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

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- **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)

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

[Martins LS, 2010]

Albino and wild-type *B.glabrata*

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.

http://www.nhm.ac.uk/discover/news/2017/may/snail-genome-provides-clues-to-controlling-devastating-disease.html

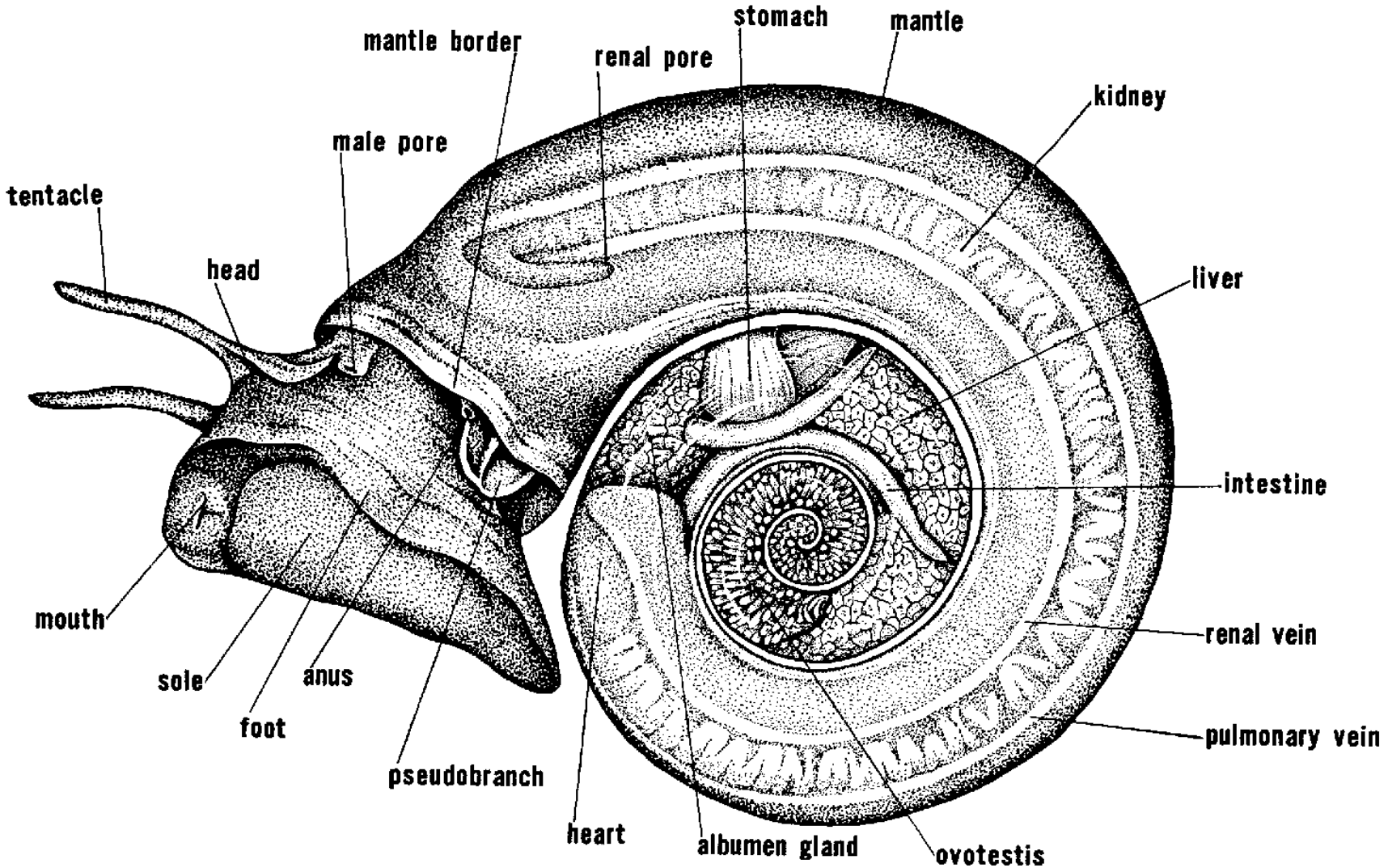
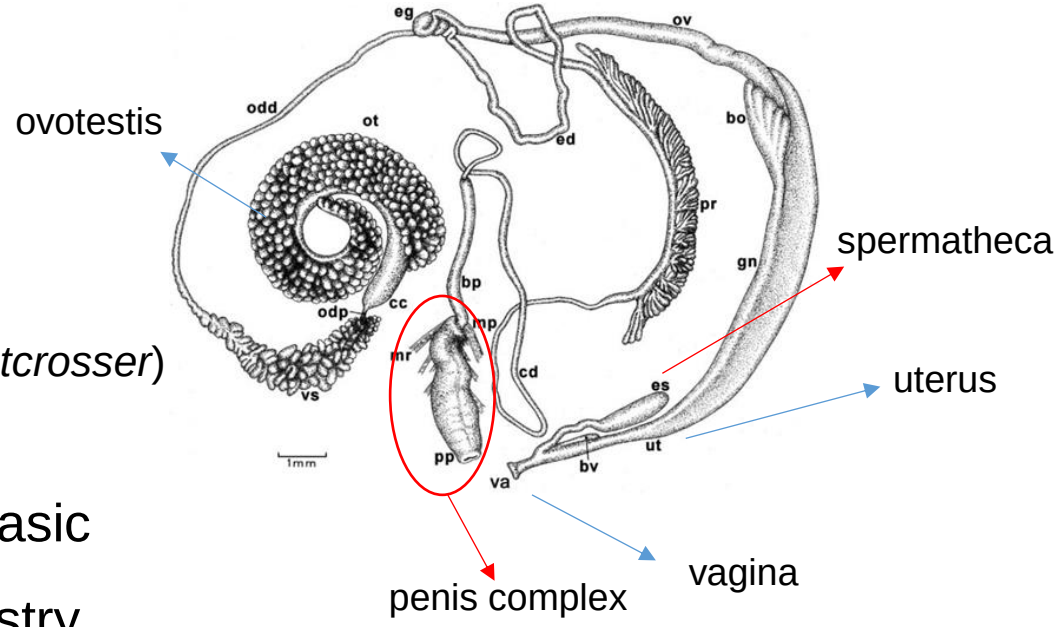


Fig. 8. A *Biomphalaria* with the shell removed and seen from the left, enlarged (after Demian)

PAHO-WHO, 1968
Scientific Publication No. 168

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



[Paraense 1975]

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

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

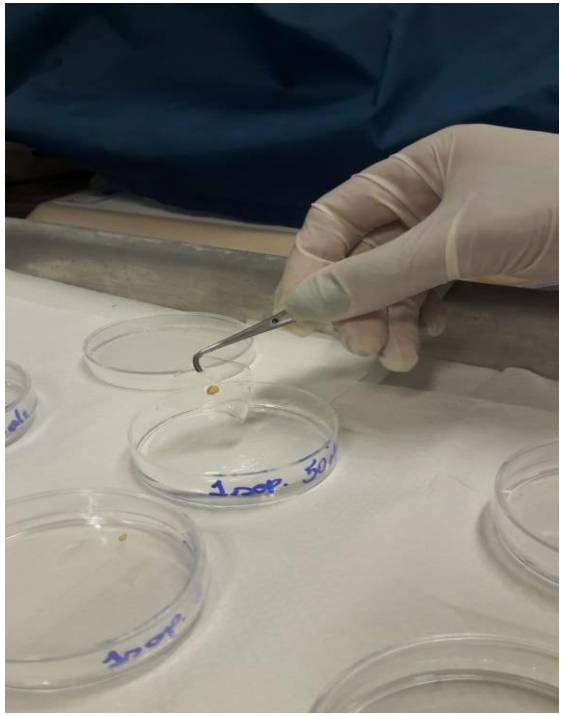
***B. glabrata* genome was sequenced and analyzed** (Adema *et al* 2017).

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

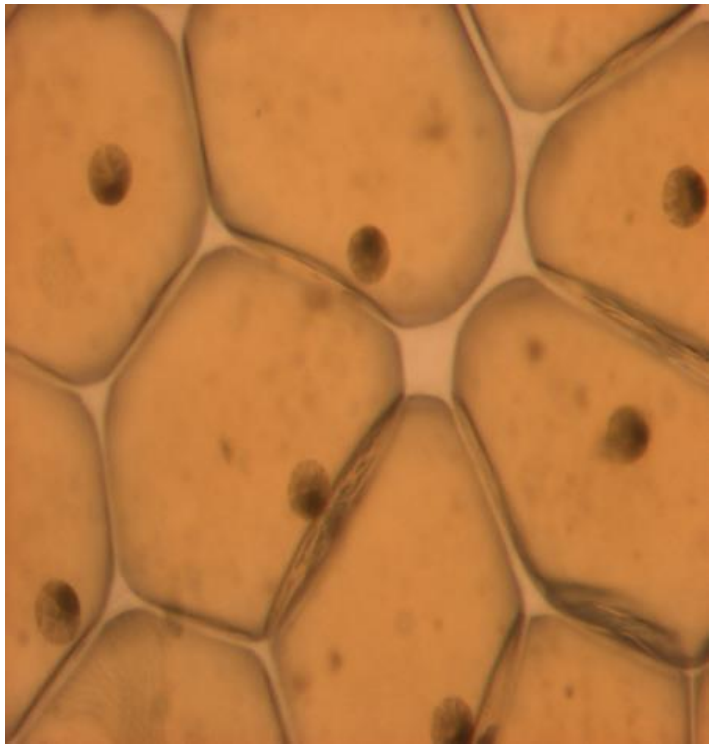
Snail DevTox Assay

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



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

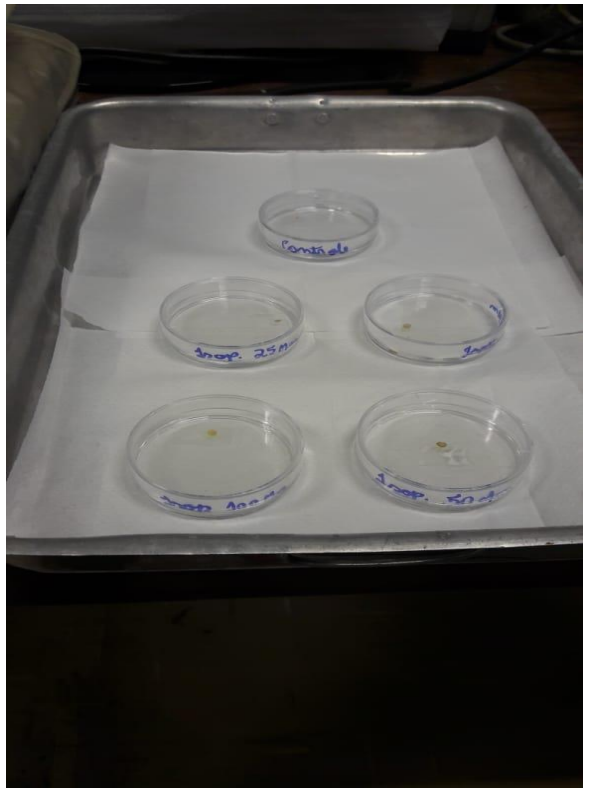
EMs are then examined under a stereomicroscope to score the embryo development stage.



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 $CaCO_3$)

Climatic photoperiod chamber: **25±1˚C;** 12-h photoperiod;

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

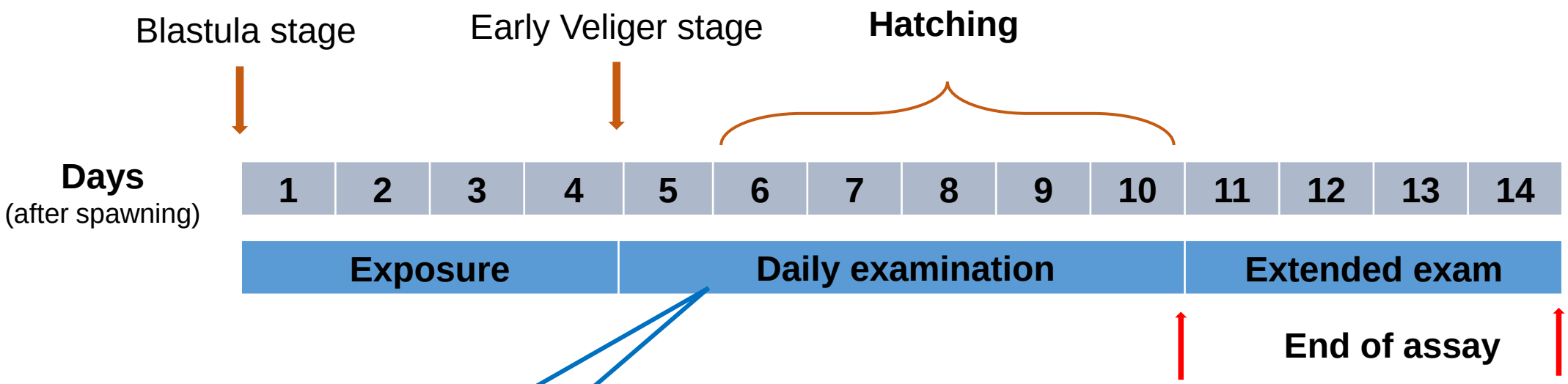
EMs are exposed to test chemicals dissolved in the assay water.

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



Evaluated endpoints

- Embryo (or snail) lethality
- Abnormal development (type of anomalies and retarded growth)
- Retarded hatching

Snail DevTox Assay

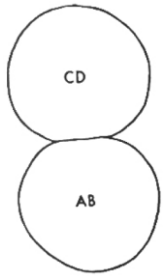
TABLE 1. Main stages of the embryonic development of *B. glabrata* at 25°C.

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

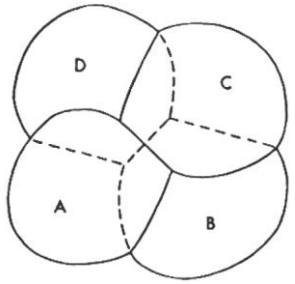
Exposure Period

[Kawano et al, 1995]

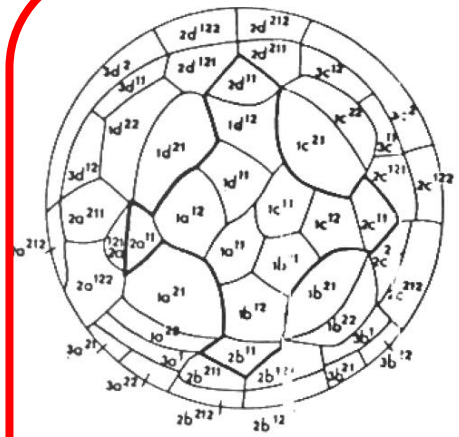
Snail DevTox Assay: *B.glabrata* embryo stages



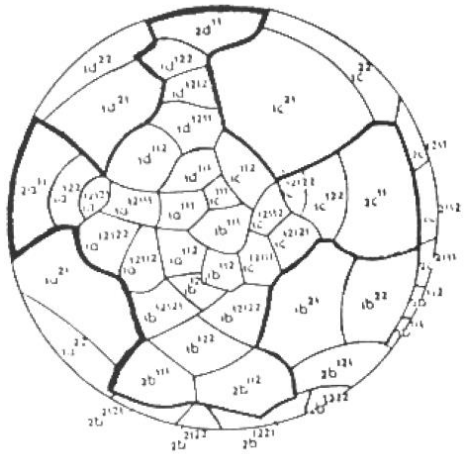
0 h: 1st cleavage



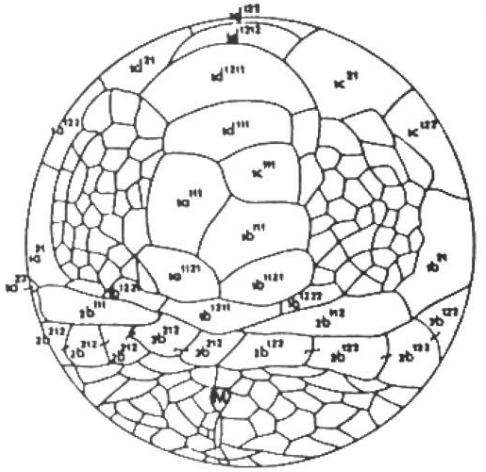
80 min:
2nd cleavage.



