced developmental stages**, no heart movements and no motility of the embryo were detected

92.5%

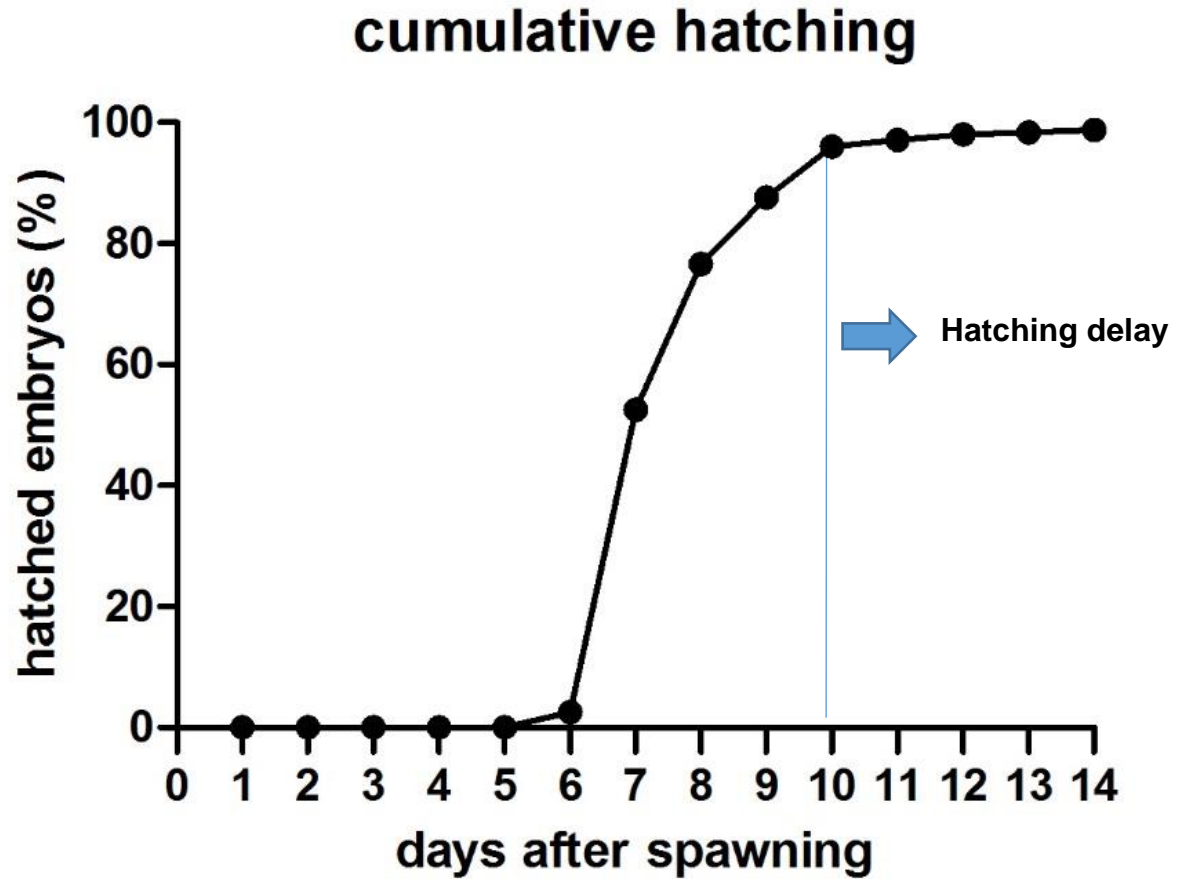
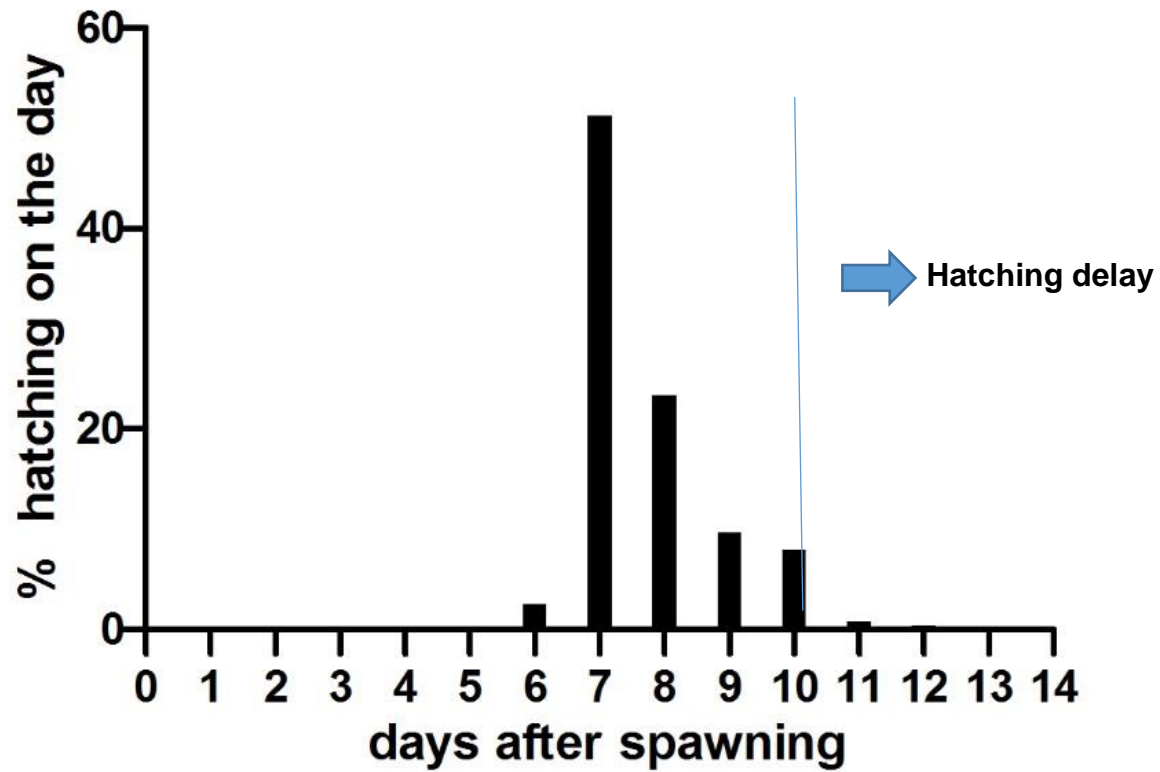
- 91.4% hatched
- 85.8% hatched with normal shape
- 5.6% hatched and malformed
- 0.4% unhatched and normal
- 0.7% unhatched and malformed



Egg mass exposed to Sodium azide 769 µM

B.glabrata historical control data

Embryos (eggs) N= 1038
Egg masses N= 69



B.glabrata historical control data

Externally-visible abnormalities

Embryos/snails with externally-visible morphological anomalies were classified as a malformed embryo/snail.

The 4 main categories of malformations are as follows*:

1. **Hydropic malformation**, embryo partly or totally swollen to a considerable degree.
2. **Shell misshapen**, shell with abnormal shape.
3. **Cephalic malformation**, embryo or snail with any anomalies in the cephalic region. Including: eye anomalies (monophthalmia, anophthalmia, eye re-duplications), tentacle anomalies (atrophic, shorter, asymmetric), mufla misshapen, and others;
4. **Nonspecific malformation**, in this group were included all clearly dysmorphic embryos with anomalies which are not classifiable as hydropic, shell or head malformation.

*Adapted from: Geilenkirchen, 1966. *J. Embryol. Exp. Morphol.* 16, 321–337 and Oliveira-Filho et al 2010.

Spontaneous occurrence of externally-visible abnormalities

Before hatching [u]

Growth retarded [Ru]

Shell misshapen [Su]

Growth retarded + Shell misshapen + Cephalic region anomaly

Shell misshapen+ Cephalic region anomaly [SCu] [RSCu]

Foot misshapen [Fu]

Hydropic malformation [Hu]

Nonspecific malformation [Nu]

After hatching

Growth retarded + Shell misshapen + Cephalic region anomaly [RSC]

Shell misshapen [S]

Shell misshapen + Cephalic region anomaly [SC]

Foot misshapen [F]

Hydropic malformation [H]

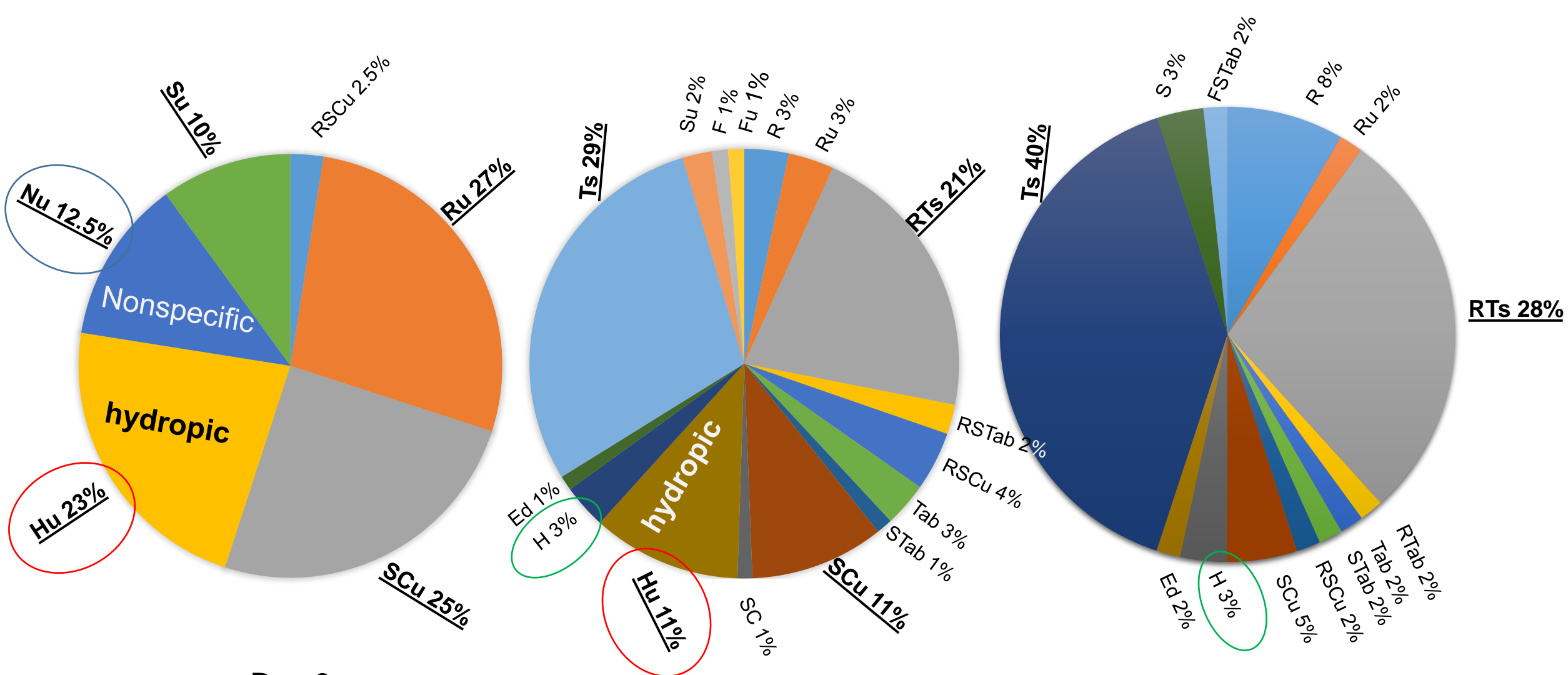
Eye duplication (left) [Ed]

Tentacle atrophy (bilat.) [Tab]

Tentacle atrophy (bilat.) + Shell misshapen [STab]

Tentacle atrophy (bilat.) + Shell misshapen + Foot misshapen [FSTab]

Tentacle shorter (bilat.) [Tsb]