15 h: Blastula
(64 blastomers)

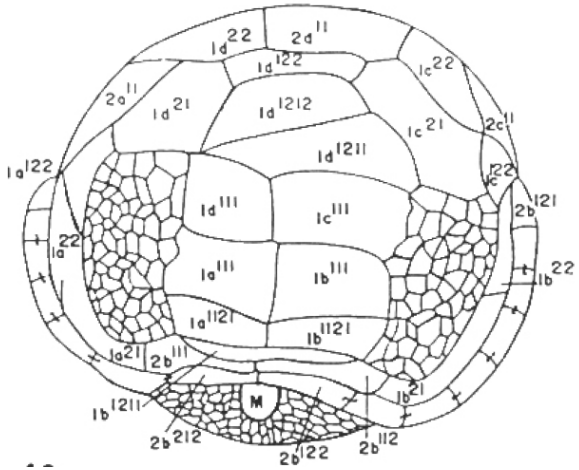


26 h: Gastrula

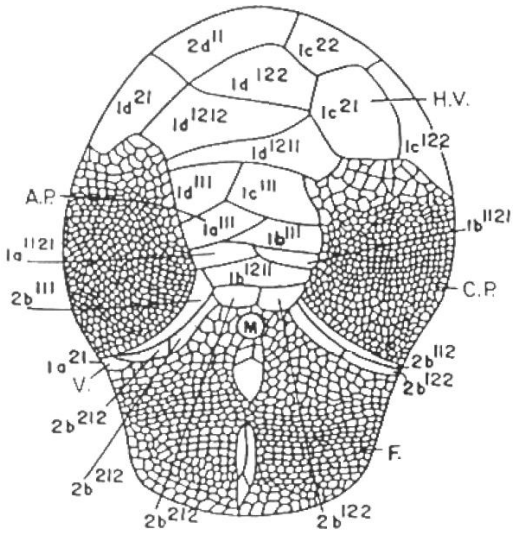


34 h: Early trochophore

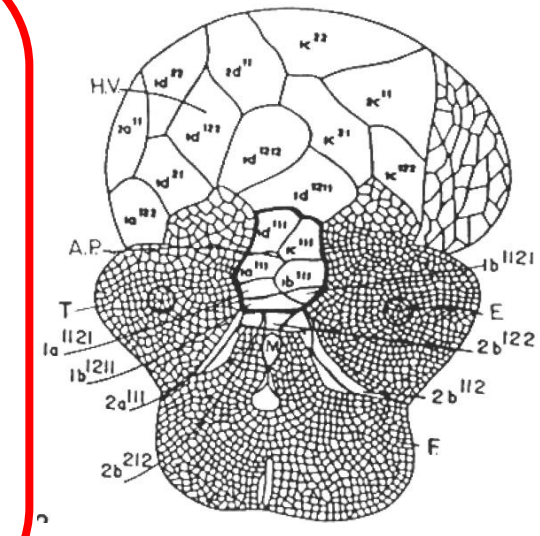
Exposed embryonic development stages



40 h: Trochophore



96 h: Early Veliger

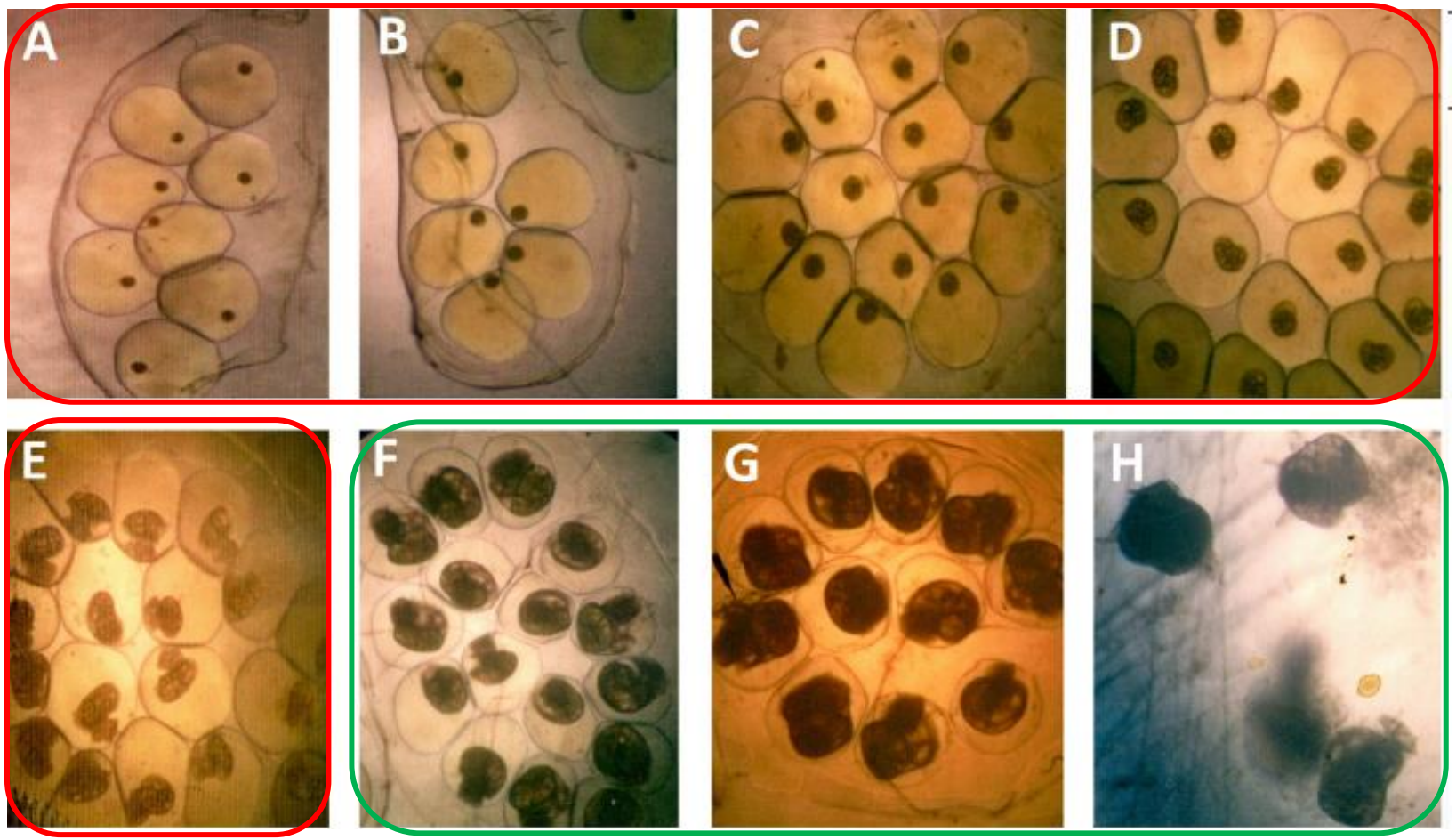


120 h: Veliger

[Kawano et al, 1995]

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.

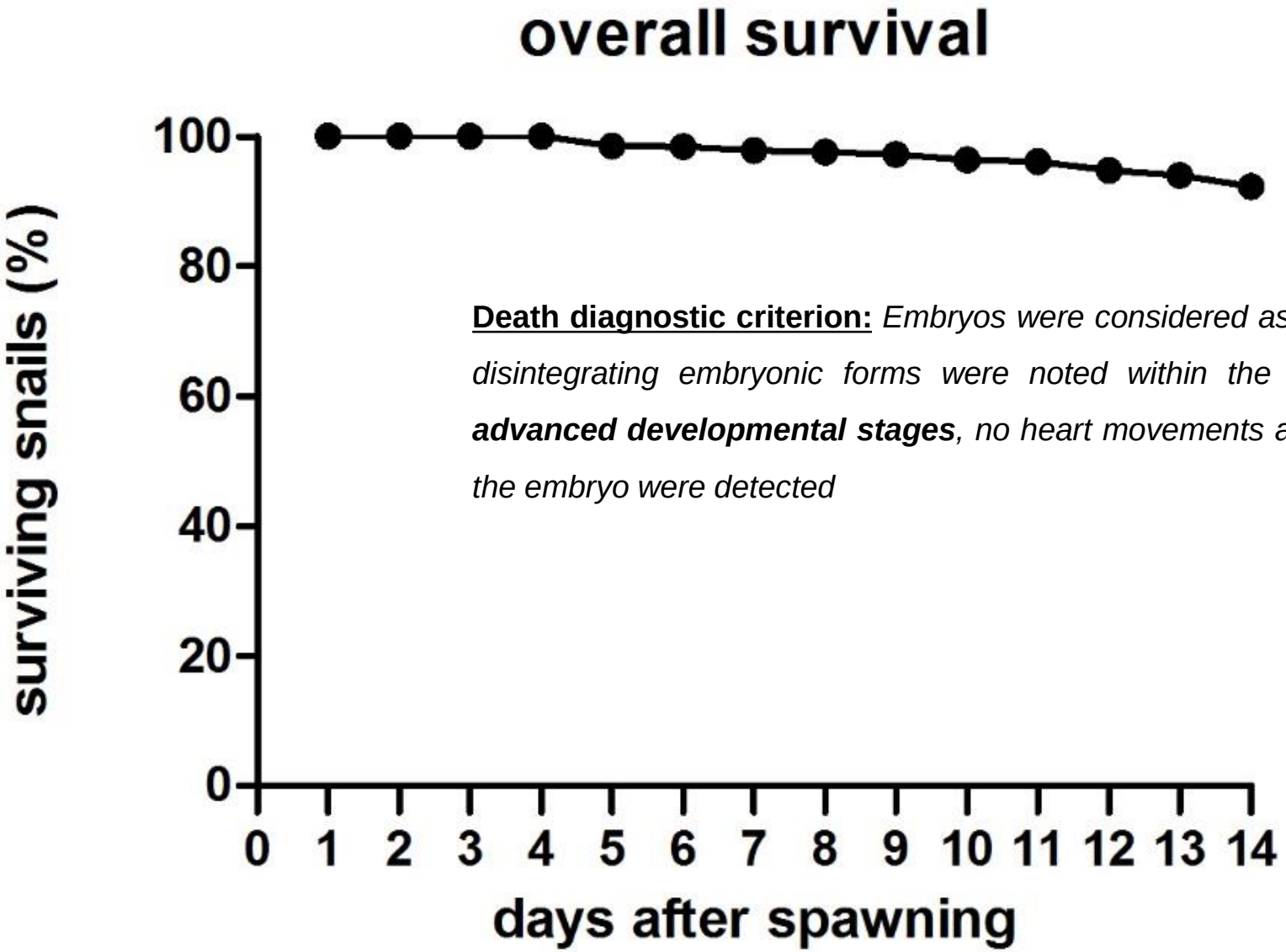
[Albuquerque et al, 2014]

The historical control data

B. glabrata DevTox assay

B.glabrata **historical control** data

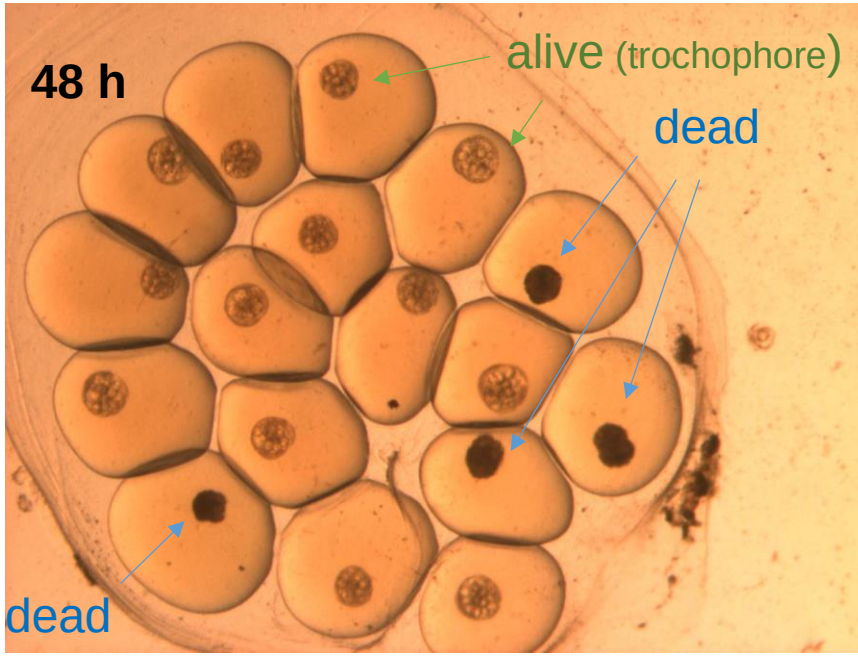
Embryos (eggs) N= 1038
Egg masses N= 69



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

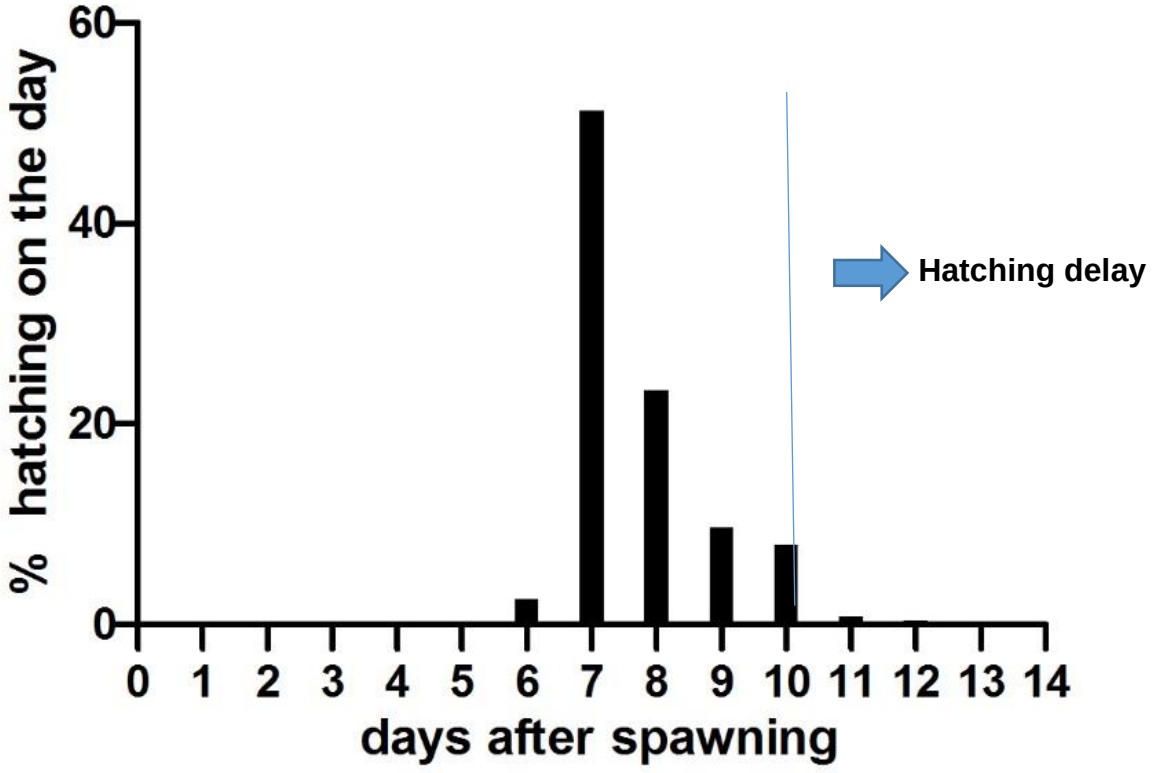
Death diagnostic criterion: *Embryos were considered as dead whenever disintegrating embryonic forms were noted within the egg and or,* ***at advanced developmental stages****, no heart movements and no motility of the embryo were detected*

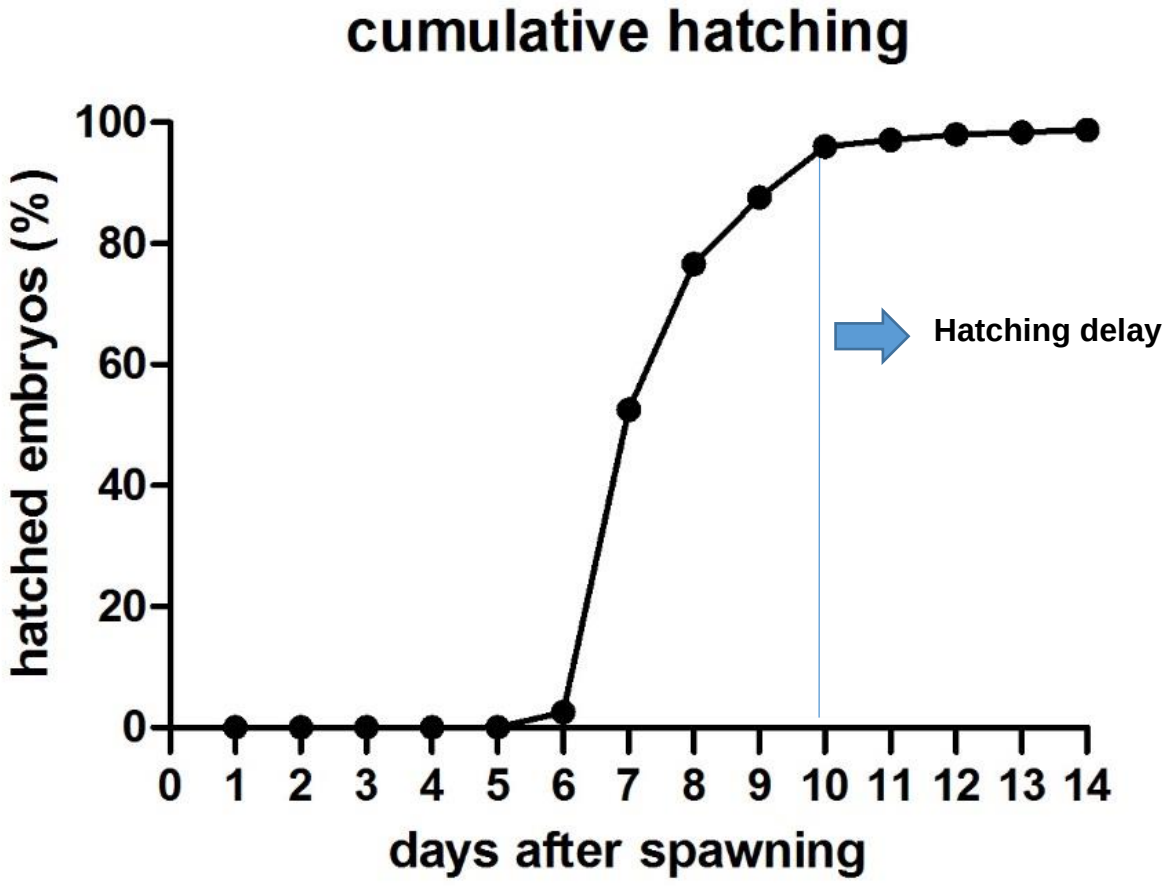


Egg mass exposed to Sodium azide 769 µM

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 missshapen**, 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.

B.glabrata **historical control** data

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

Spontaneous occurrence of externally-visible abnormalities

Before hatching [u]

Growth retarded [Ru]

Shell misshapen [Su]

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

Shell misshapen+ Cephalic region anomaly [SCu]

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]

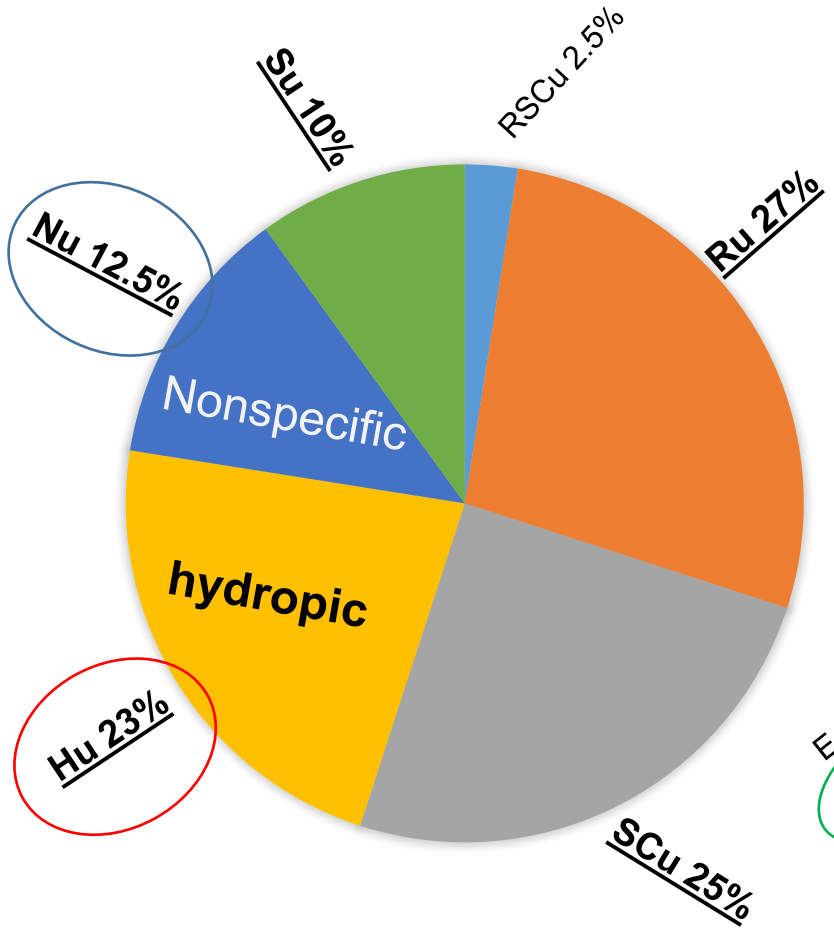
Eye duplication (left) [Ed]

Tentacle atrophy (bilat.) [Tab]

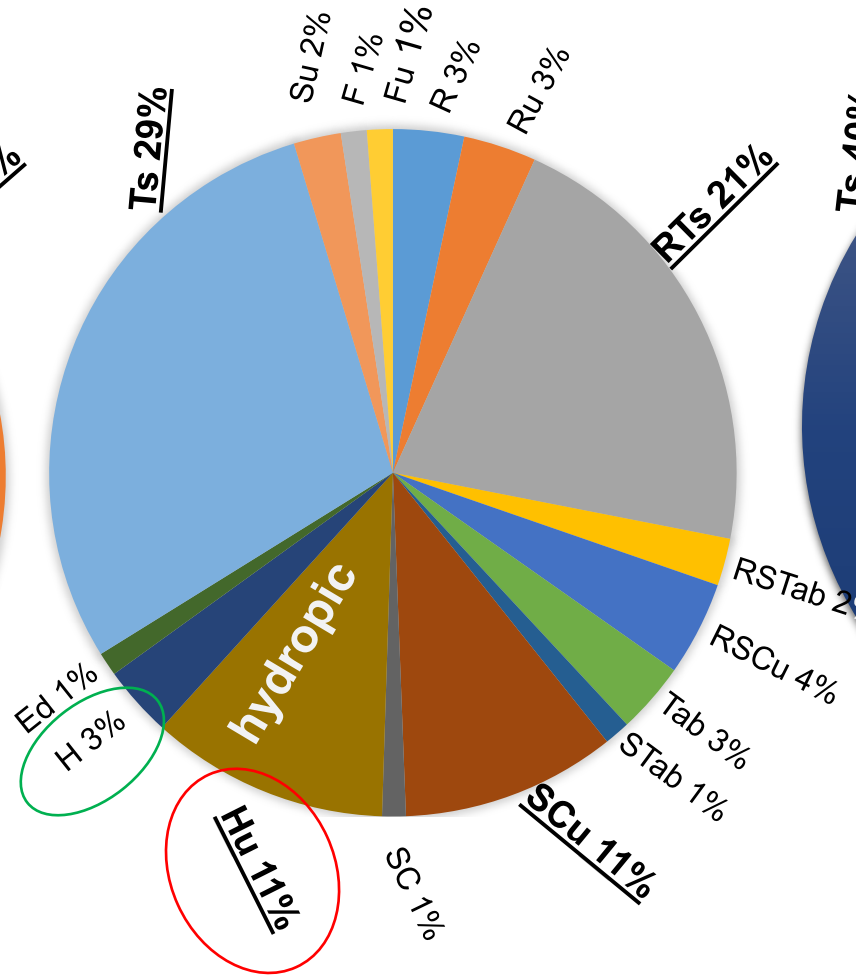
Tentacle atrophy (bilat.) + Shell misshapen [STab]

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

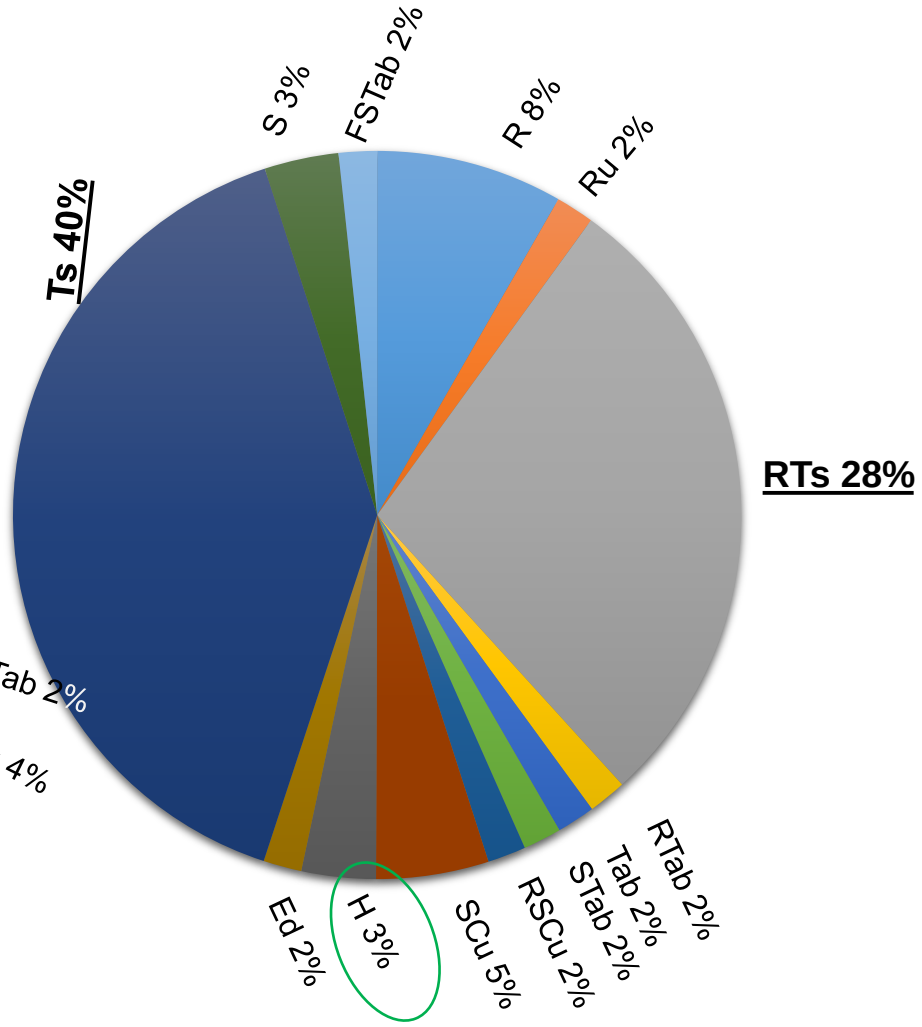
Tentacle shorter (bilat.) [Tsb]



Day 6
Total malformed; N=40



Day 10
Total malformed; N=90



Day 14
Total malformed; N=60

B.glabrata **historical control** data

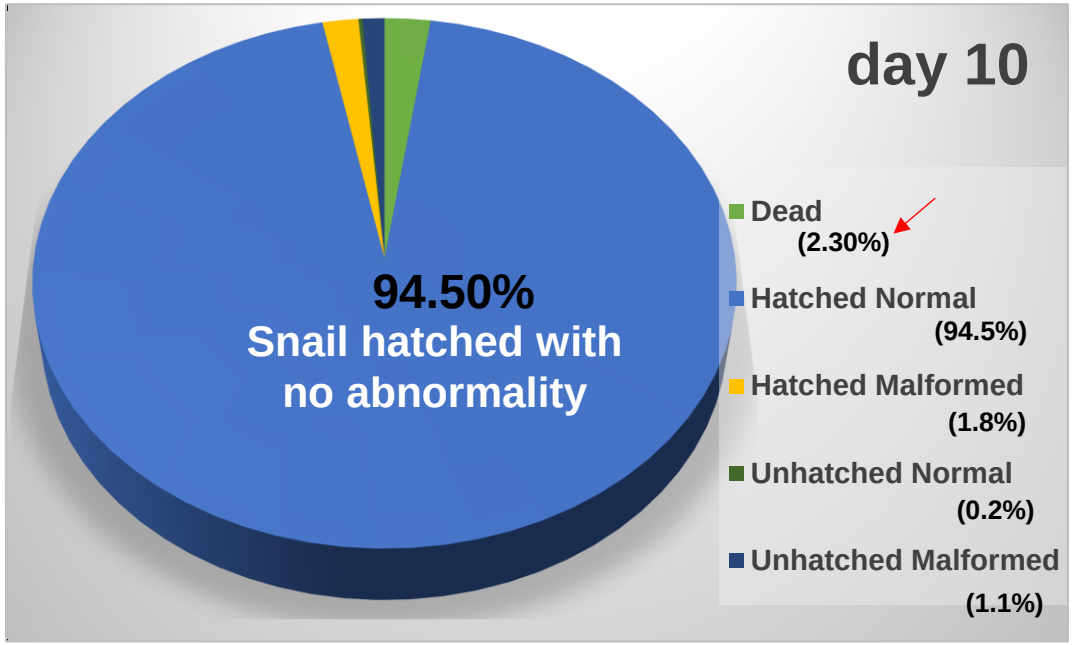
Embryos N= 1038
Egg masses N=69

B. glabrata historical control data

25±1˚C synthetic softwater (40-48 mg/L as $CaCO_3$)