***B.glabrata* historical control data**

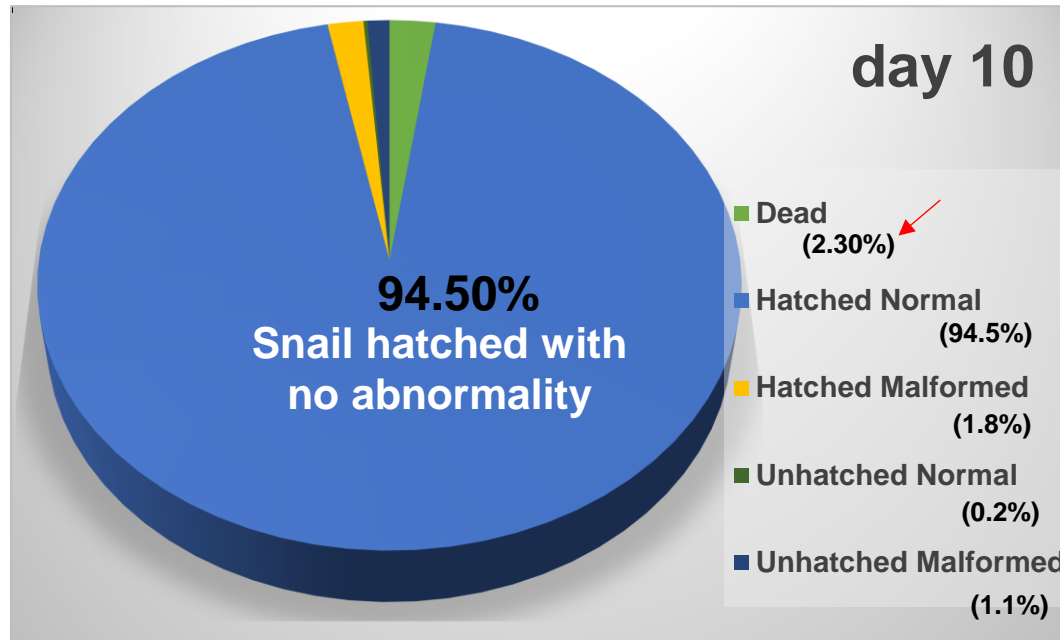
Embryos N= 1038
Egg masses N=69

***B. glabrata* historical control data**

25±1°C synthetic softwater (40-48 mg/L as CaCO₃)

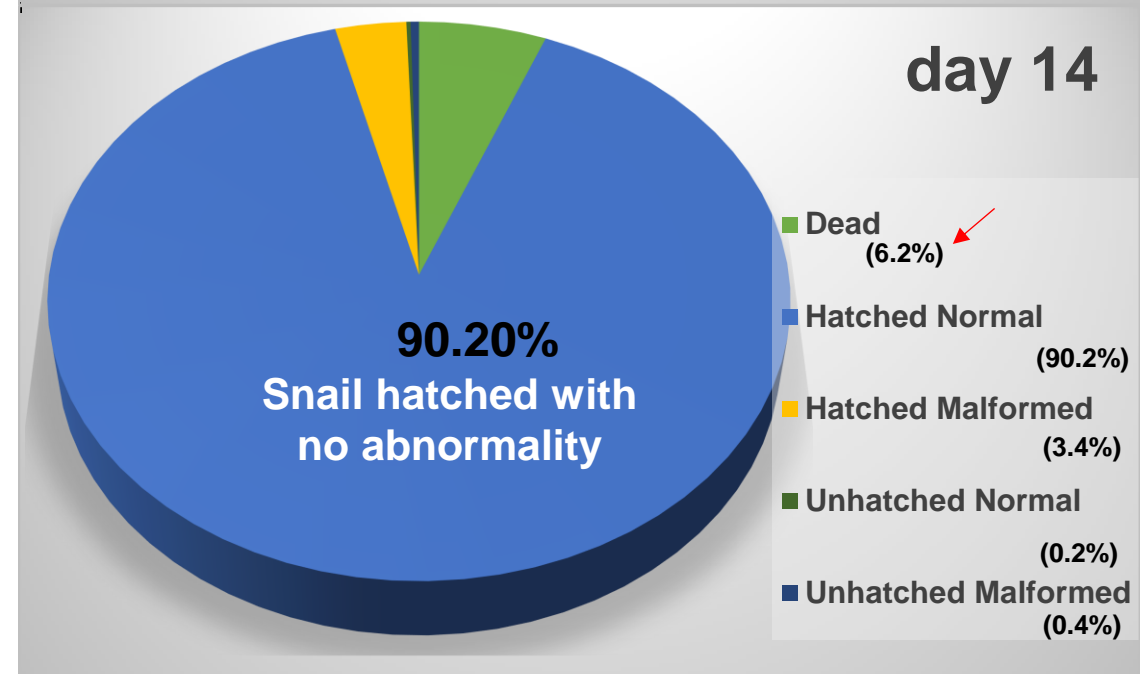
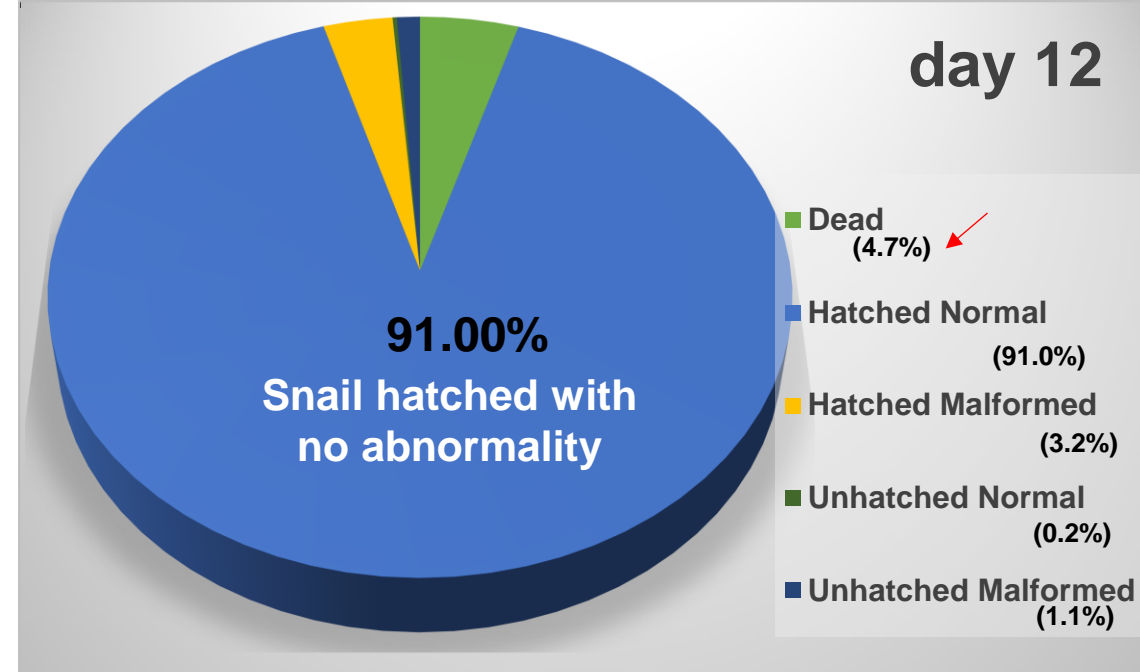
pH 7.0±0.2; 12-h photoperiod

climatic photoperiod chamber



Embryos (eggs): N= 4075 (100%)

Egg masses: N= 239



Embryos (eggs): N= 1748 (100%)

Egg masses: N= 107

B. glabrata historical control data

Embryos (eggs) N= 1038
Egg masses N= 69

Day 6

Egg masses (EM) with malformed embryos

N= 21 (30.4%)

Malformed embryos (M)

N= 40 (3.8%)

% Malformed per EM
median (range)

EM with M
10% (3.3-27.2%)

EM (all)
0% (0-27.2%)

Day 10

Egg masses (EM) with malformed snails

N= 31 (44.9%)

Malformed snails (M)

N= 90 (8.7%)

% Malformed per EM
median (range)

EM with M
16.6% (2.2-60%)

EM (all)
0% (0-60%)

Day 14

Egg masses (EM) with malformed snails

N= 27 (39.1%)

Malformed snails (M)

N= 60 (5.8%)

% Malformed per EM
median (range)

EM with M
11% (2.2-60%)

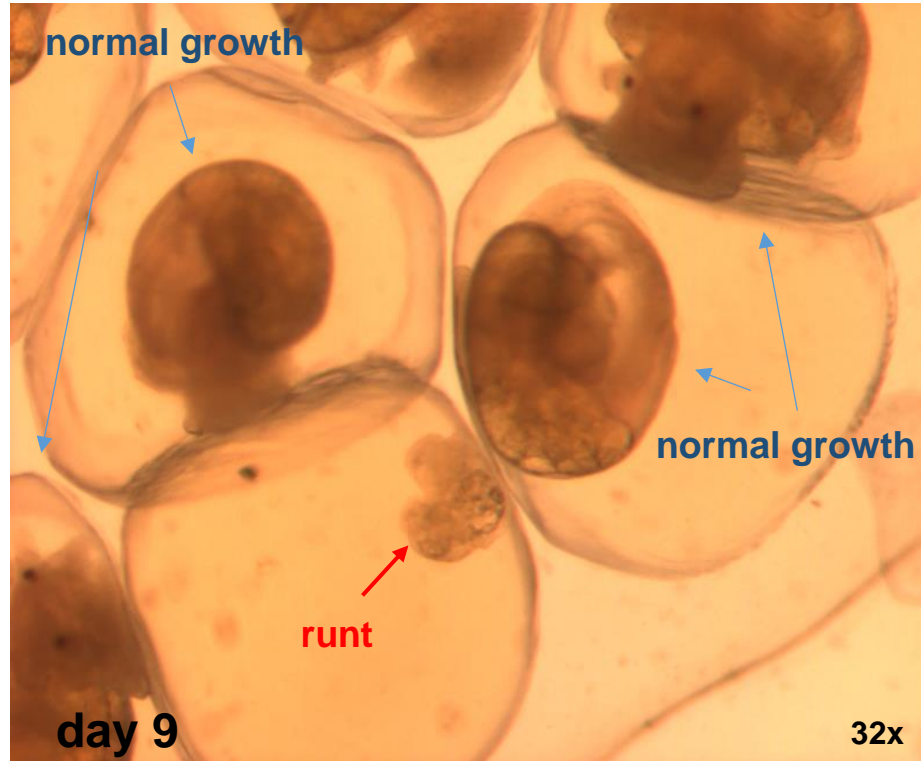
EM (all)
0% (0-60%)

Evaluation taking the egg mass (treatment unit) as the unit of analysis.

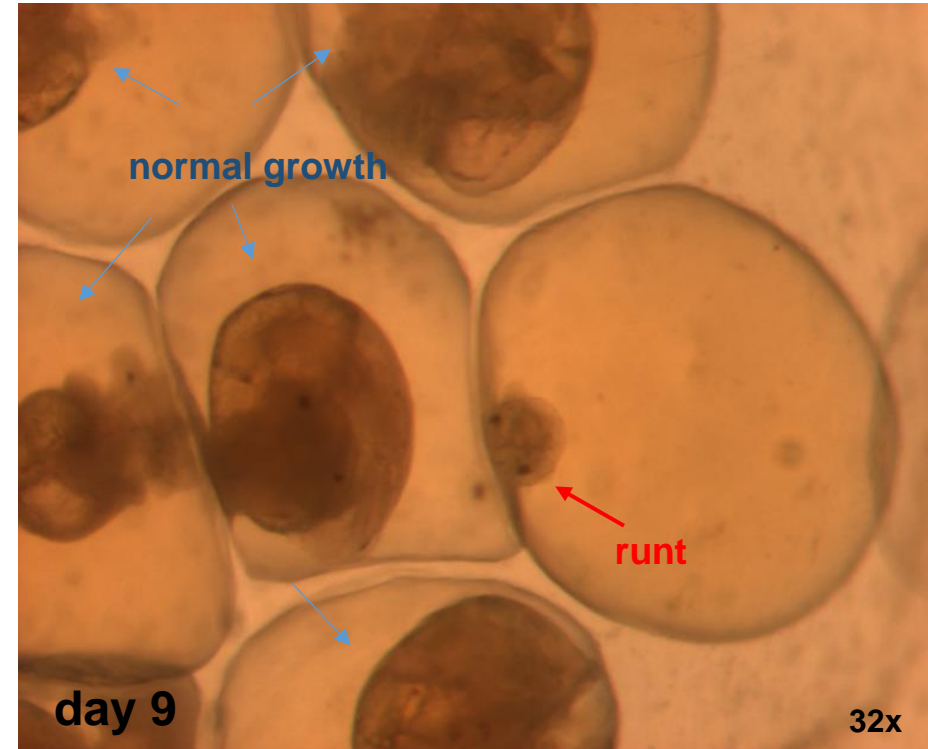
Among EMs with at least 01 malformed embryo, the median % of abnormal embryos is ca 10%