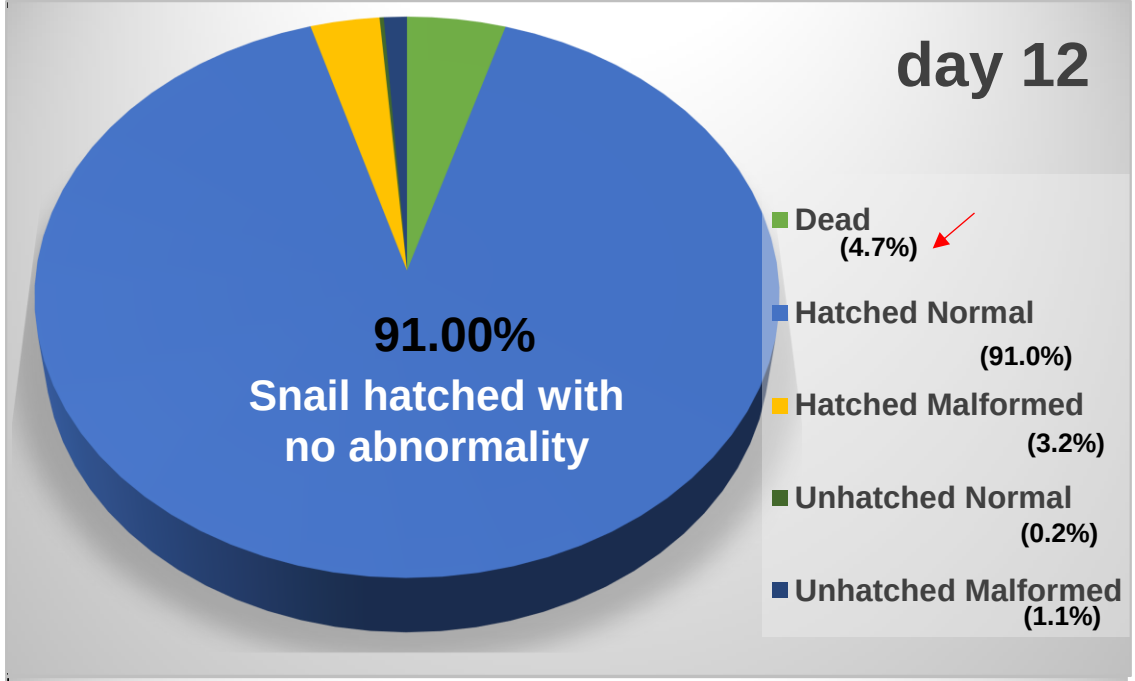
pH 7.0±0.2; 12-h photoperiod

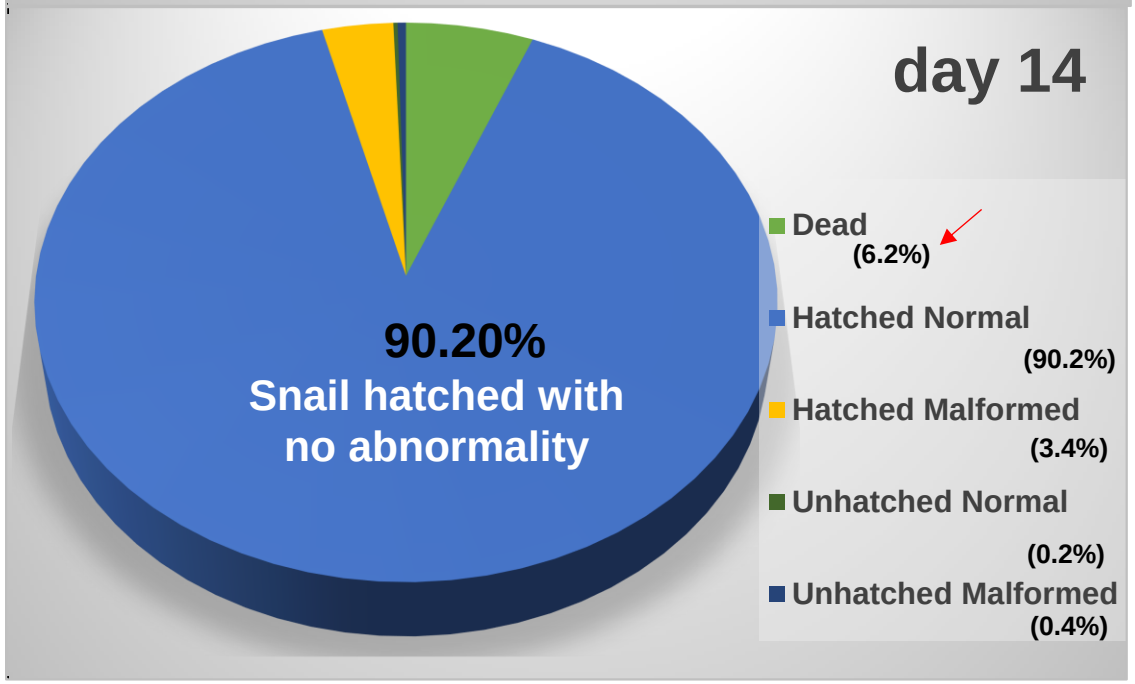
climatic photoperiod chamber



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

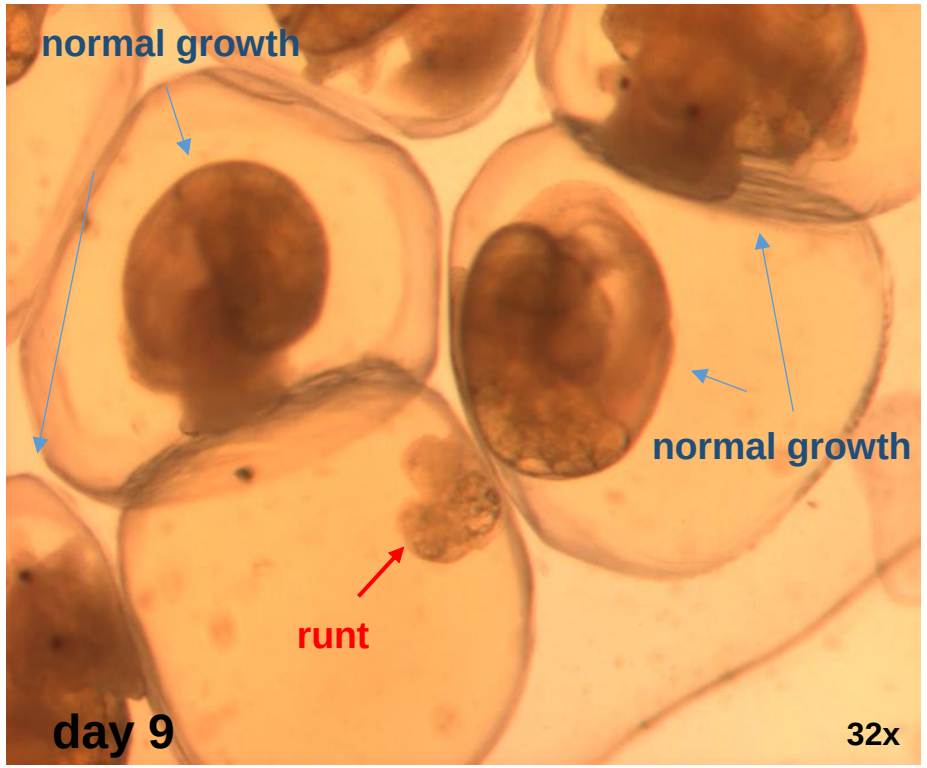
Day 6	Day 10	Day 14
Egg masses (EM) with malformed embryos N= 21 (30.4%)	Egg masses (EM) with malformed snails N= 31 (44.9%)	Egg masses (EM) with malformed snails N= 27 (39.1%)
Malformed embryos (M) N= 40 (3.8%)	Malformed snails (M) N= 90 (8.7%)	Malformed snails (M) N= 60 (5.8%)
% Malformed per EM median (range) EM with M 10% (3.3-27.2%) EM (all) 0% (0-27.2%)	**% Malformed per EM** median (range) EM with M 16.6% (2.2-60%) EM (all) 0% (0-60%)	**% 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.

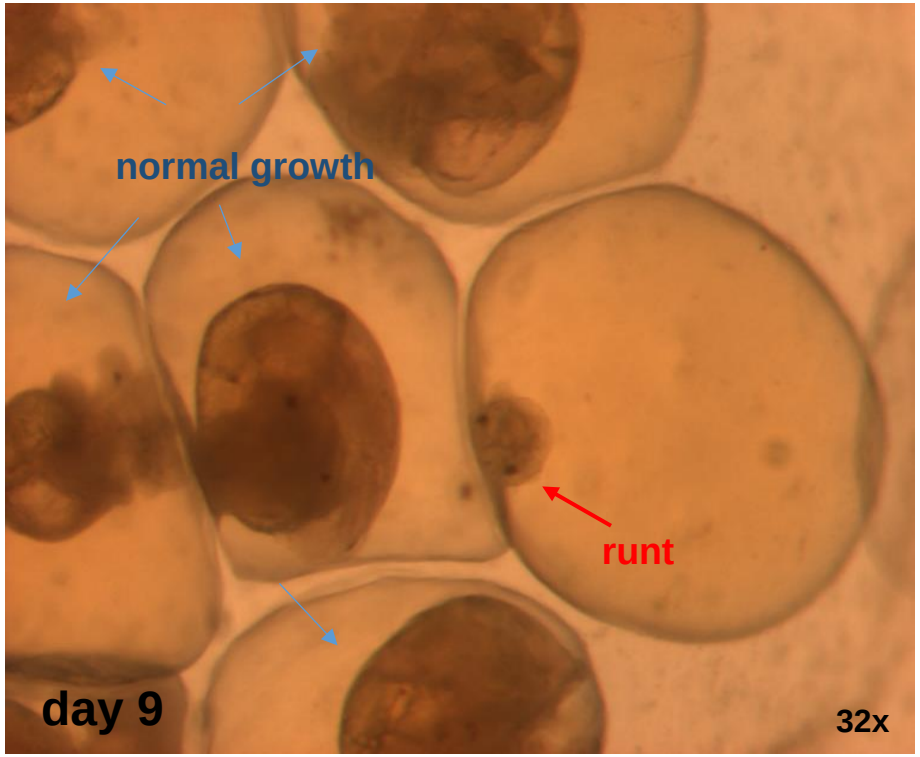
Among EMs with at least 01 malformed embryo, the median % of abnormal embryos is ca 10%

>50% EMs have no abnormal embryo

Growth retardation



Egg mass exposed to Glycerin 100 mM

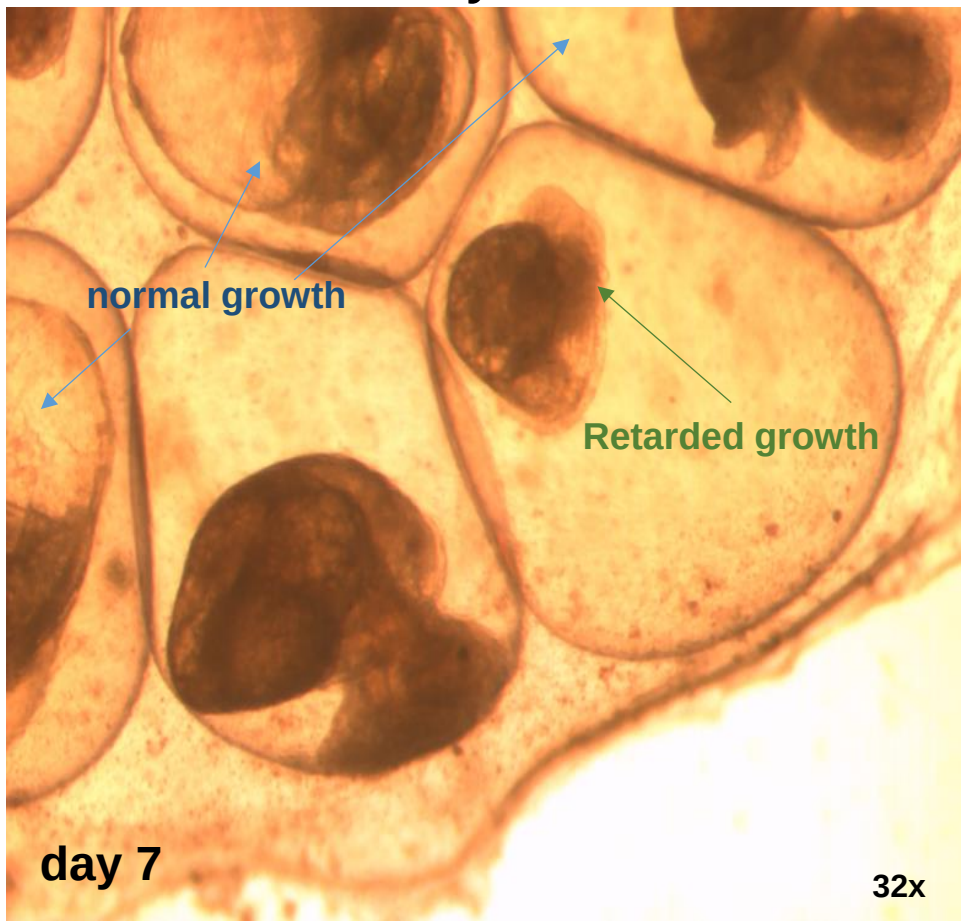


Egg mass exposed to Glycerin 100 mM

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.

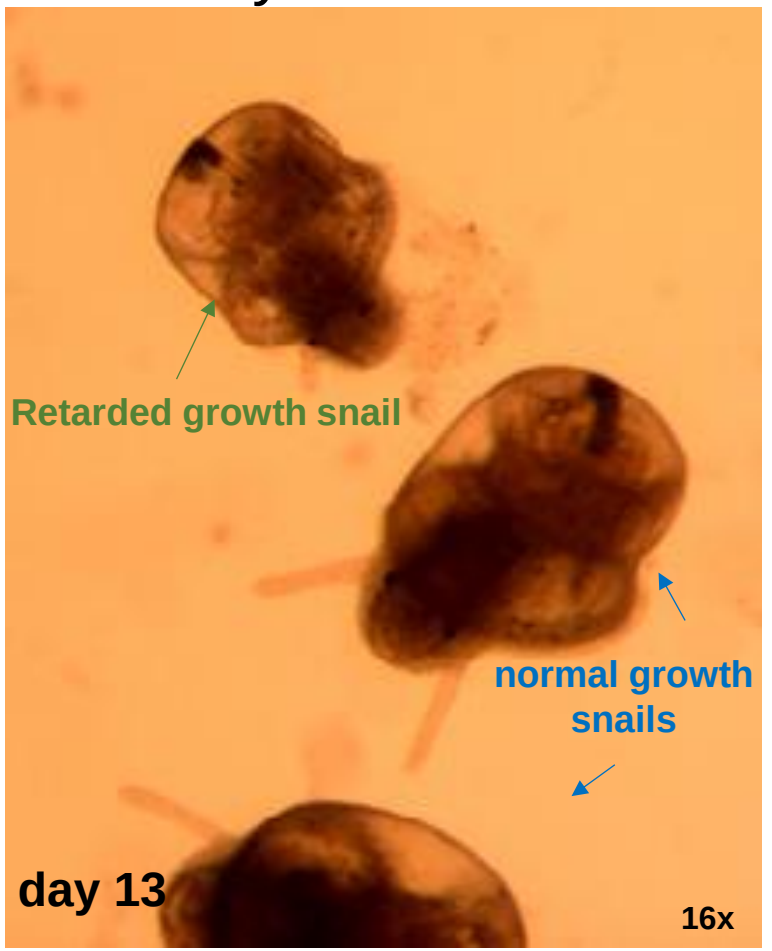
Growth retardation

embryos



Untreated egg mass

newly hatched snails



Untreated egg mass

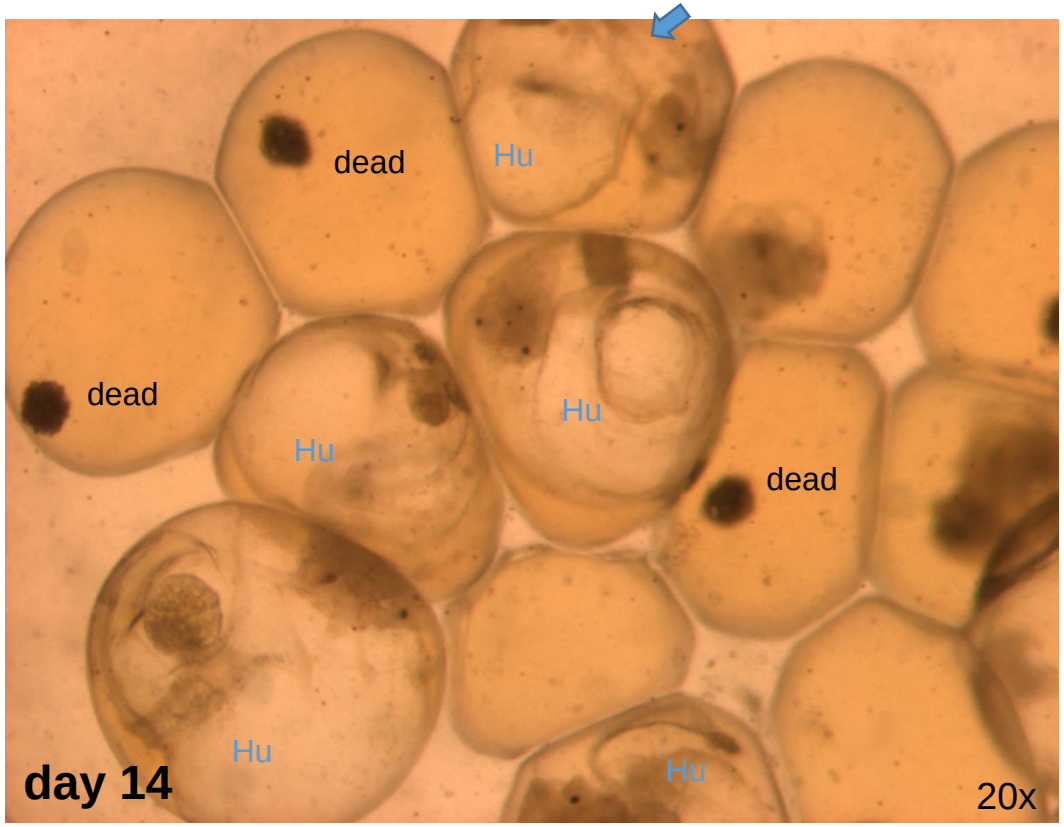
Retarded growth embryos / snails are larger than runts yet smaller than the other embryos / snails of the same egg mass. They are alive and may or may not be malformed. Retarded growth embryo generally hatch.