>50% EMs have no abnormal embryo

Growth retardation



Egg mass exposed to Glycerin 100 mM

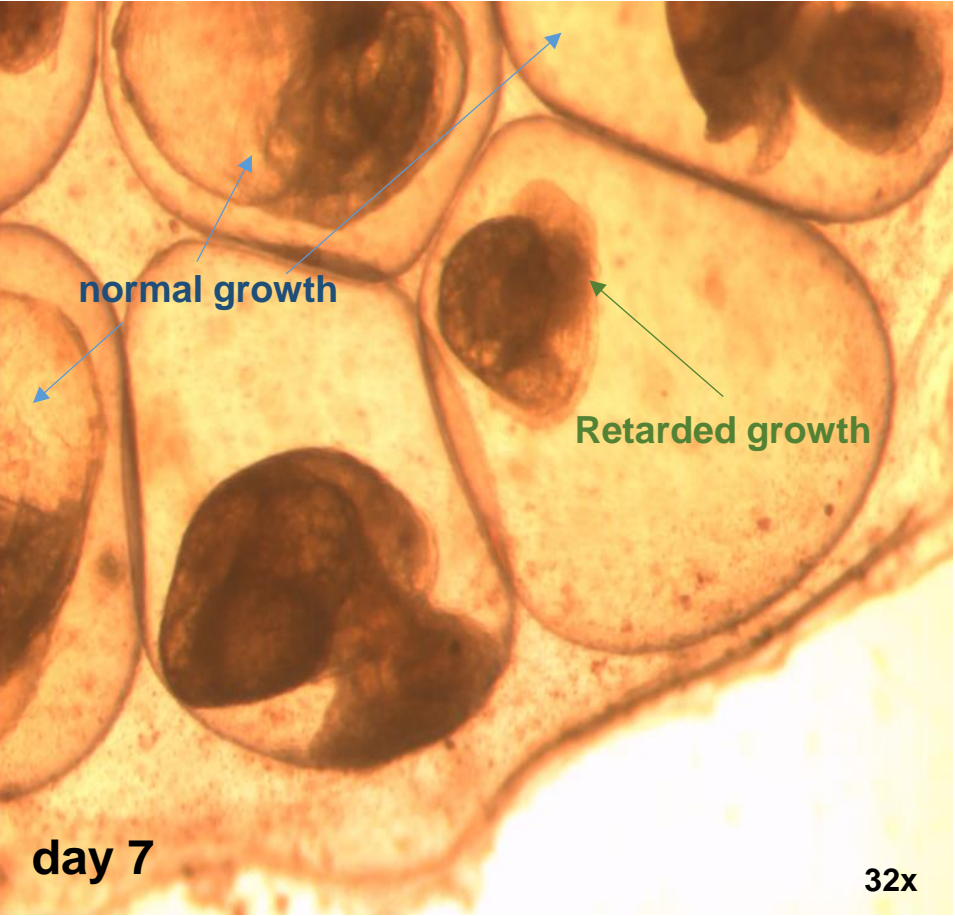


Egg mass exposed to Glycerin 100 mM

Runts: very small embryos compared to others of the same egg mass. They are alive and normally shaped. Runts do not hatch and die within the egg.

Growth retardation

embryos



Untreated egg mass

newly hatched snails



Untreated egg mass

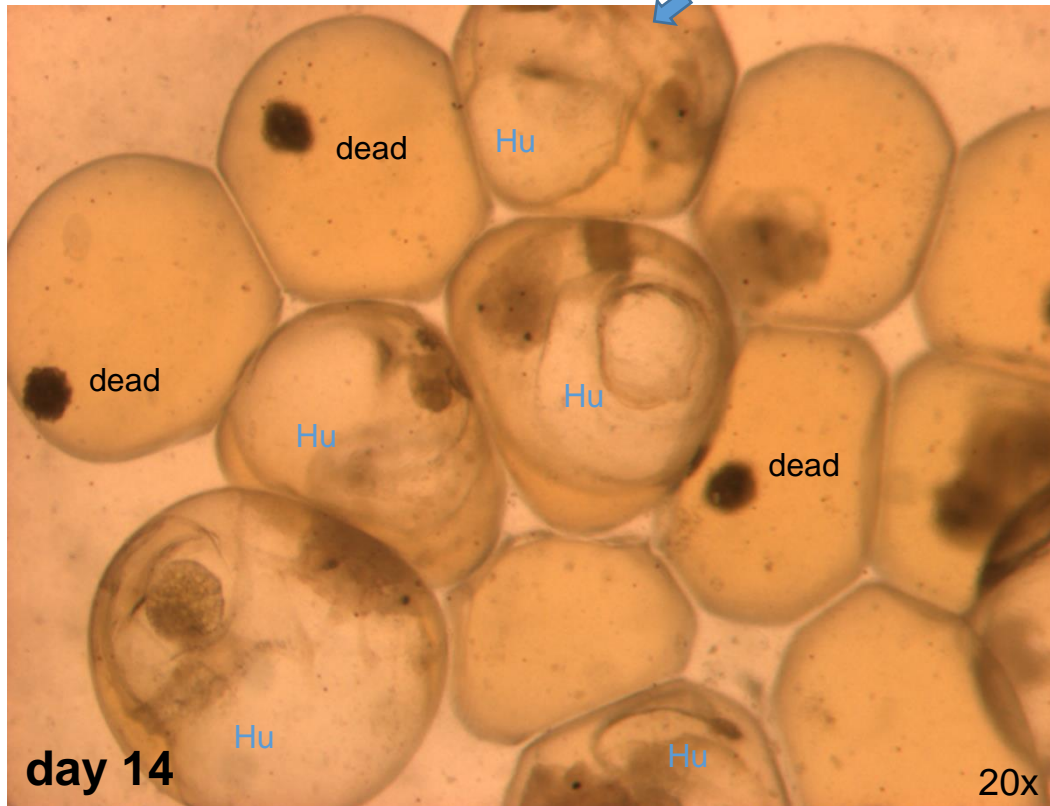
Retarded growth embryos / snails are larger than runts yet smaller than the other embryos / snails of the same egg mass. They are alive and may or may not be malformed. Retarded growth embryo generally hatch.

B.glabrata snail DevTox assay

Externally-visible abnormalities: Hydropic malformation (H)

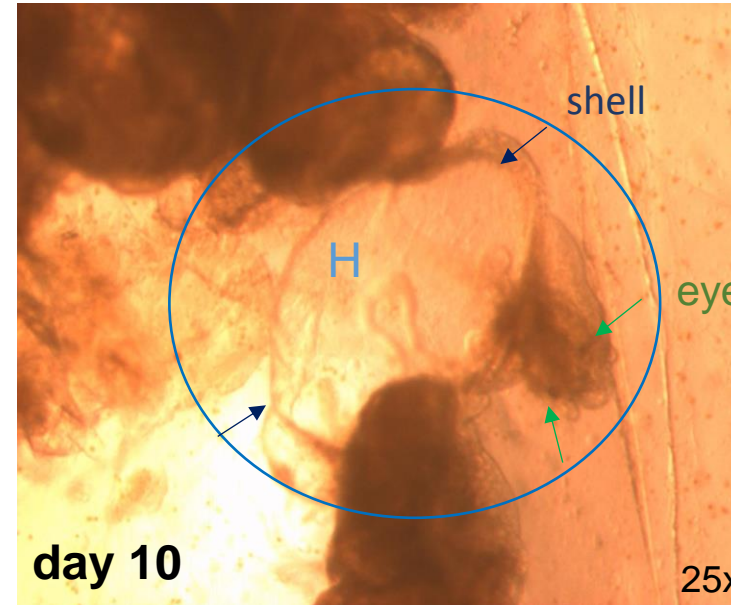
Before hatching

Hydropic (Hu)



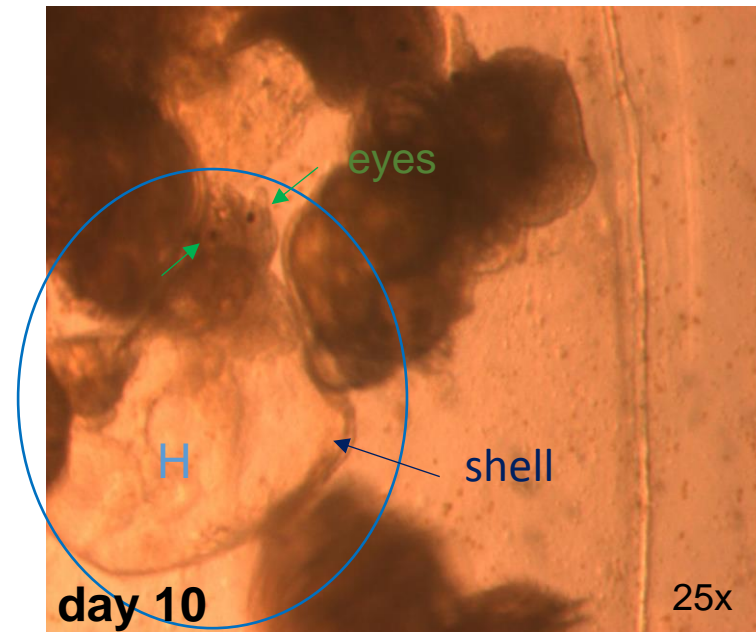
Hydropic embryos within the eggs

Egg masses exposed to methanol 1000 mM



After hatching

Hydropic (H)



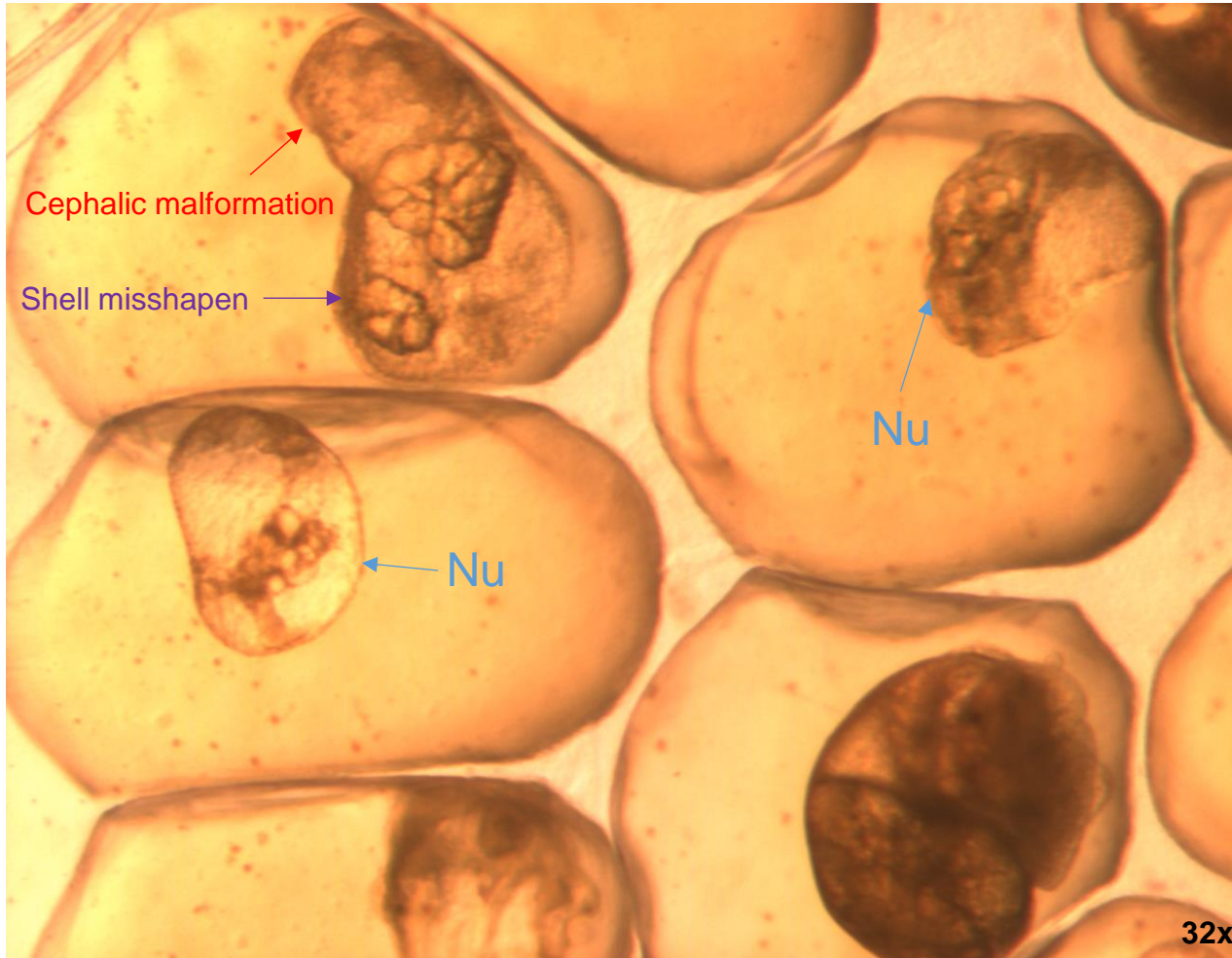
Hydropic snails

EM exposed to DSS

Hydropic malformed embryos seldom hatch. If these snails successfully hatch, they die soon after hatching.