B.glabrata snail DevTox assay

Externally-visible abnormalities: **Hydropic malformation** (H)

Before hatching

Hydropic (Hu)

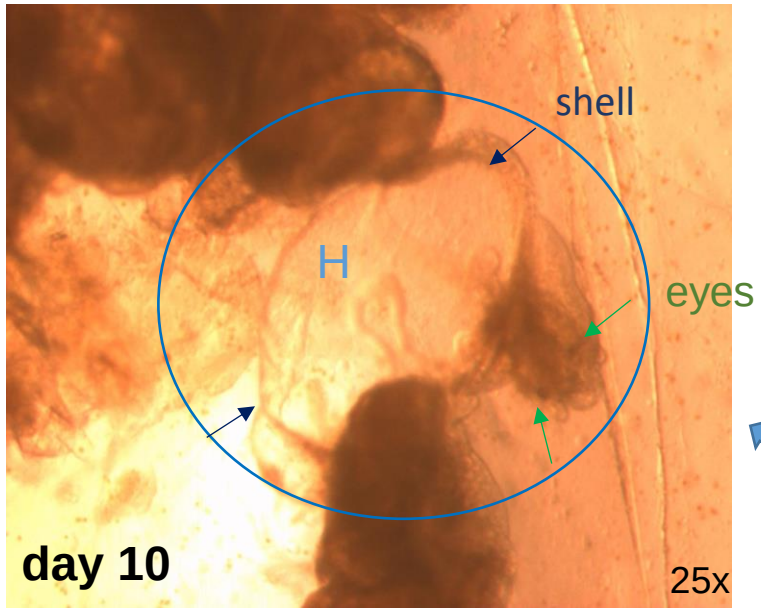


Hydropic embryos within the eggs

Egg masses exposed to methanol 1000 mM

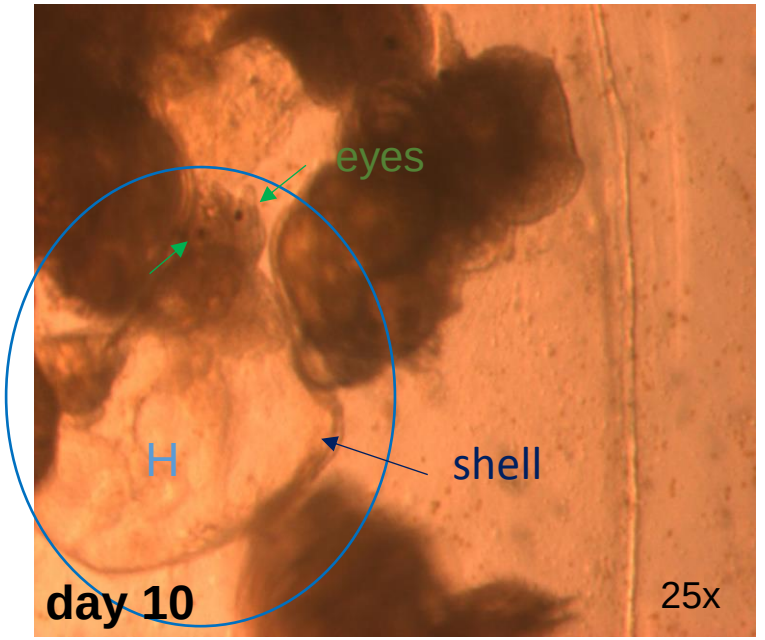
After hatching

Hydropic (H)



Hydropic snails

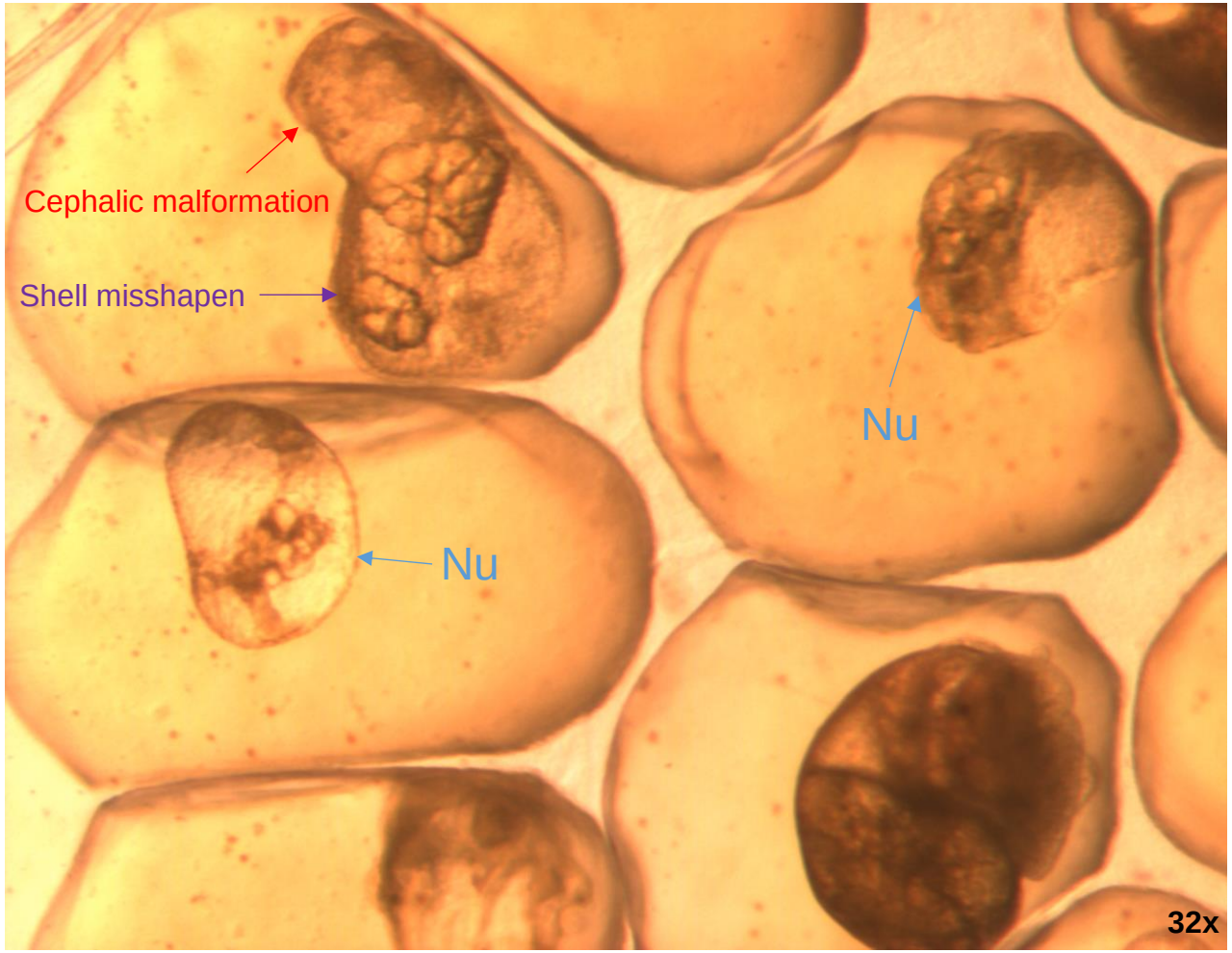
EM exposed to DSS



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 within the eggs

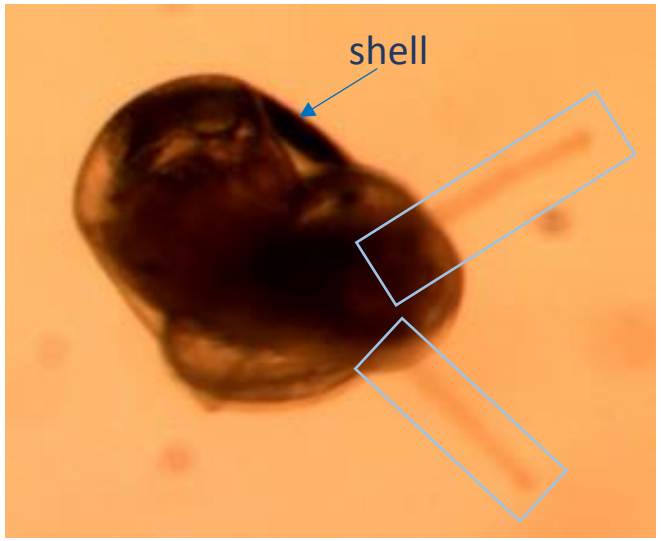
Egg mass exposed to sodium azide

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

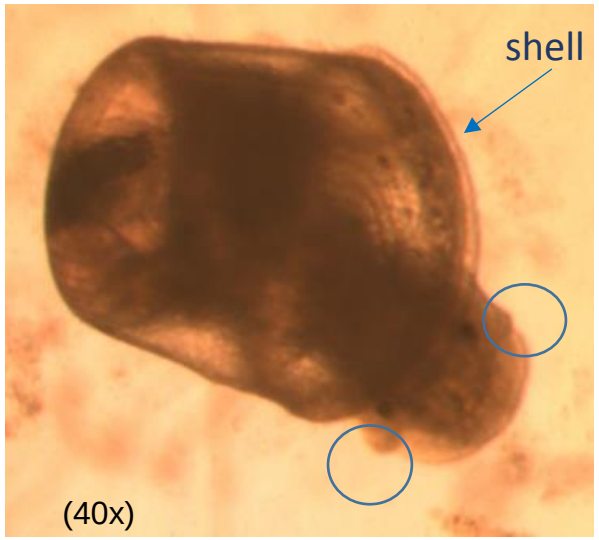
Nu: *"strikingly dysmorphic developed embryos with anomalies which are not classifiable as hydropic, shell or head malformation".*

B.glabrata snail DevTox assay

Externally-visible abnormalities: **Tentacle malformations** (T)

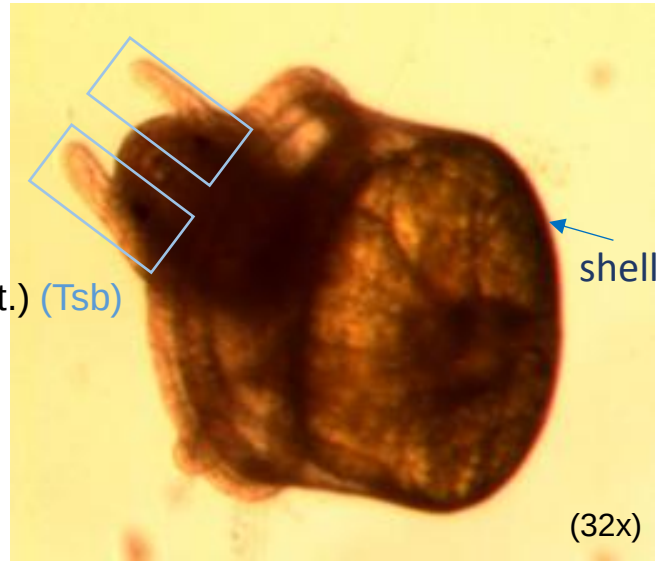


Tentacles with normal shape and size
Untreated control

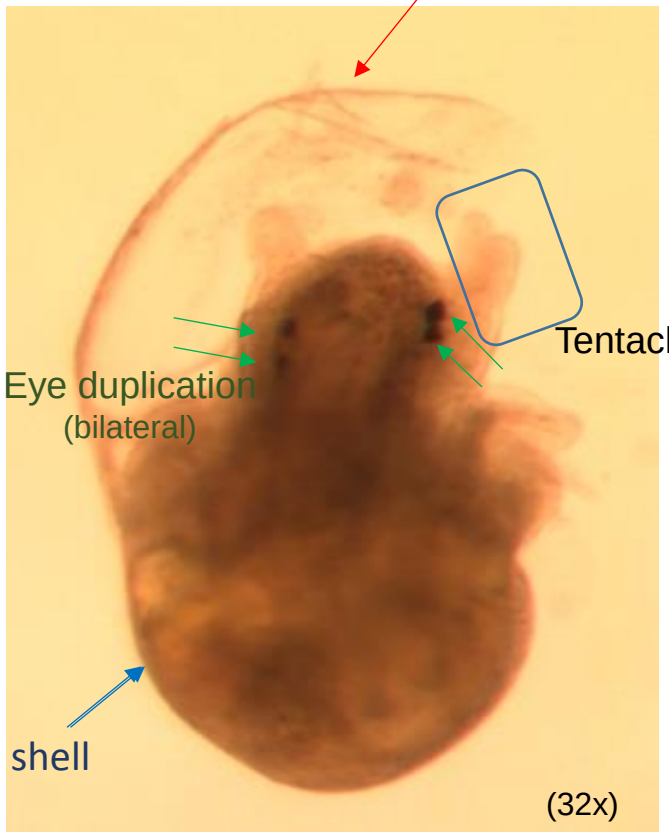


Tentacles atrophy (bilat.) (Tab)
Treated with Isopropyl alcohol 75 mM

Tentacles shorter (bilat.) (Tsb)



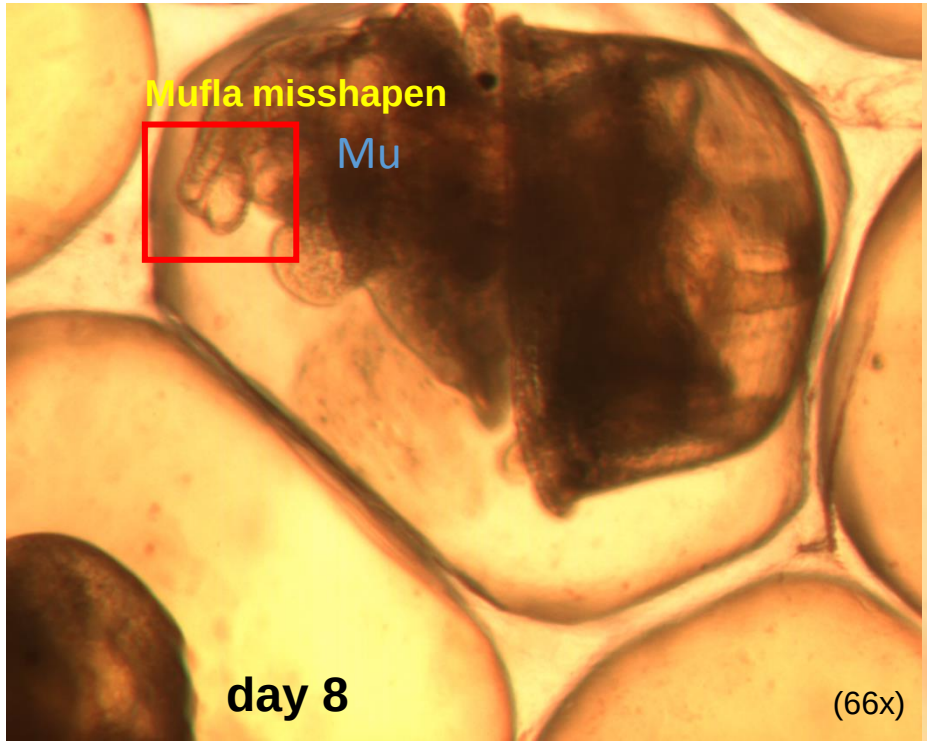
Treated with Isopropyl alcohol 50 mM



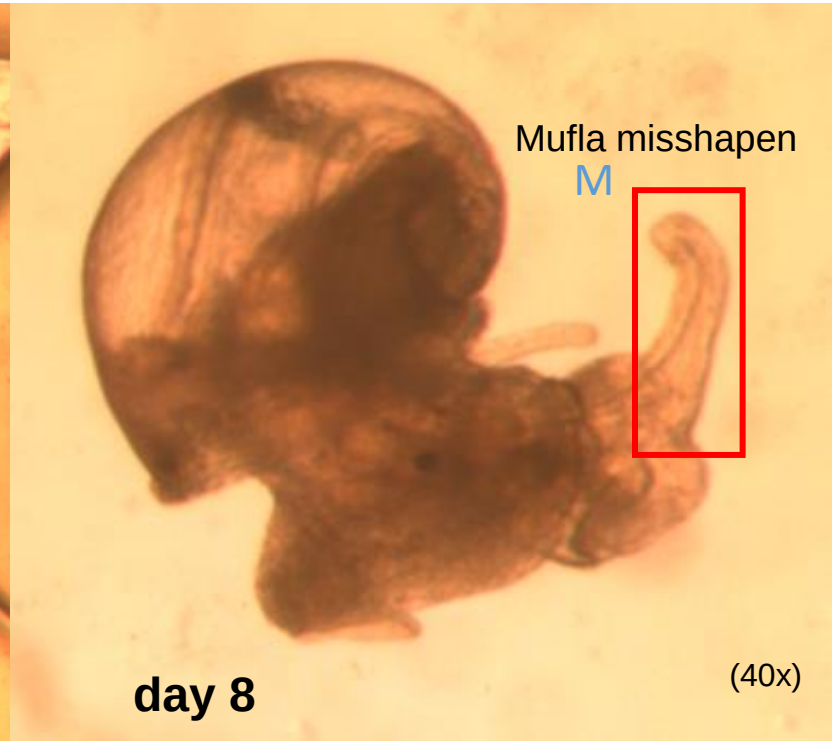
Treated with methanol 500 mM

B.glabrata snail DevTox assay

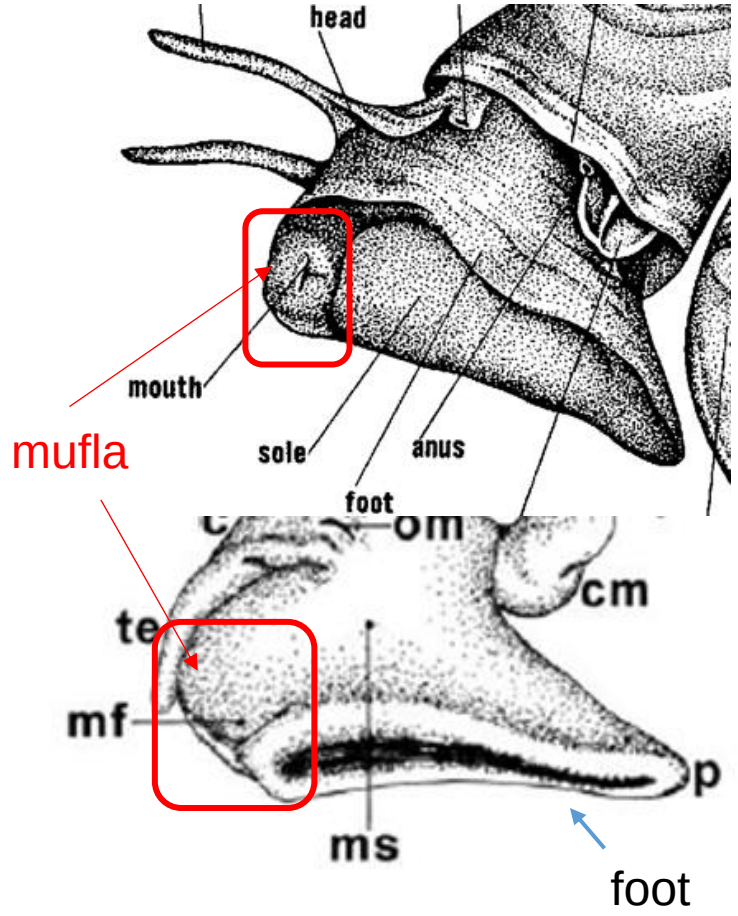
Externally-visible abnormalities: **Mufla malformation** (M)