B.glabrata snail DevTox assay

Externally-visible abnormalities: Nonspecific malformation (N)



Nonspecifically malformed embryos die within the egg (Nu). These embryos never hatch.

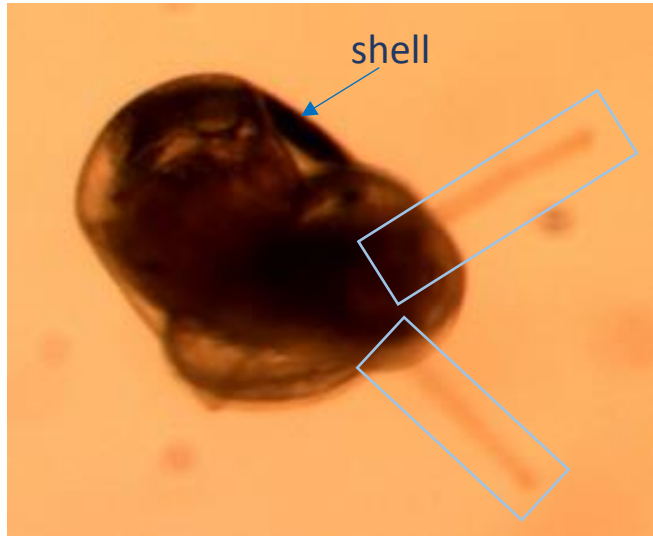
Nu: “strikingly dysmorphic developed embryos with anomalies which are not classifiable as hydropic, shell or head malformation”.

Nonspecifically-malformed embryos within the eggs

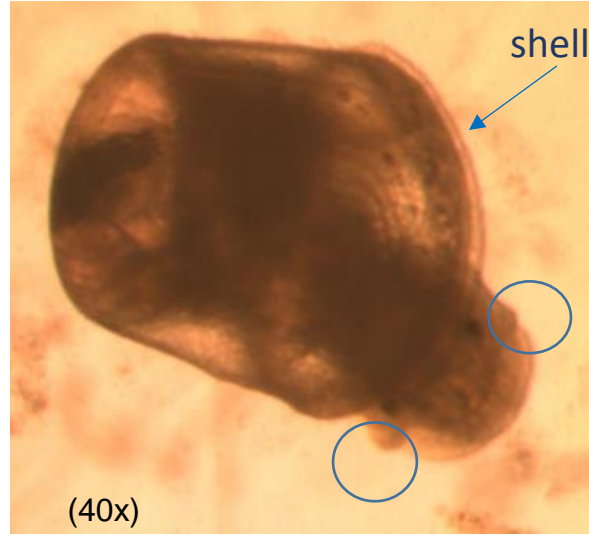
Egg mass exposed to sodium azide

B.glabrata snail DevTox assay

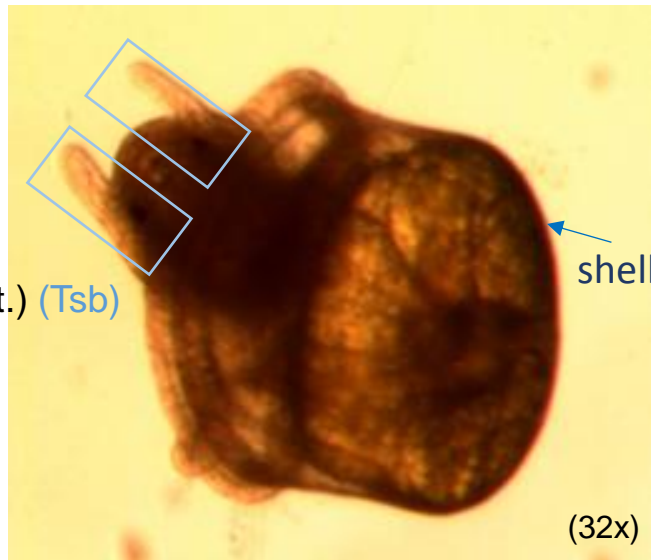
Externally-visible abnormalities: Tentacle malformations (T)



Tentacles with normal shape and size
Untreated control

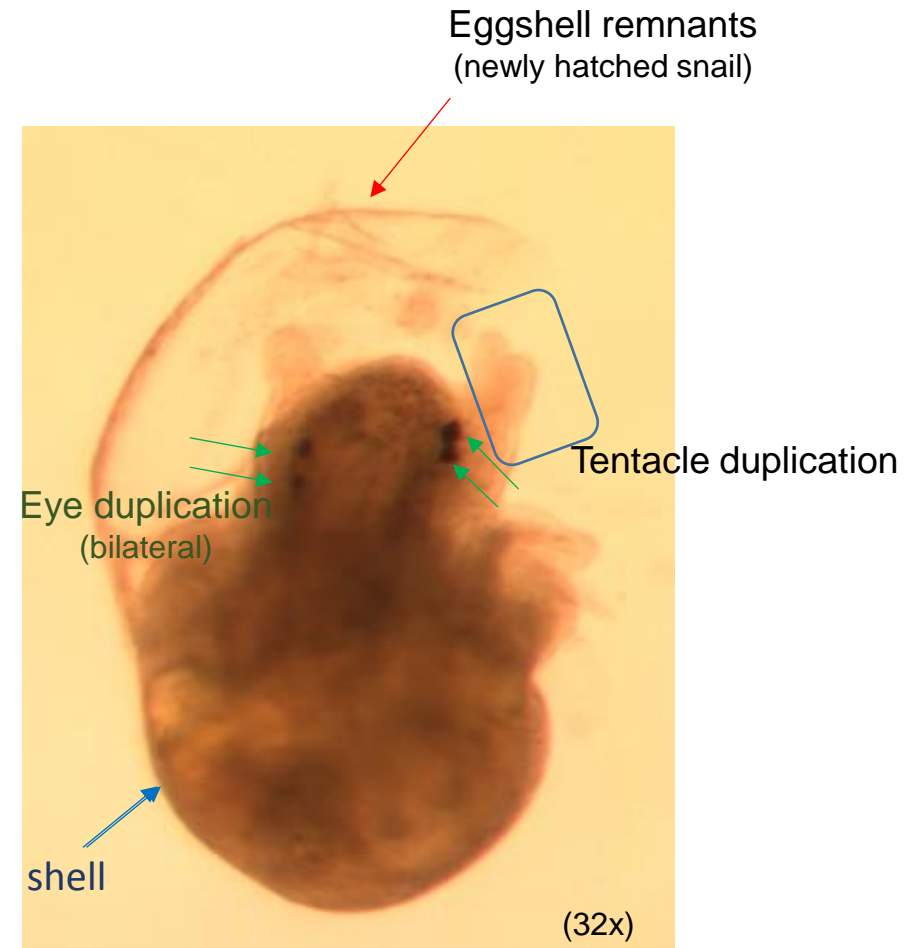


(40x)
Tentacles atrophy (bilat.) (Tab)
Treated with Isopropyl alcohol 75 mM



Tentacles shorter (bilat.) (Tsb)

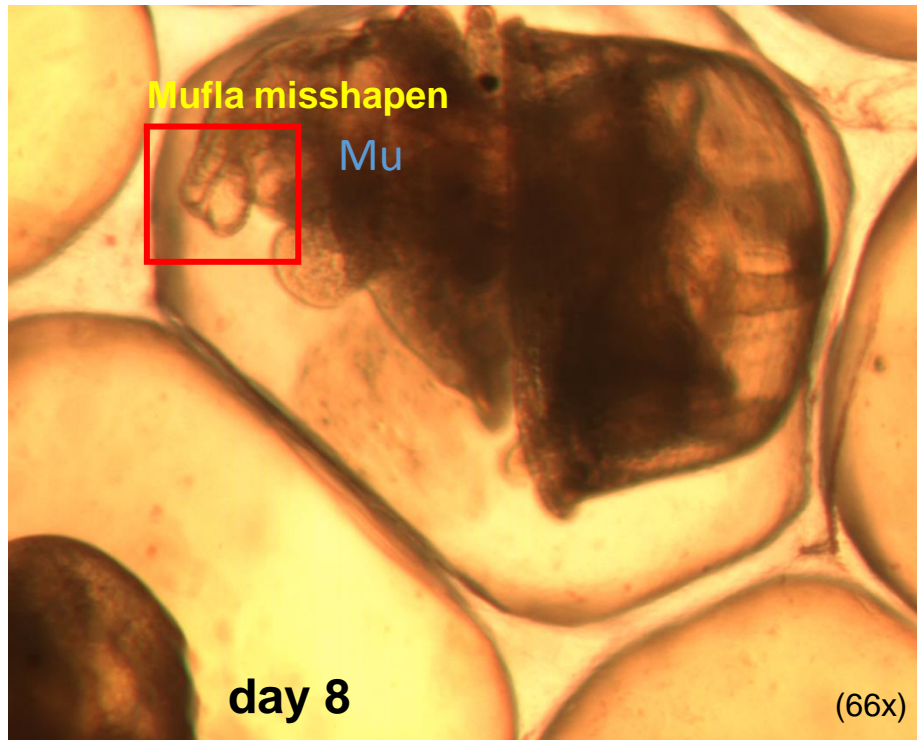
(32x)
Treated with Isopropyl alcohol 50 mM



Eggshell remnants
(newly hatched snail)
Eye duplication
(bilateral)
Tentacle duplication
(32x)
Treated with methanol 500 mM

B.glabrata snail DevTox assay

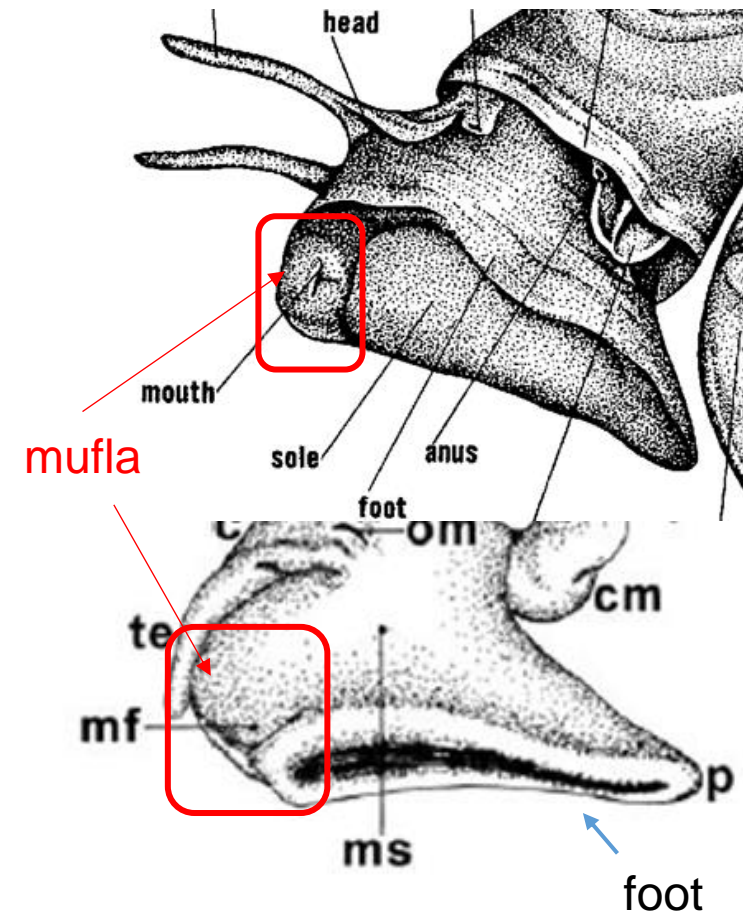
Externally-visible abnormalities: Mufla malformation (M)



Treated with methanol 100 mM (unhatched)



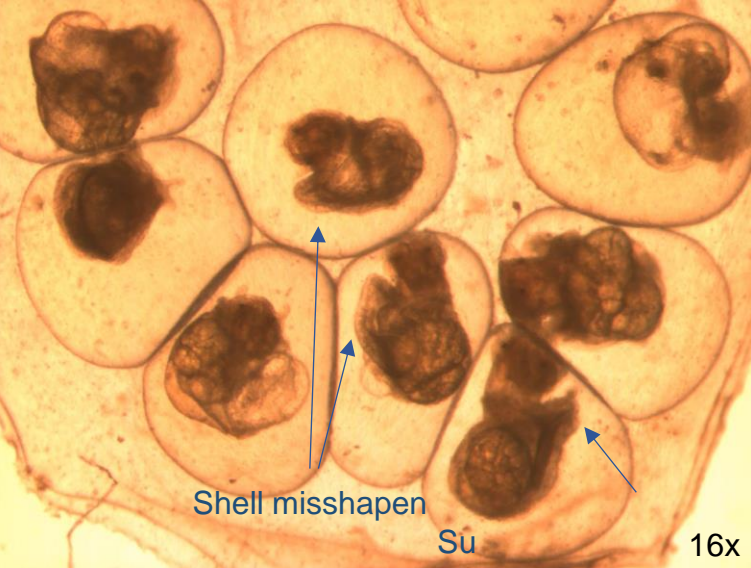
Treated with methanol 100 mM (hatched)