Treated with methanol 100 mM (unhatched)



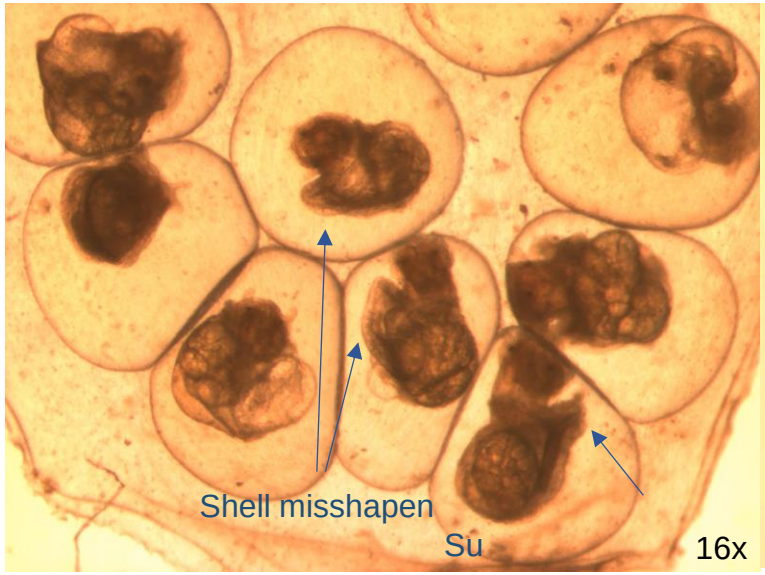
Treated with methanol 100 mM (hatched)



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

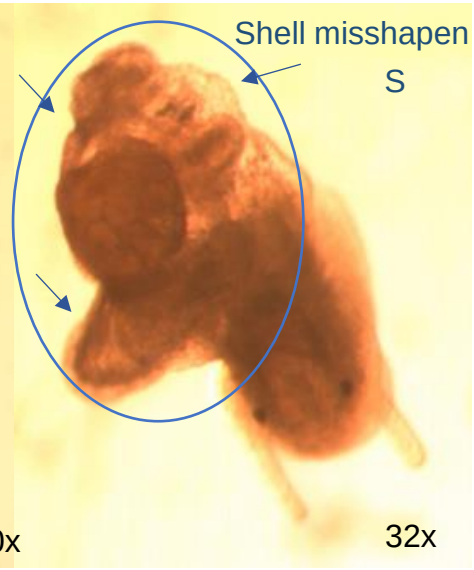
B.glabrata snail DevTox assay

Externally-visible abnormalities: **Shell malformation** (S)

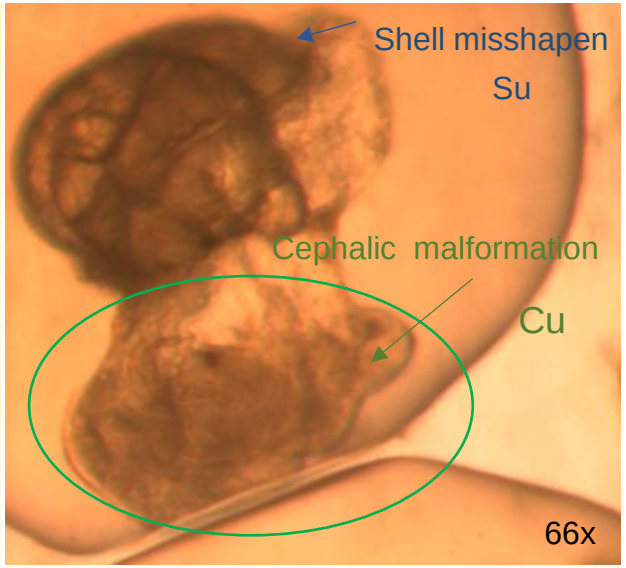


Treated with *E.milii* 50 ppm

Treated with *E.milii* 50 ppm



Treated with DSS



Treated with $CuSO_4$

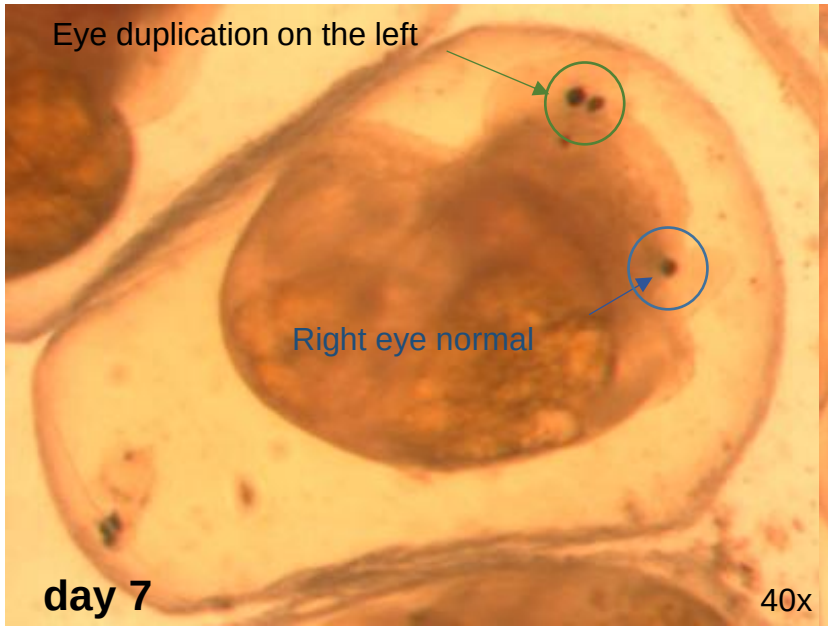
Shell anomaly **within the egg**

Shell anomaly **after hatching**

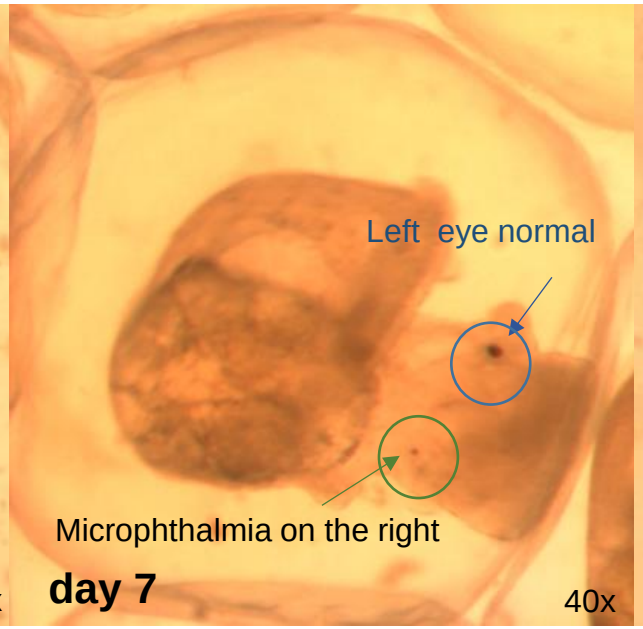
Shell anomaly **within the egg**

B.glabrata snail DevTox assay

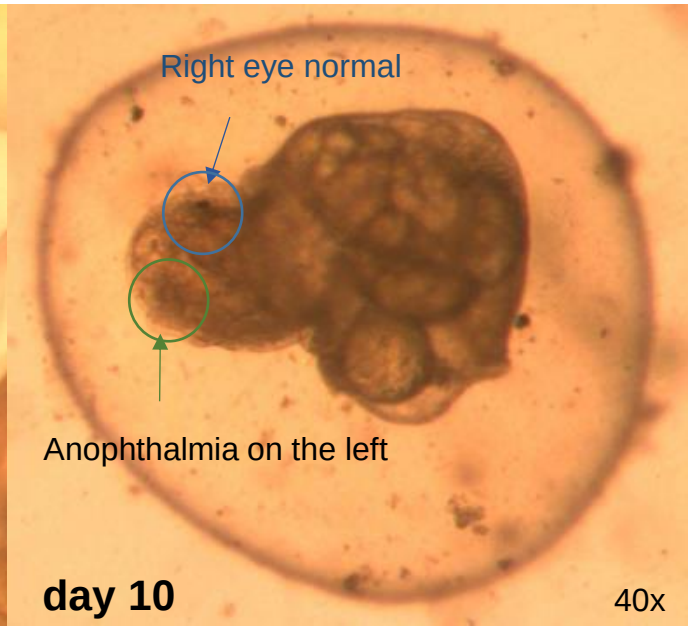
Externally-visible abnormalities: **Eye malformations** (E)



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**: <u>Foot misshapen</u>** (F)

EM treated with methanol 250 mM

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

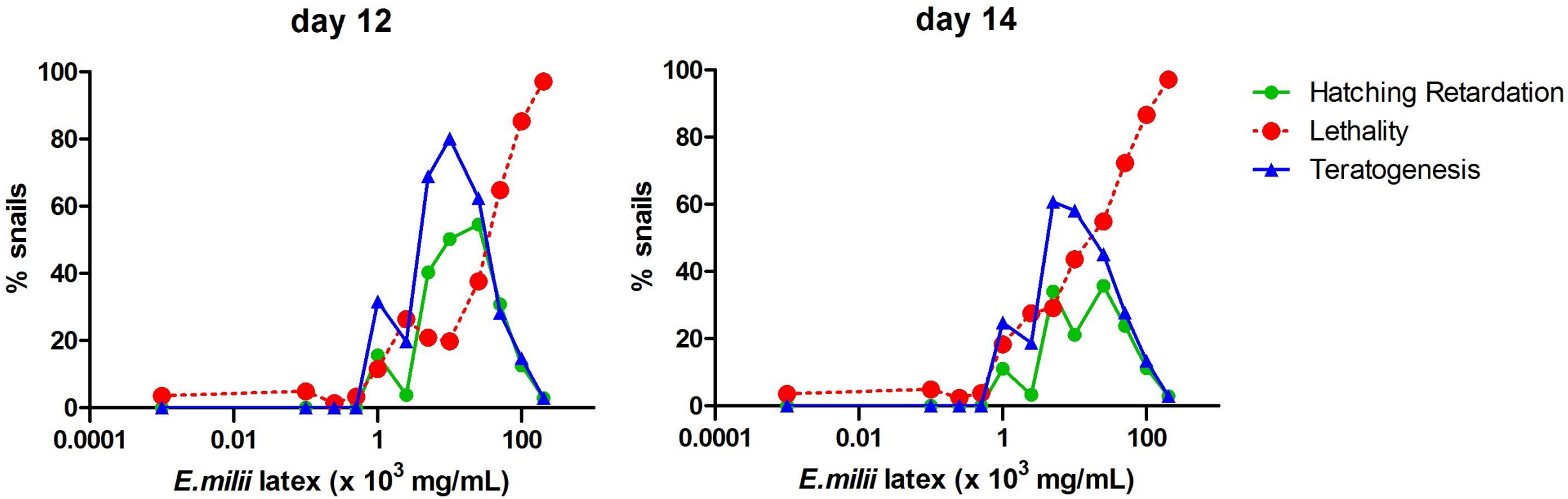
Snail **DevTox** Assay results

Dose-response curves for DevTox endpoints

on days 12 and 14 after spawning

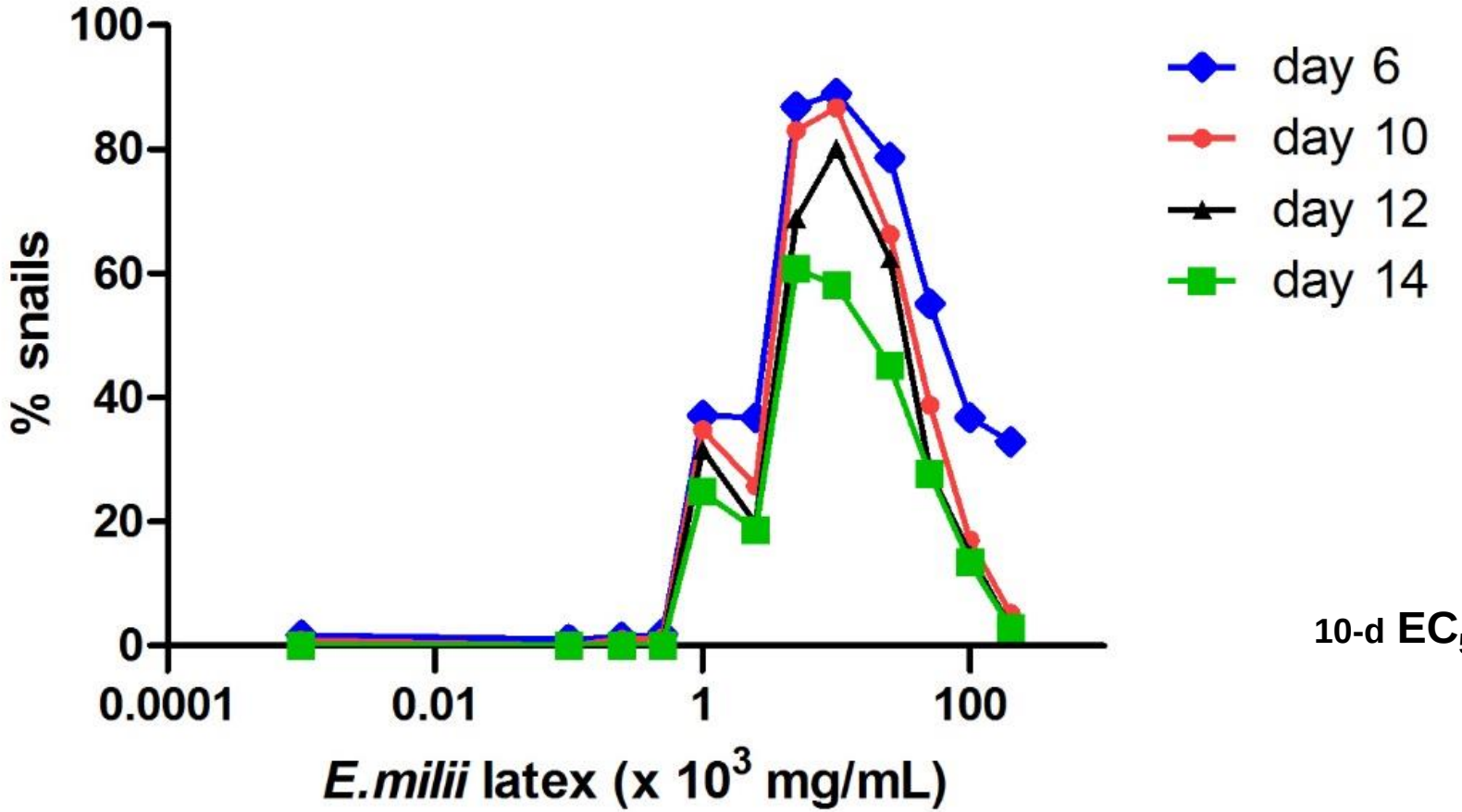
(exposure days1-4)

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



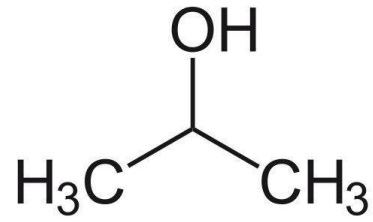
Teratogenesis - malformed embryo/snails on days 6, 10, 12 and 14

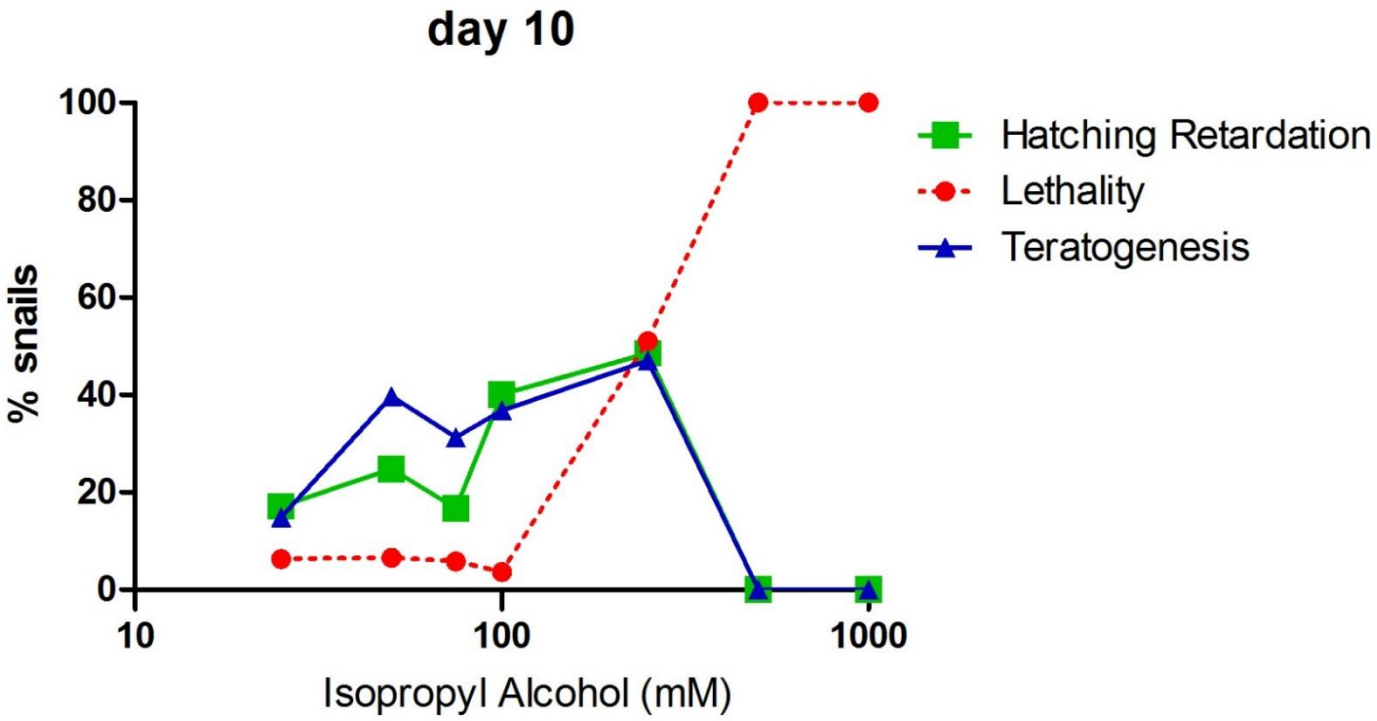
(exposure: days 1-4 post spawning)

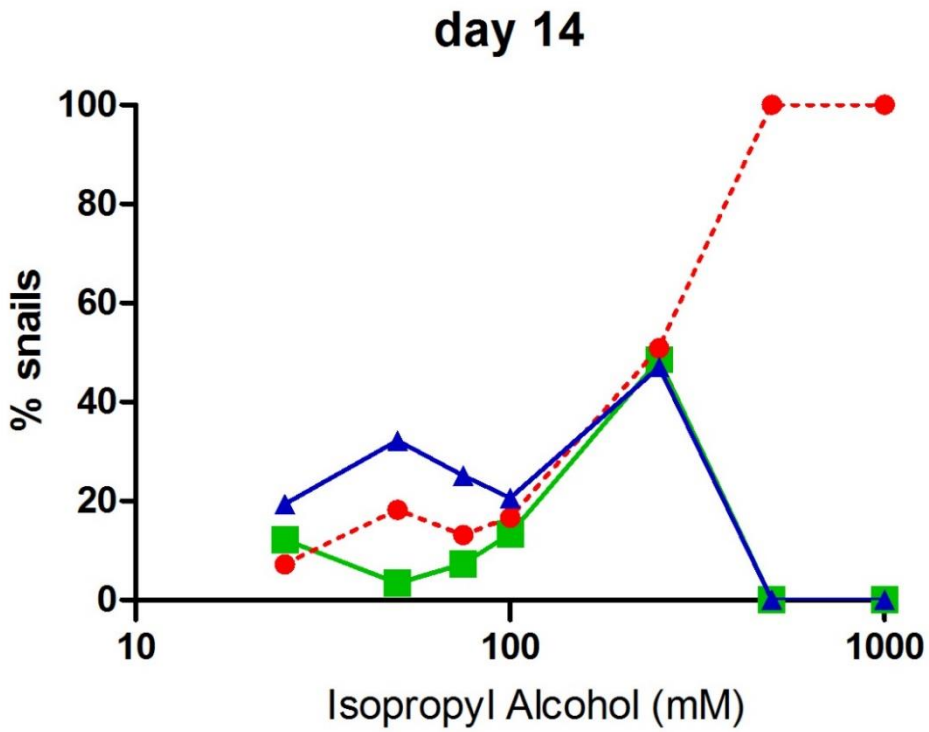


$^{10\text{-d}}EC_{50}$ = 2040 µg/L

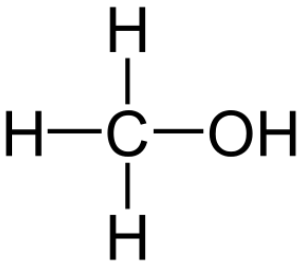
Isopropanol

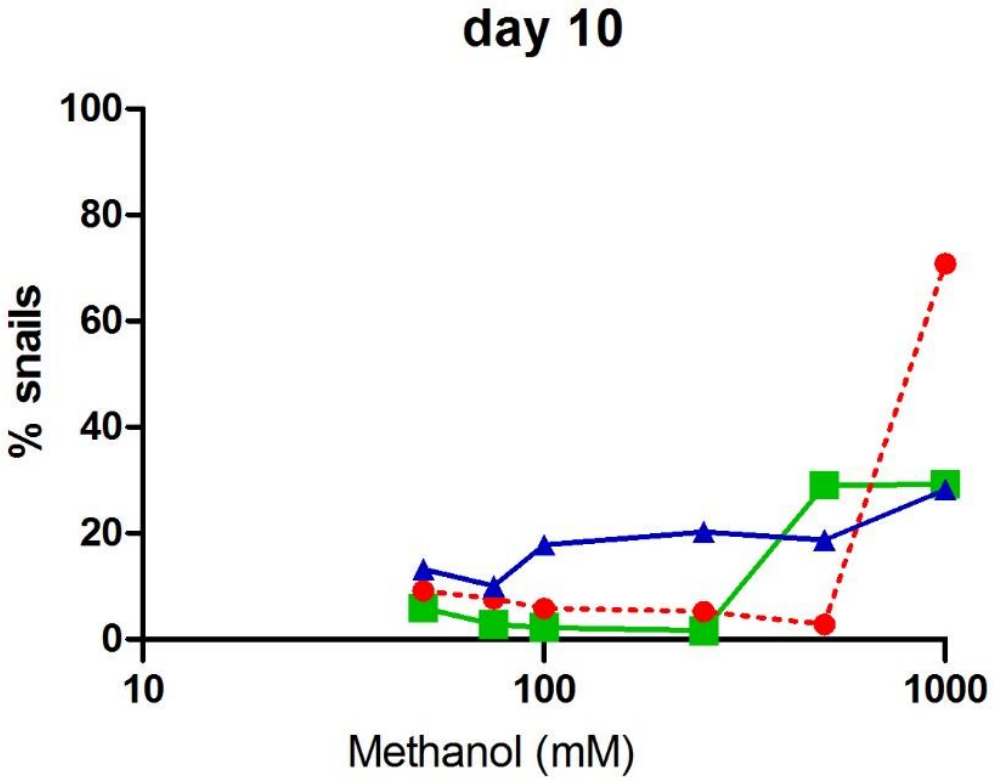


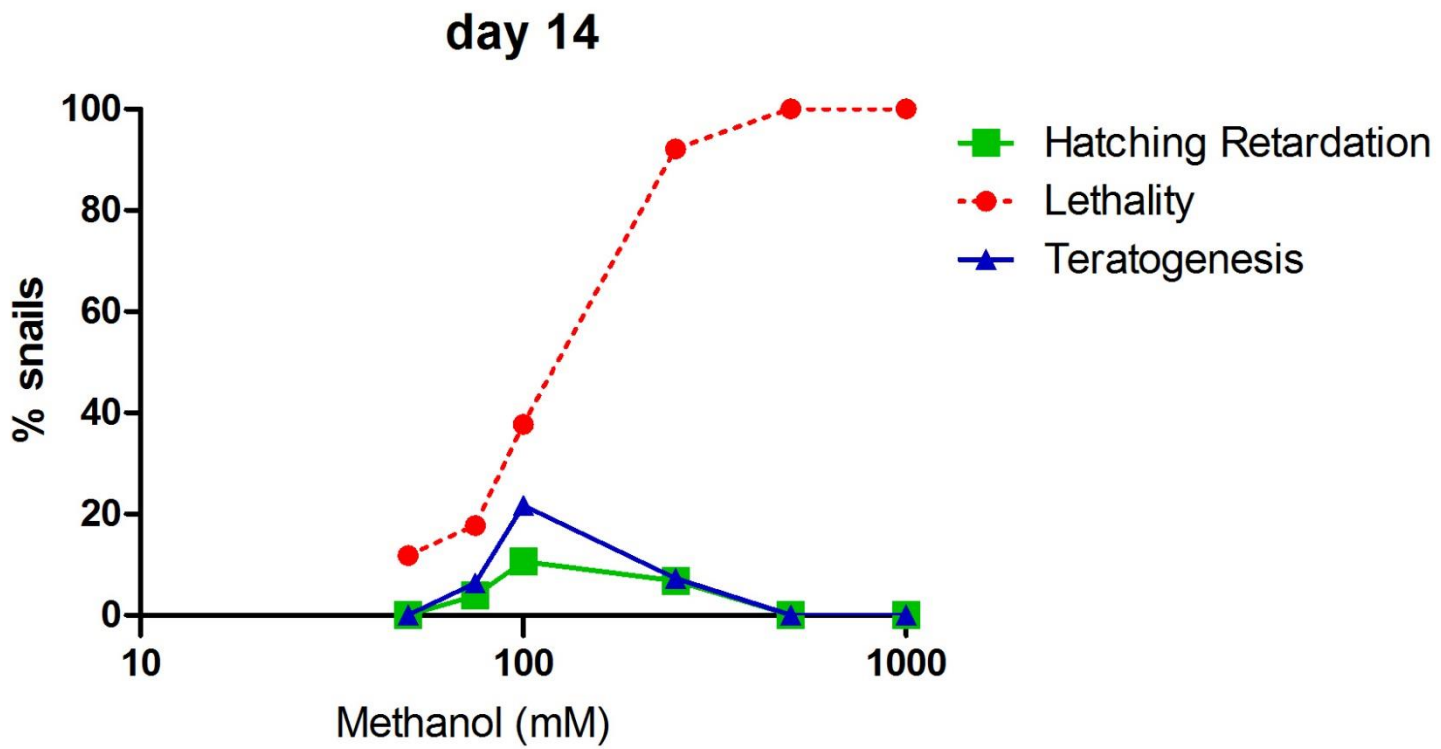




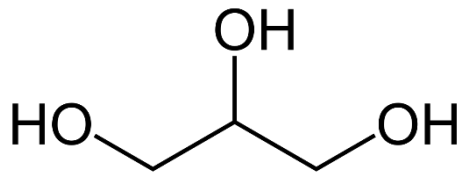
Methanol

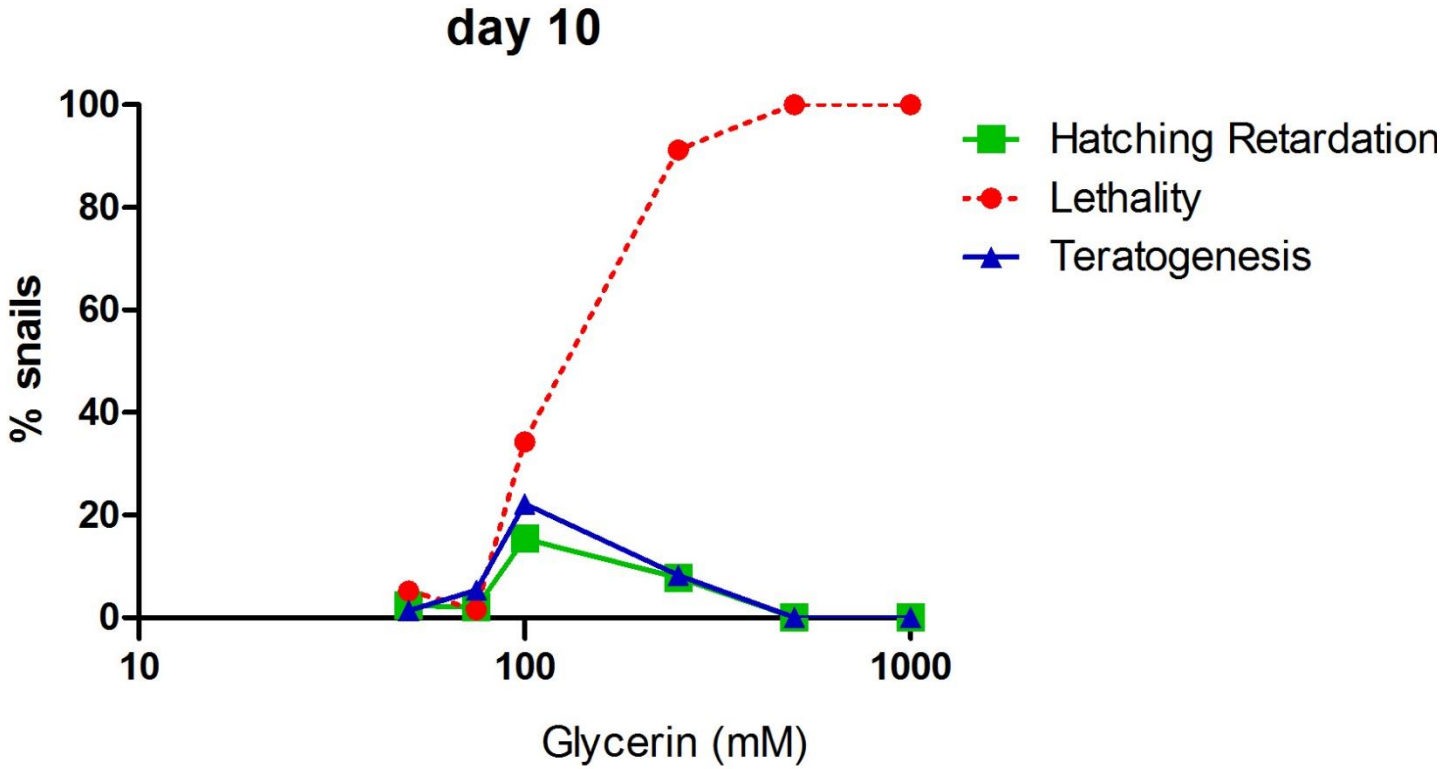

H
H—C—OH
H

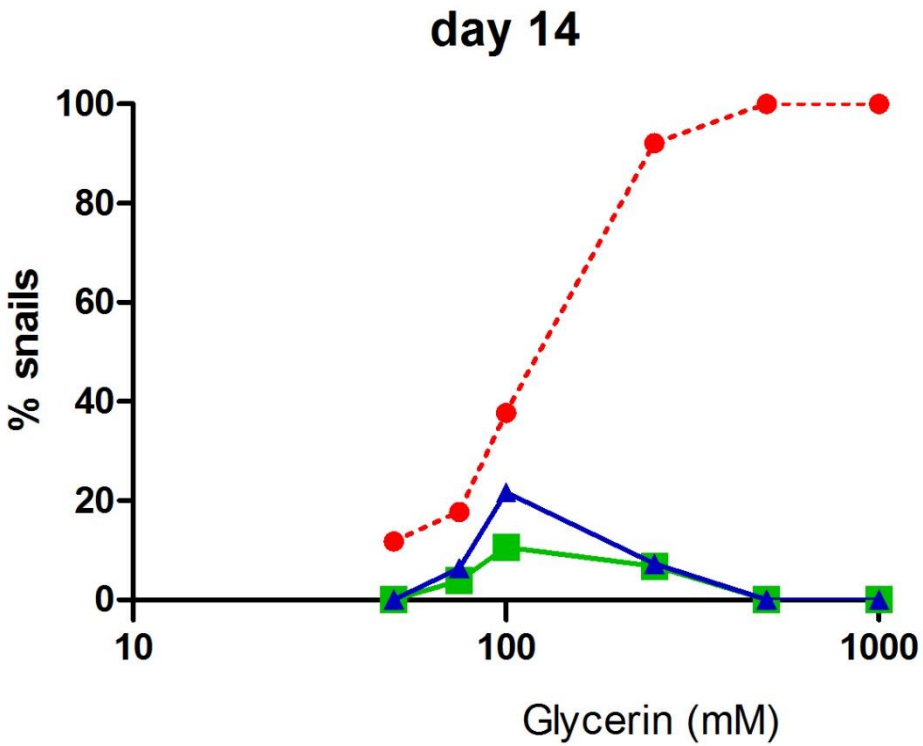

day 10
100
80
60
40
20
0
% snails
10
100
1000
Methanol (mM)


day 14
100
80
60
40
20
0
% snails
10
100
1000
Methanol (mM)
Hatching Retardation
Lethality
Teratogenesis