Mufla: a snail cephalic region between the tentacles and around the mouth

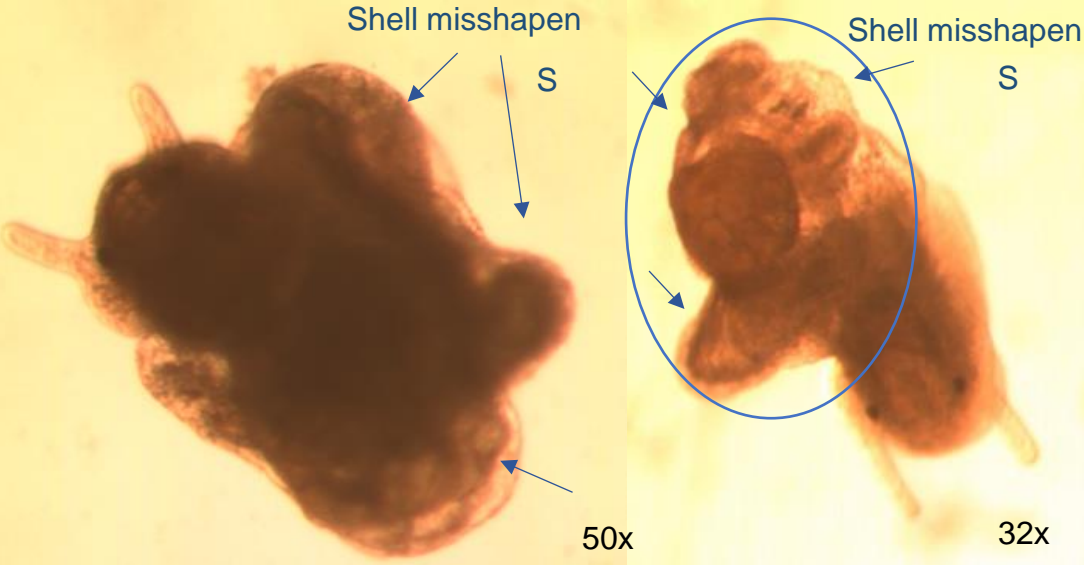
B.glabrata snail DevTox assay

Externally-visible abnormalities: Shell malformation (S)



Treated with *E.milii* 50 ppm

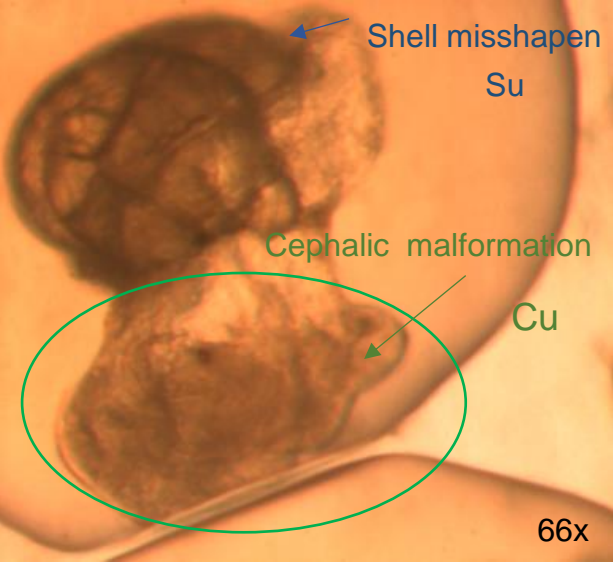
Shell anomaly **within the egg**



Treated with *E.milii* 50 ppm

Treated with DSS

Shell anomaly **after hatching**

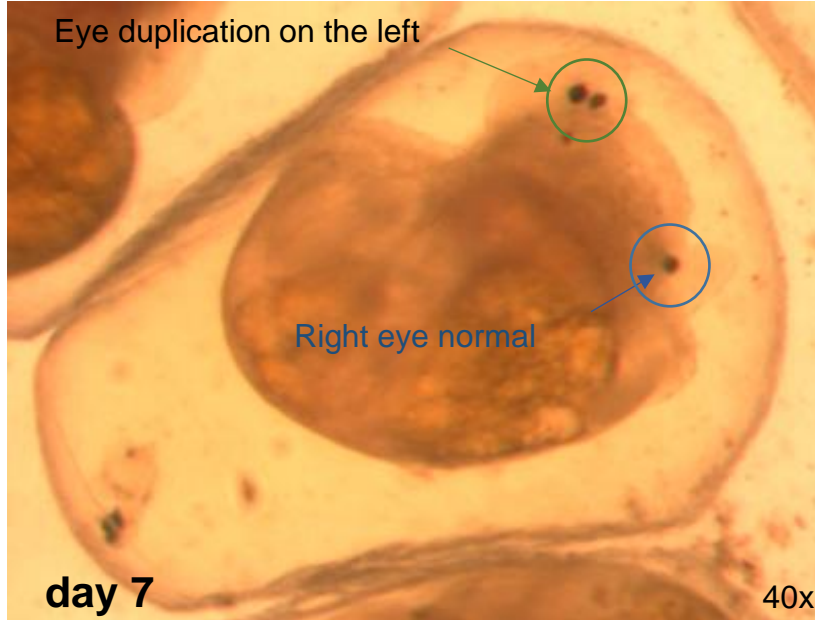


Treated with CuSO₄

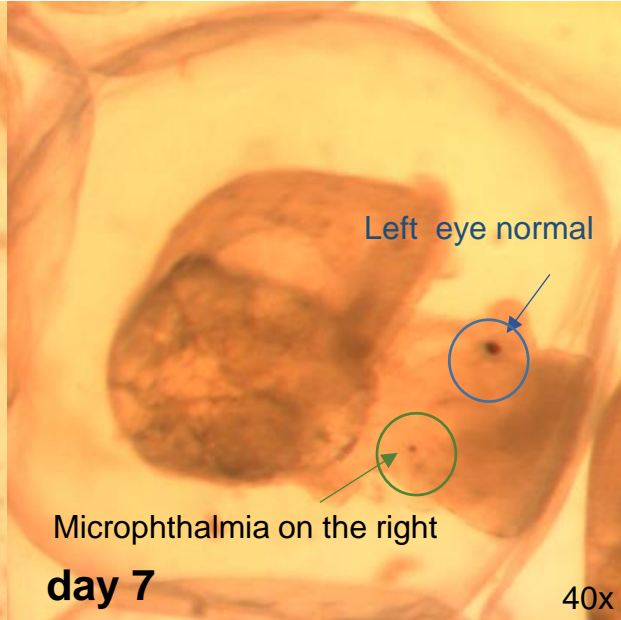
Shell anomaly **within the egg**

B.glabrata snail DevTox assay

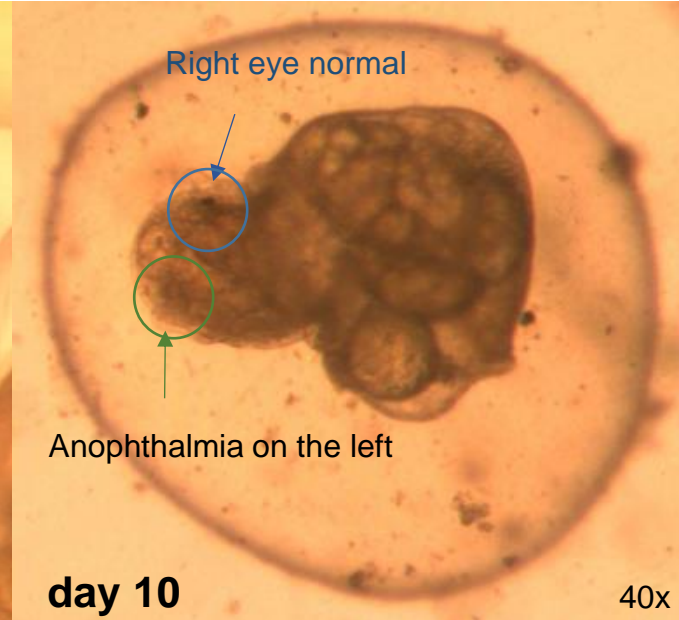
Externally-visible abnormalities: Eye malformations (E)



Treated with *E.milii* 100 ppm



Treated with methanol 500 mM



Treated with *E.milii* 100 ppm

***B.glabrata* snail DevTox assay**

Externally-visible abnormalities: **Foot misshapen** (F)



Newly hatched snail: the malformed snail (still within the egg mass gelatinous matrix) is breaking free from the eggshell.

EM treated with methanol 250 mM

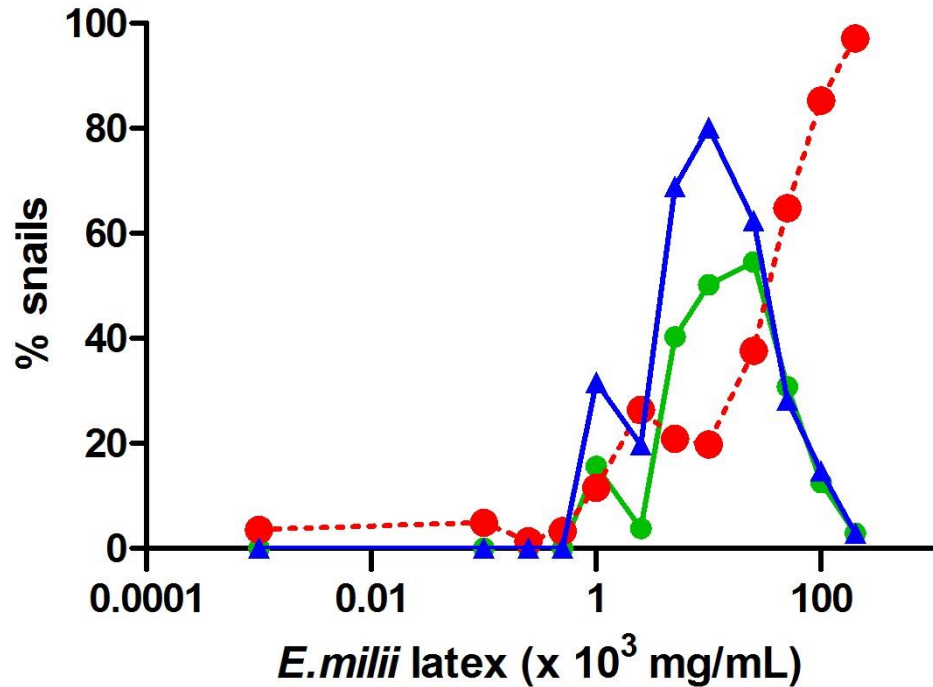
Snail **DevTox** Assay results

Dose-response curves for DevTox endpoints on days 12 and 14 after spawning (exposure days 1-4)

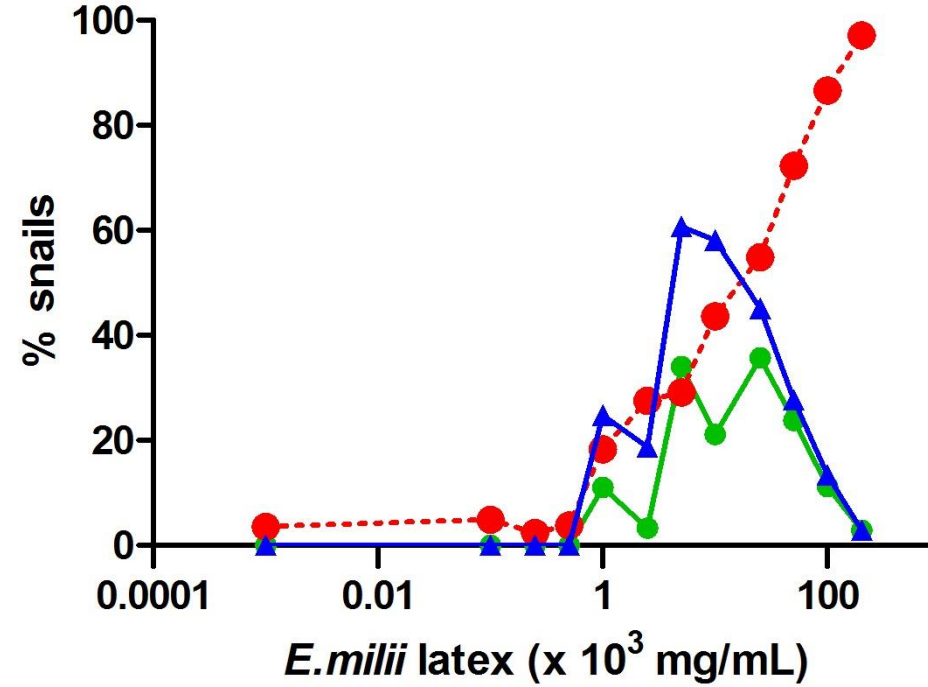


Christ's crown – *Euphorbia milii*
Plant molluscicide (latex)

day 12



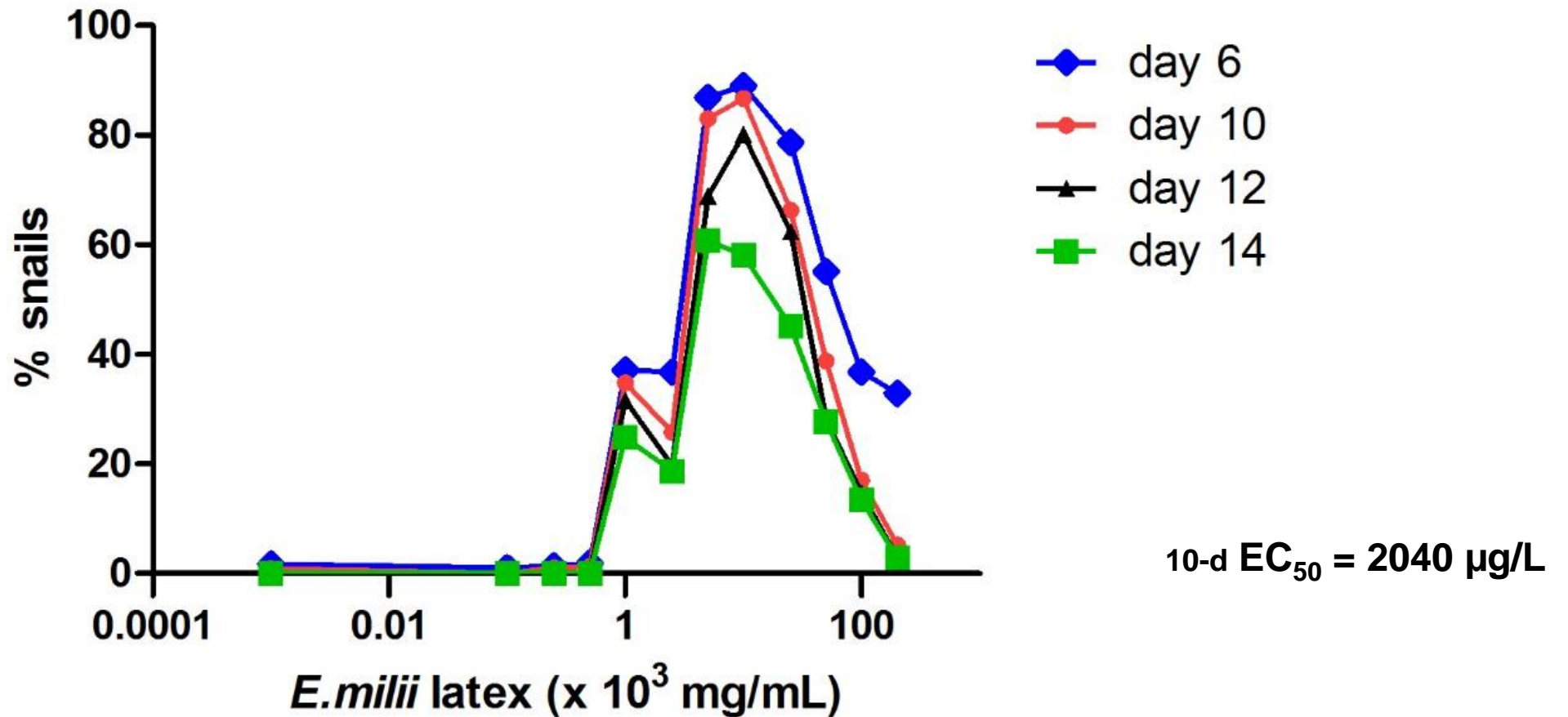
day 14



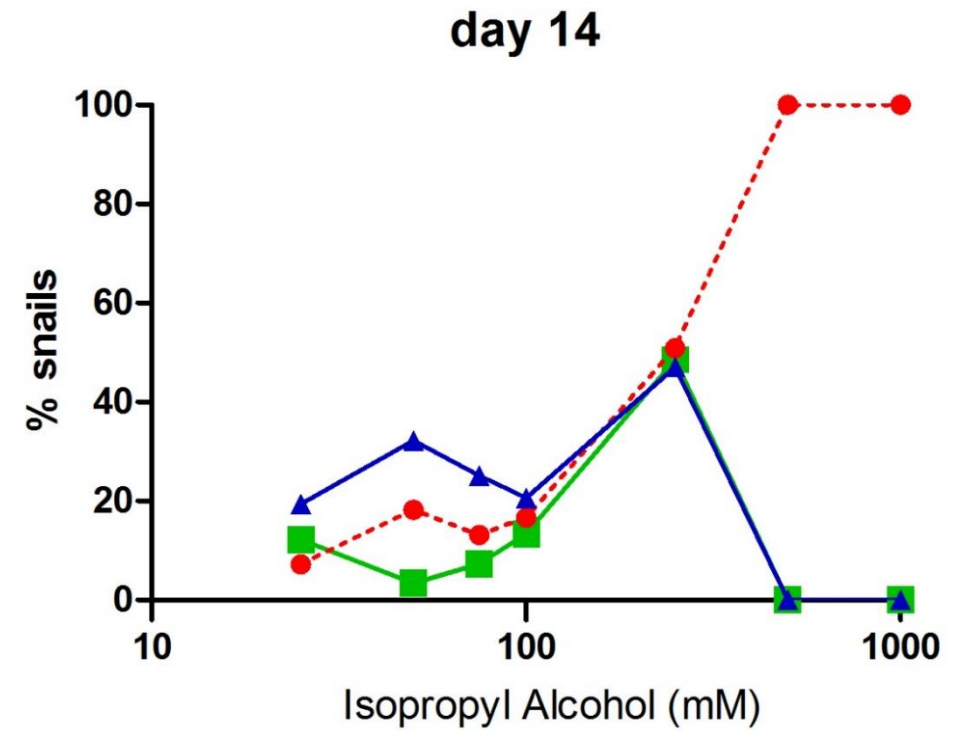
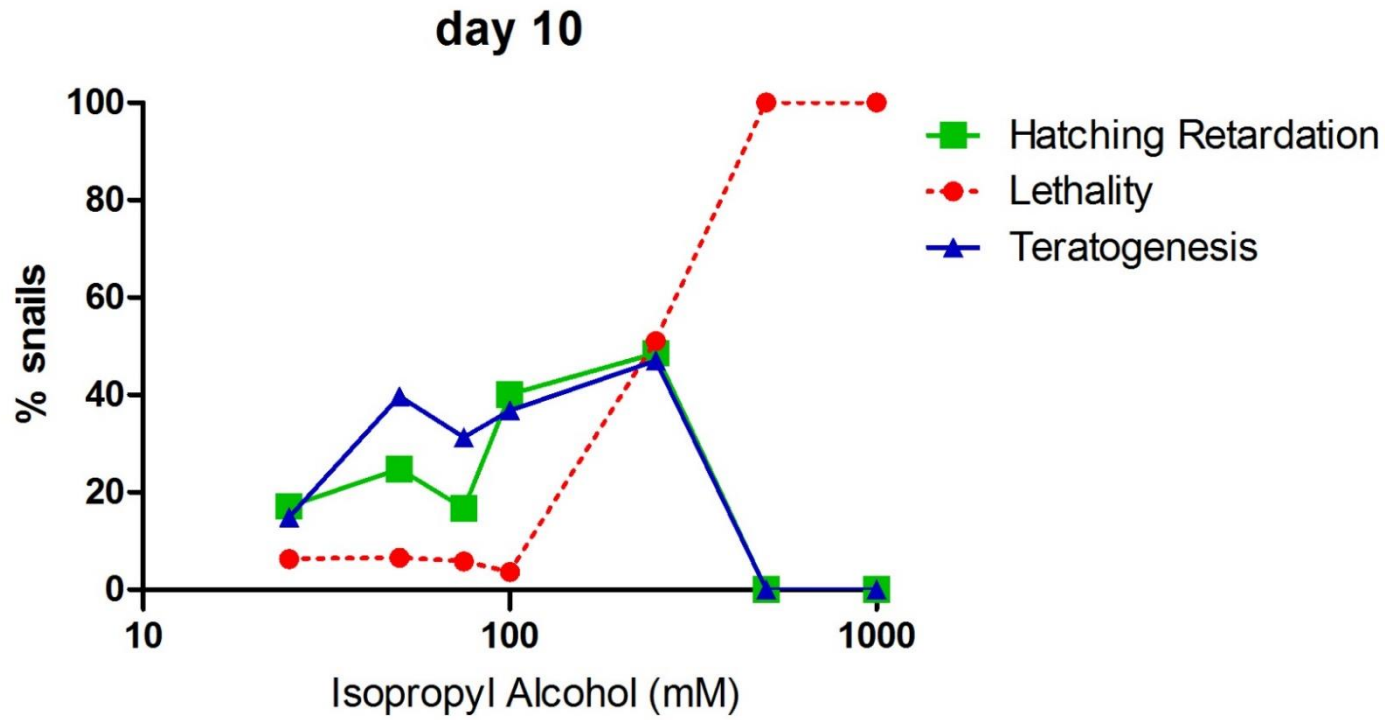
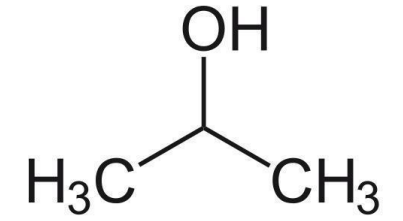
- Hatching Retardation
- Lethality
- ▲ Teratogenesis

Teratogenesis - malformed embryo/snails on days 6, 10, 12 and 14

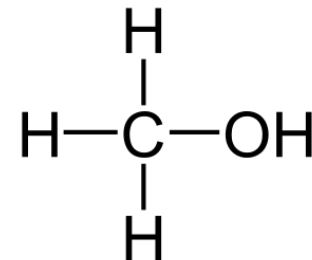
(exposure: days 1-4 post spawning)