Glycerin

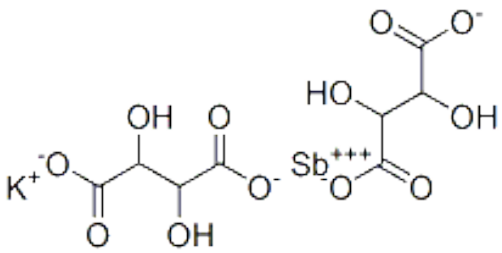

OH
HO
OH

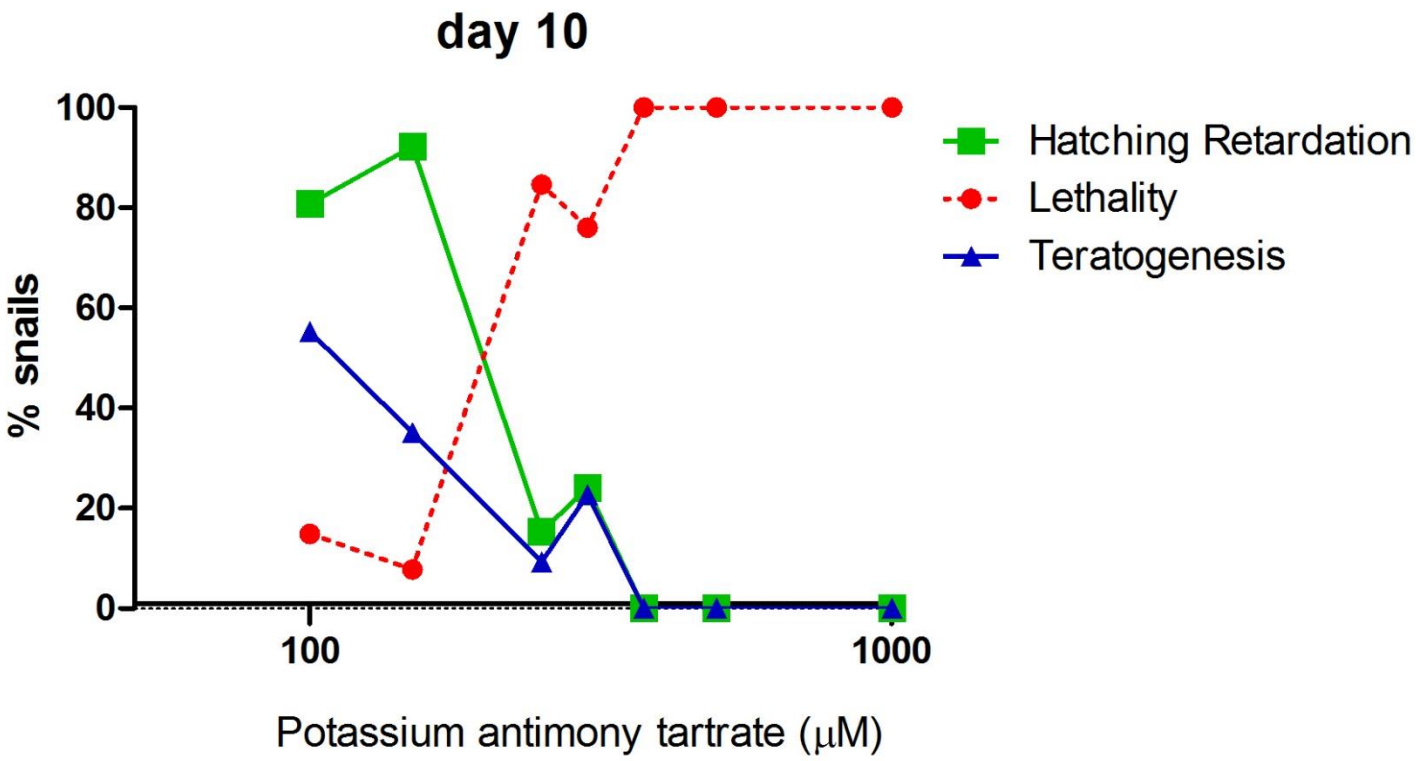

day 10
100
80
60
40
20
0
% snails
10
100
1000
Glycerin (mM)
Hatching Retardation
Lethality
Teratogenesis

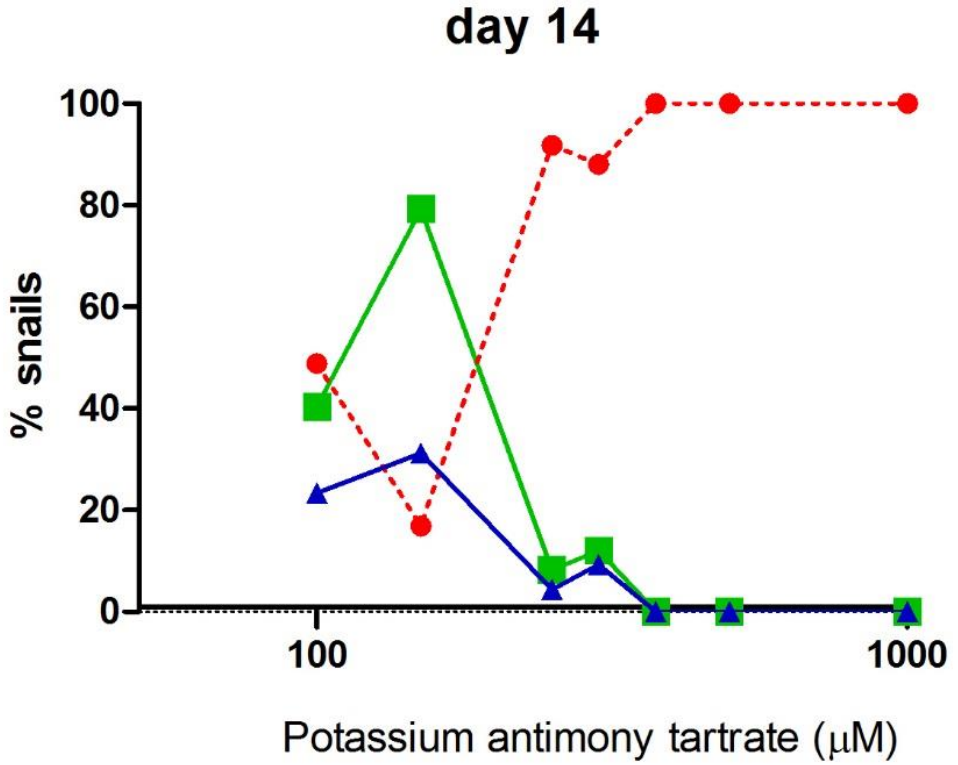

day 14
100
80
60
40
20
0
% snails
10
100
1000
Glycerin (mM)

Potassium antimony tartrate

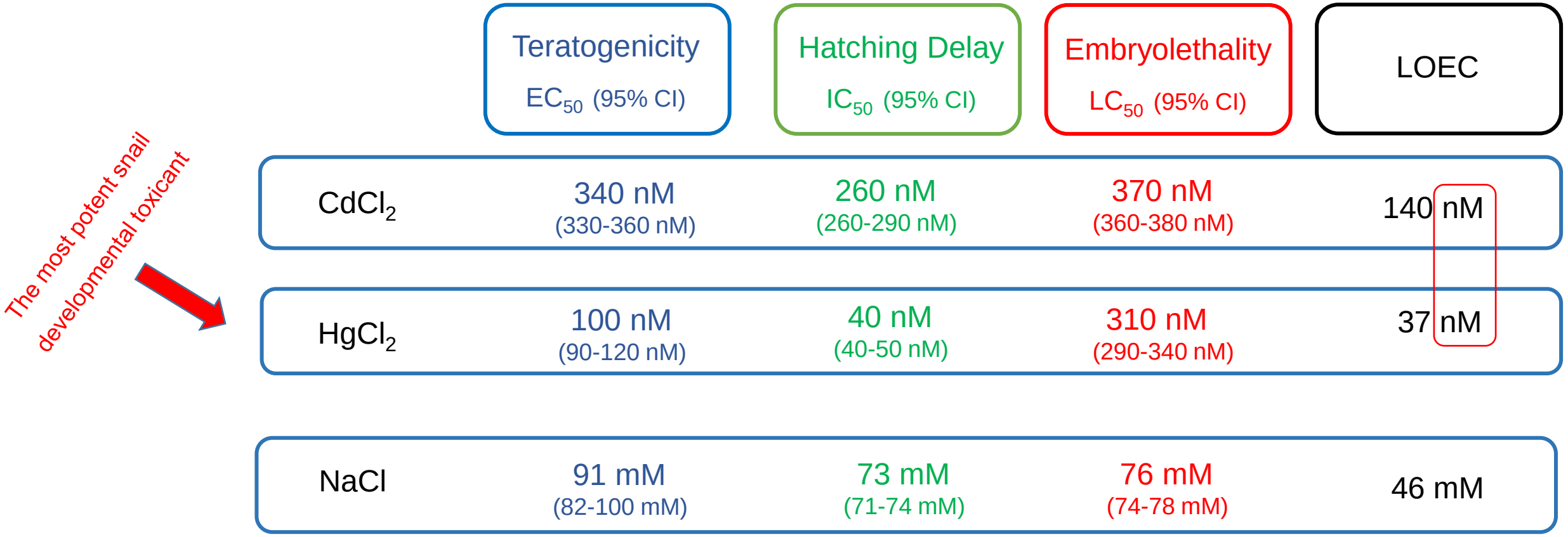
Sb^{3+}



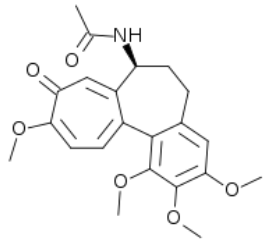


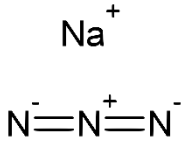


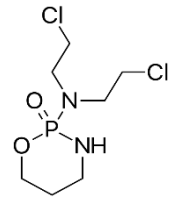
Developmental toxicity of cadmium, mercury and sodium chloride



Developmental toxicity of sodium azide, cyclophosphamide and colchicine



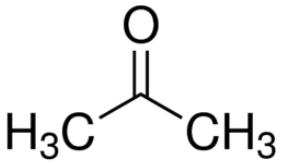
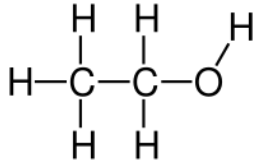
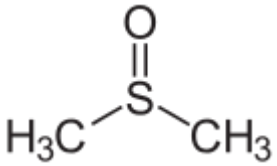




	Teratogenicity EC_{50} (95% CI)	Hatching Delay IC_{50} (95% CI)	Embryolethality LC_{50} (95% CI)	LOEC
Colchicine	1.1 µM (1.0-1.2 µM)	0.9 µM (0.8-1.0 µM)	2.2 µM (2.1-2.3 µM)	0.2 µM
Sodium Azide	100 µM (90-100 µM)	140 µM (130-160 µM)	160 µM (150-170 µM)	48 µM
Cyclophosphamide	13 mM (12-15 mM)	5 mM (5-6 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)

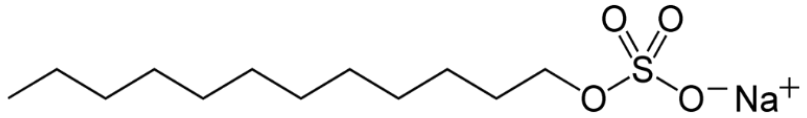
	Teratogenicity EC_{50} (95% CI)	Hatching Delay IC_{50} (95% CI)	Embryolethality LC_{50} (95% CI)	LOEC
 Acetone	170 mM (170-180 mM)	180 mM (180-190 mM)	170 mM (160-180 µM)	30 mM
 Ethanol	NC	260 mM (220-300 mM)	270 mM (250-280 mM)	17 mM
 DMSO	NC	120 mM (110-130 mM)	160 mM (150-160 mM)	14 mM

If these solvents are used to dissolve a test substance, final concentrations in the assay softwater water should not to excceed 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)	Embryolethality LC_{50} (95% CI)	LOEC
DSS	100 µM	140 µM (130-140 µM)	200 µM (190-210 µM)	12 µM
H_2O_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**
E.milii 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

DSS: Dodecyl sodium sulfate

* [Oliveira-Filho et al, 2010]

	Xenopus laevis (FETAX)		*B.glabrata* DevTox assay	
	LC_{50}	**EC_{50}**	**LC_{50}**	**EC_{50}**
$CdCl_2$	32 µM*	3.7 µM*	0.37 µM	0.34 µM
Ethanol	239 mM[+]	152 mM[+]	270 mM	NT
$CuCl_2$	0.9 mg/L[#]	0.4 mg/L[#]	-	-
$CuSO_4$	-	-	2.2 mg/L[++]	NT[++]
H_2O_2	598 µM[§]	536 µM[§]	470 µM	NT
$HgCl_2$	601 nM**	513 nM**	310 nM	100 nM

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

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

The frog and the snail assays gave rise to fairly comparable LC_{50} and EC_{50} concentrations for these chemicals

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**

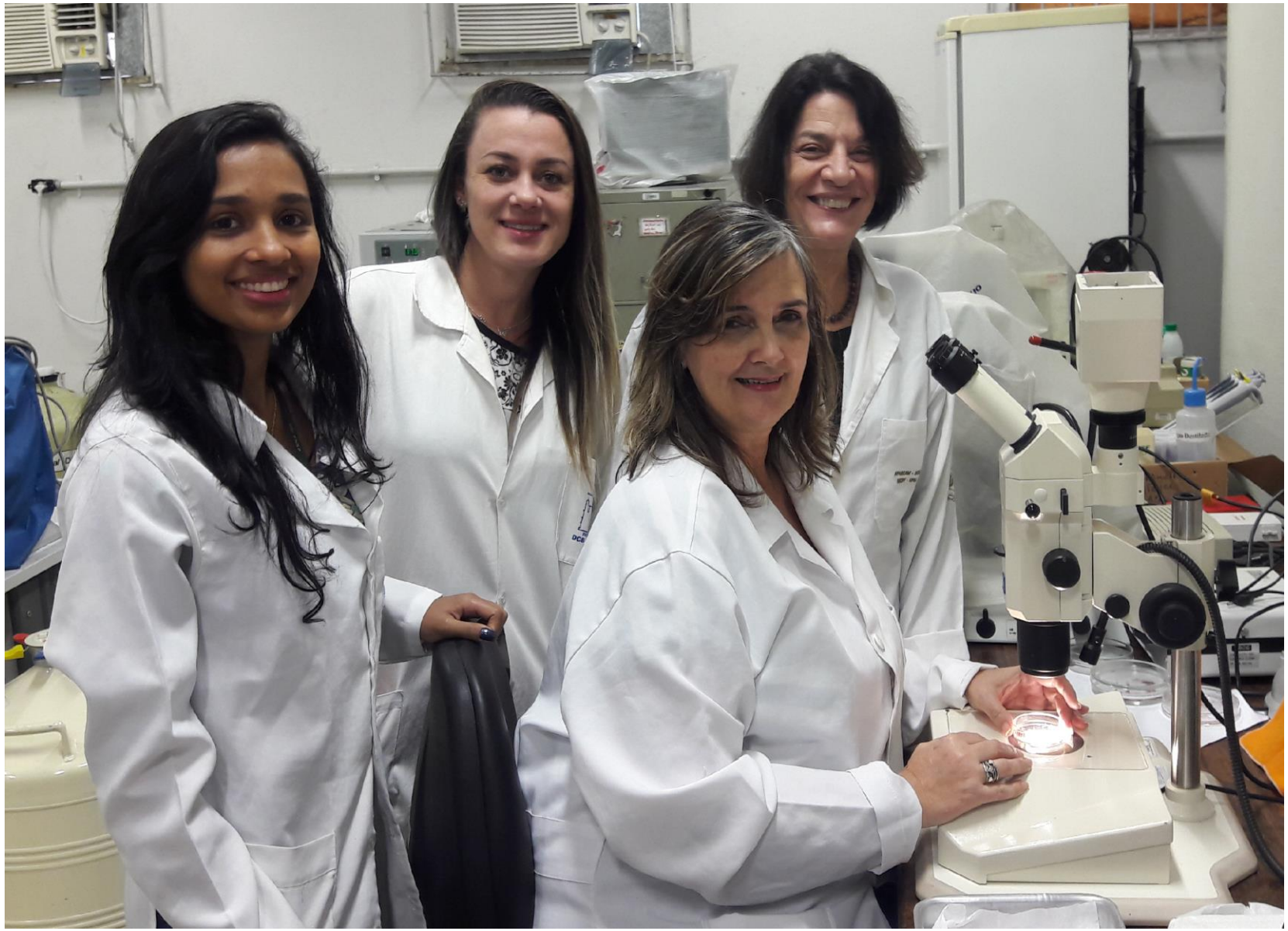
It may also be used for Developmental Biology (mechanistic) studies

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

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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.