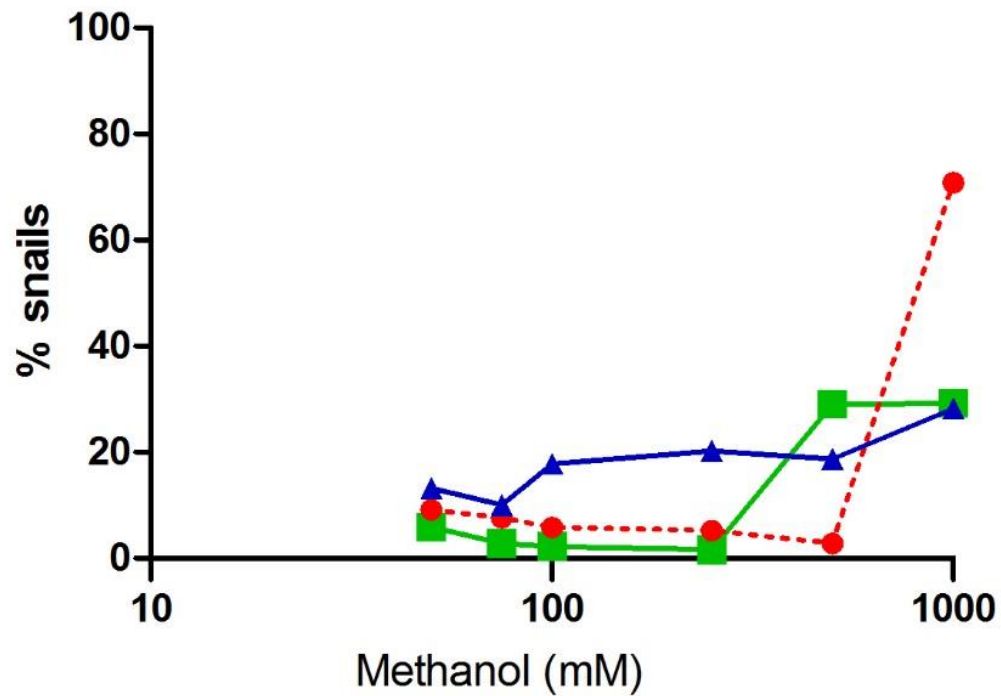
Isopropanol



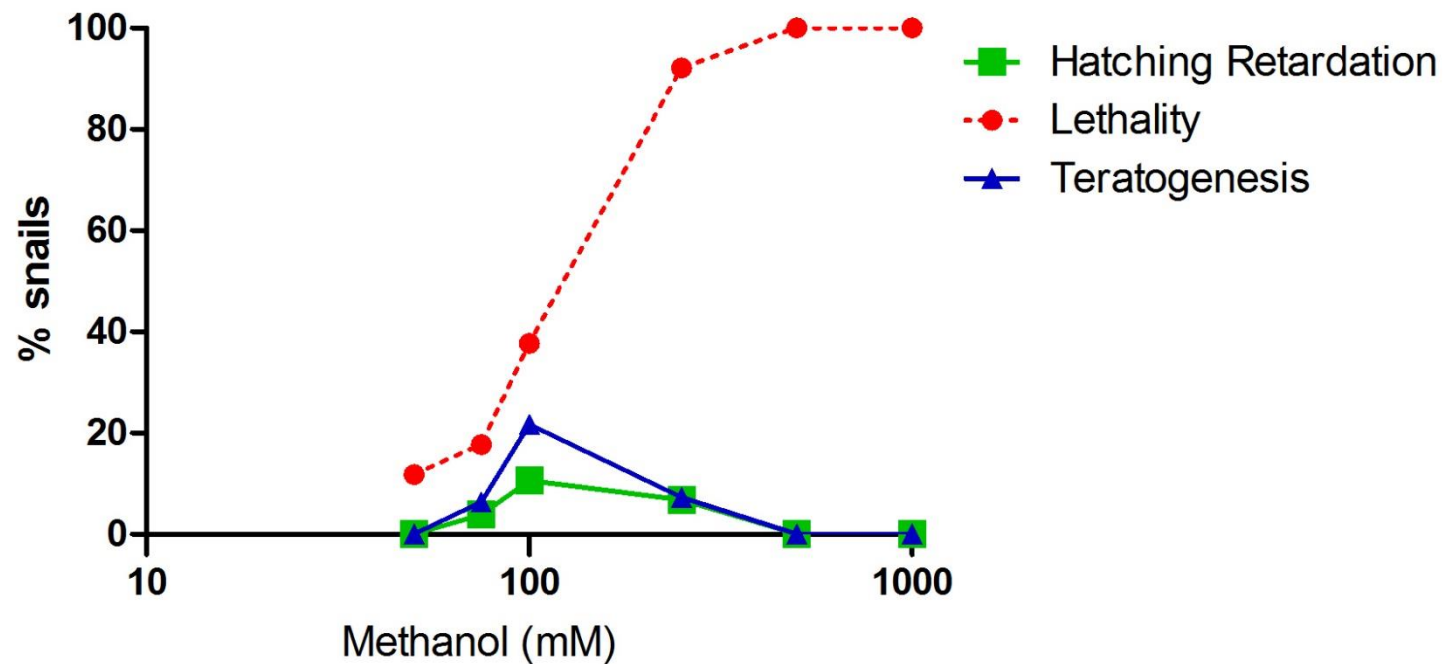
Methanol



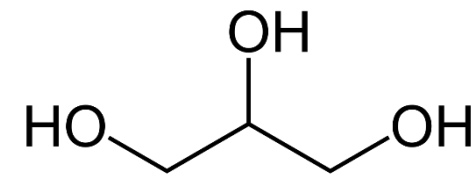
day 10



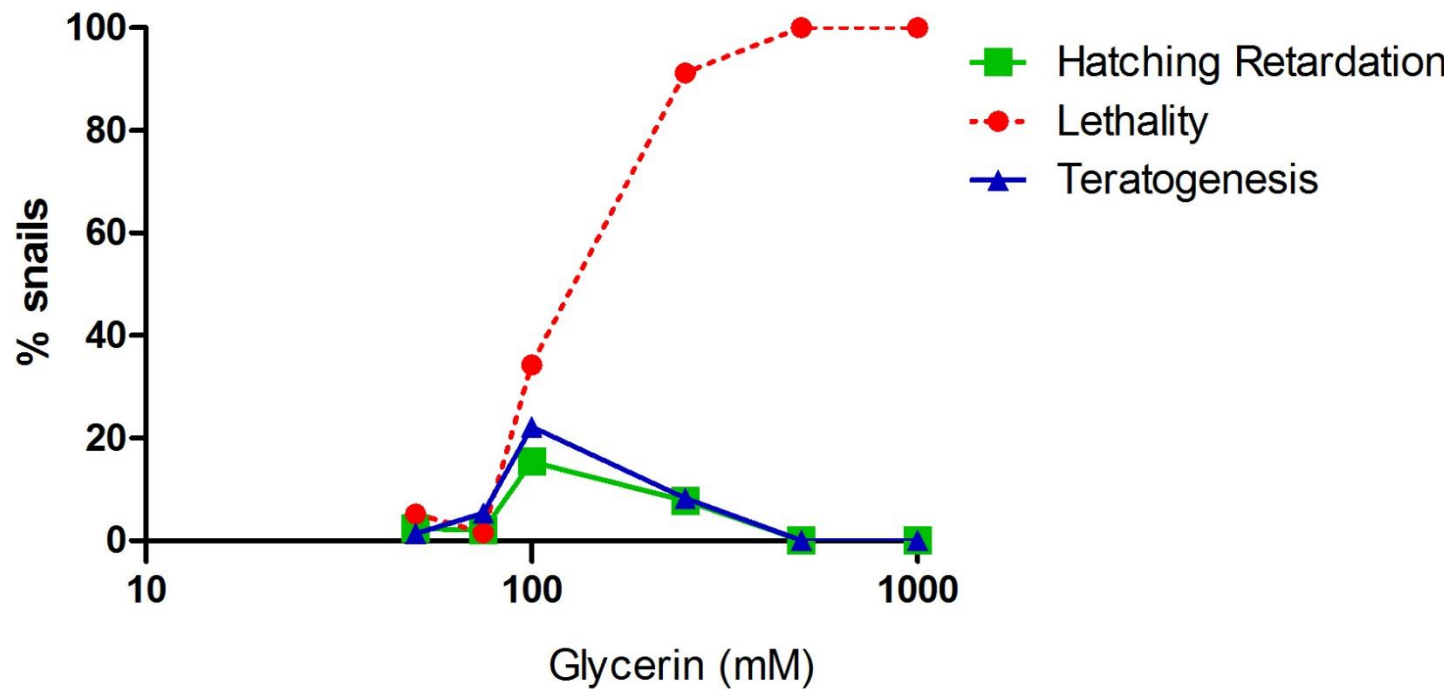
day 14



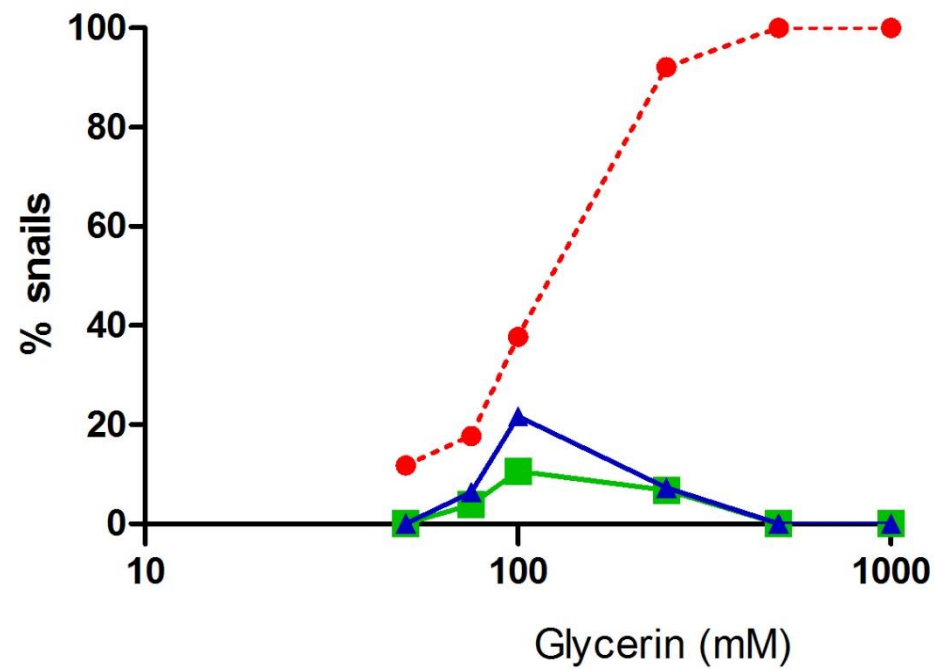
Glycerin



day 10

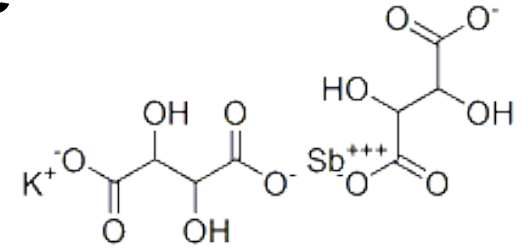


day 14

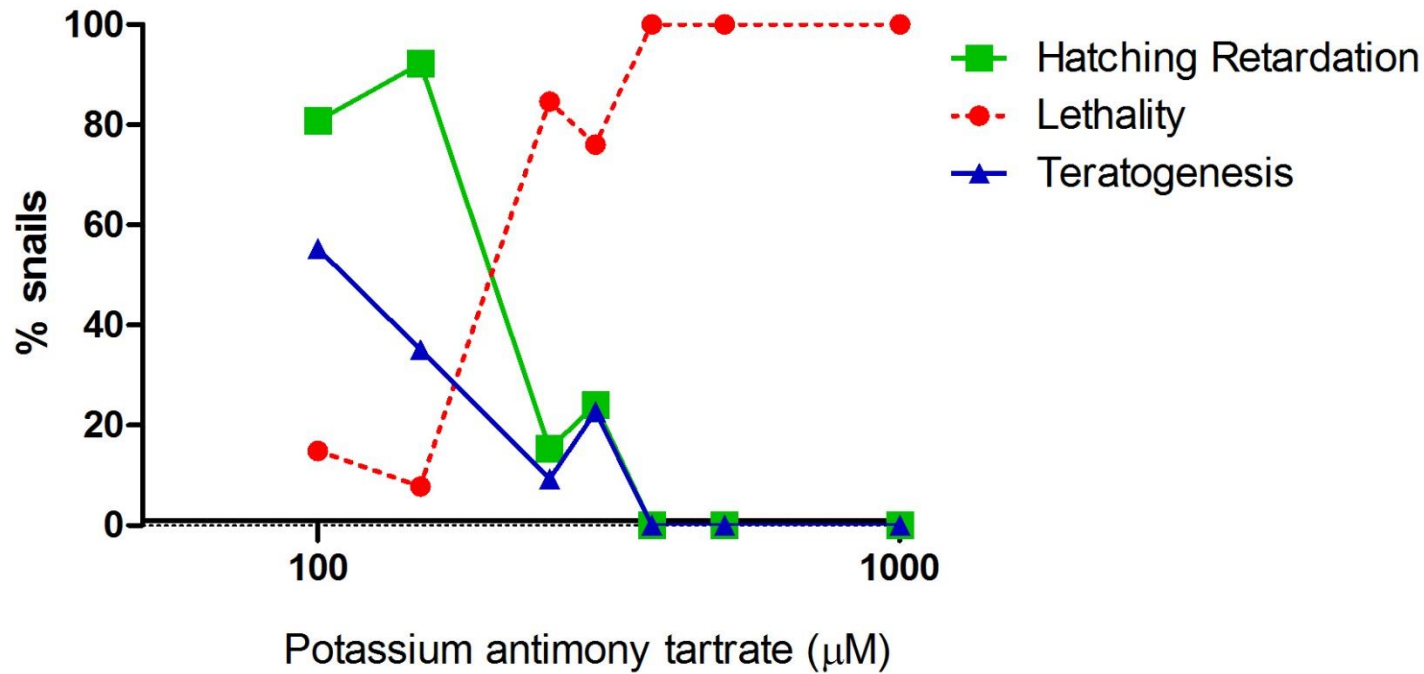


Potassium antimony tartrate

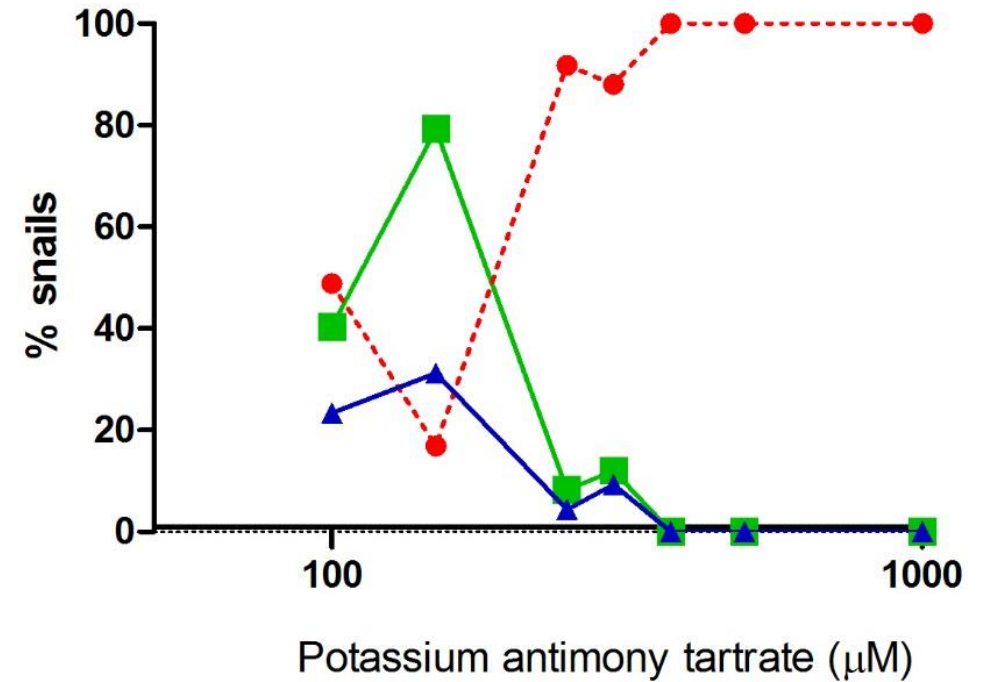
Sb³⁺



day 10




day 14



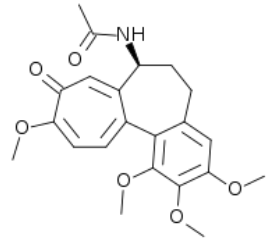
Developmental toxicity of cadmium, mercury and sodium chloride

The most potent snail developmental toxicant

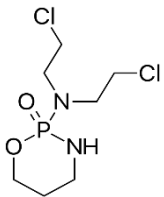
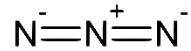


	Teratogenicity EC ₅₀ (95% CI)	Hatching Delay IC ₅₀ (95% CI)	Embryoletality LC ₅₀ (95% CI)	LOEC
CdCl ₂	340 nM (330-360 nM)	260 nM (260-290 nM)	370 nM (360-380 nM)	140 nM
HgCl ₂	100 nM (90-120 nM)	40 nM (40-50 nM)	310 nM (290-340 nM)	37 nM
NaCl	91 mM (82-100 mM)	73 mM (71-74 mM)	76 mM (74-78 mM)	46 mM

Developmental toxicity of sodium azide, cyclophosphamide and colchicine



Na⁺



Teratogenicity

EC₅₀ (95% CI)

Hatching Delay

IC₅₀ (95% CI)

Embryoletality

LC₅₀ (95% CI)

LOEC

Colchicine

1.1 μM
(1.0-1.2 μM)

0.9 μM
(0.8-1.0 μM)

2.2 μM
(2.1-2.3 μM)

0.2 μM

Sodium Azide

100 μM
(90-100 μM)

140 μM
(130-160 μM)

160 μM
(150-170 μM)

48 μM

Cyclophosphamide

13 mM
(12-15 mM)

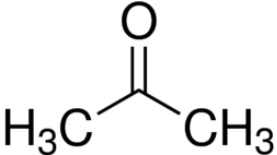
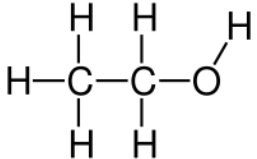
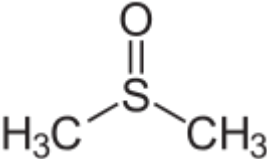
5 mM
(5-6 mM)

15 mM
(14-15 mM)

1.8 mM

Apparently the snail embryo has no metabolic competence to activate cyclophosphamide

Developmental toxicity of acetone, ethanol and dimethyl sulfoxide (DMSO)

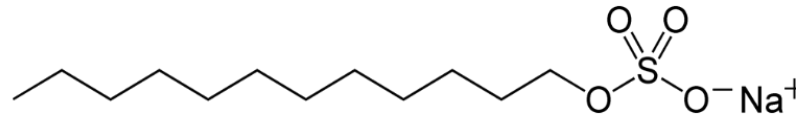
	Teratogenicity EC ₅₀ (95% CI)	Hatching Delay IC ₅₀ (95% CI)	Embryoletality LC ₅₀ (95% CI)	LOEC
 Acetone	170 mM (170-180 mM)	180 mM (180-190 mM)	170 mM (160-180 μM)	30 mM
 Ethanol	NC	260 mM (220-300 mM)	270 mM (250-280 mM)	17 mM
 DMSO	NC	120 mM (110-130 mM)	160 mM (150-160 mM)	14 mM

If these solvents are used to dissolve a test substance, final concentrations in the assay softwater water should not to exceed 30 mM for acetone, 17 mM for etanol and 14 mM for DMSO.

Developmental toxicity of Dodecyl sodium sulfate (DSS) and Hydrogen peroxide

	Teratogenicity EC ₅₀ (95% CI)	Hatching Delay IC ₅₀ (95% CI)	Embryoletality LC ₅₀ (95% CI)	LOEC
DSS	100 μM	140 μM (130-140 μM)	200 μM (190-210 μM)	12 μM
H ₂ O ₂	NC	390 μM	470 μM (460-480 μM)	120 μM

DSS: Dodecyl sodium sulfate



***B.glabrata* DevTox assay – day 10**

Teratogenic Index (TI) = LC_{50}/EC_{50}	
Test substance	TI
<i>E.milii</i> latex*	16.7
HgCl ₂	3.1
Colchicine	2
DSS	2
Sodium azide	1.6
Cyclophosphamide	1.2
CdCl ₂	1.1
Acetone	1
NaCl	0.8

Teratogenic in the snail assay

Not teratogenic in the snail assay

	<i>Xenopus laevis</i> (FETAX)		<i>B.glabrata</i> DevTox assay	
	LC ₅₀	EC ₅₀	LC ₅₀	EC ₅₀
CdCl₂	32 µM*	3.7 µM*	0.37 µM	0.34 µM
Ethanol	239 mM ⁺	152 mM ⁺	270 mM	NT
CuCl₂	0.9 mg/L [#]	0.4 mg/L [#]	-	-
CuSO₄	-	-	2.2 mg/L ⁺⁺	NT ⁺⁺
H₂O₂	598 µM [§]	536 µM [§]	470 µM	NT
HgCl₂	601 nM ^{**}	513 nM ^{**}	310 nM	100 nM

A comparison of the vertebrate frog FETAX with the invertebrate Snail **DevTox** assay

The frog and the snail assays gave rise to fairly comparable LC₅₀ and EC₅₀ concentrations for these chemicals

NT: Not teratogenic, almost no anomalies. (-) not tested

FETAX assay: 23±1°C; exposure after removing the jelly coat with 2% (w/v) cysteine solution (pH 8.1)

Snail assay: 25±1°C; exposure via the egg mass jelly coat

Both assays: Exposure for 96 h

*Sunderman et al, 1991

+ Fort et al, 2004

Martini et al, 2012

++ Oliveira-Filho et al, 2010

** Prati et al, 2002

§ Vismara et al, 2006

Concluding remarks

The Snail DevTox Assay seems to be a feasible alternative test system for screening chemicals of developmental toxicity.

Snail **DevTox** Assay

- **inexpensive**
- **fast** and **easy** to perform
- requires **simple** lab equipment and infra-structure
- test many **chemicals** over a wide concentration range in a relatively **short period** of time
- possible 3-Rs **alternative for** the use of **vertebrates**

It may also be used for Developmental Biology (mechanistic) studies

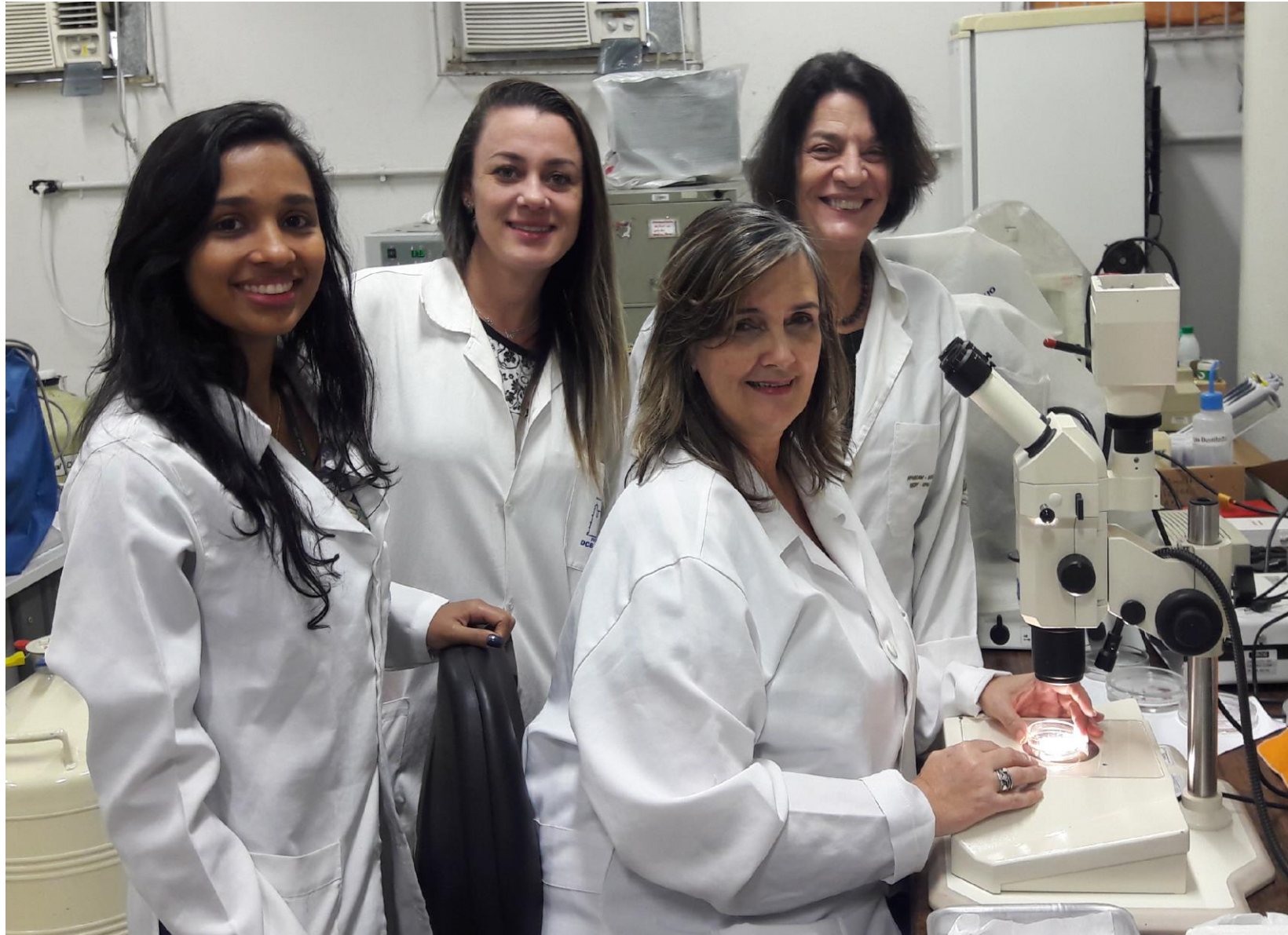
Snail DevTox Assay

Further research steps:

- ➔ Optimization and **standardization** of a Snail DevTox **test protocol** (*in progress*)
- ➔ Study of within and between laboratory reproducibility of results using a standardized test protocol
- ➔ Comparative study of Snail DevTox Assay with tests based on other invertebrate and nonmammalian vertebrate species



Laboratory of Environmental Toxicology
National School of Public Health
Oswaldo Cruz Foundation – FIOCRUZ
Rio de Janeiro - Brazil



Snail **DevTox** Assay group

Gabrielle da Silveira Lopes

Hellen da Silva Leal

Rosangela R. de Carvalho

Maria Regina Gomes Carneiro

Thank you so much for your attention !

In memory of Dr Toshie Kawano, an outstanding Brazilian malacologist whose invaluable contribution to the knowledge of *Biomphalaria glabrata* embryology and genetics made this and many other recent studies possible.

