A snail's tail: In search of the mechanism for tributyltin-induced imposex

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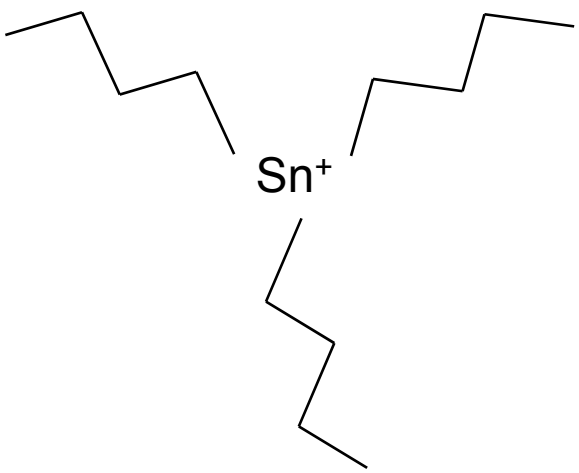
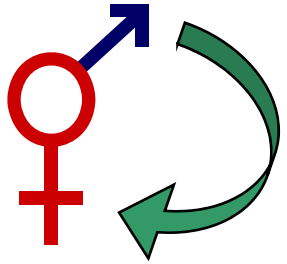
Tributyltin (TBT)



- **Biocide** in antifouling paints
- **Fungicide**
- **Catalyst**
- **Protectant** against microbial decomposition

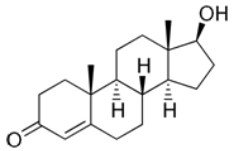
imposex:
imposition of male **sex** characteristics onto female neogastropods

TBT



imposex

+ penis,
vas deferens,
seminiferous tubules



testosterone

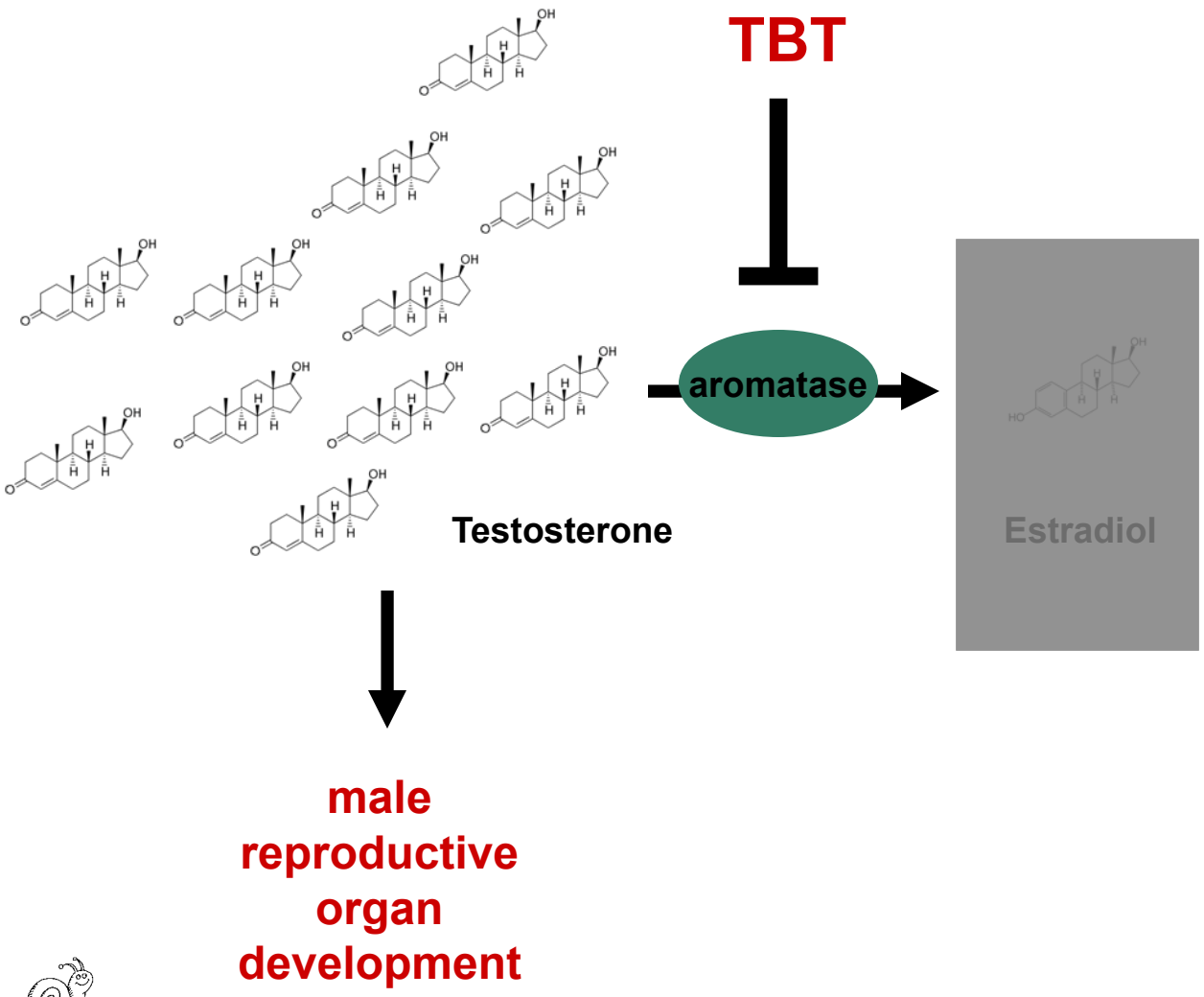
Hypothesis for TBT's MOA

- elevates free testosterone levels

TBT → ↑ free testosterone → male reproductive tract development

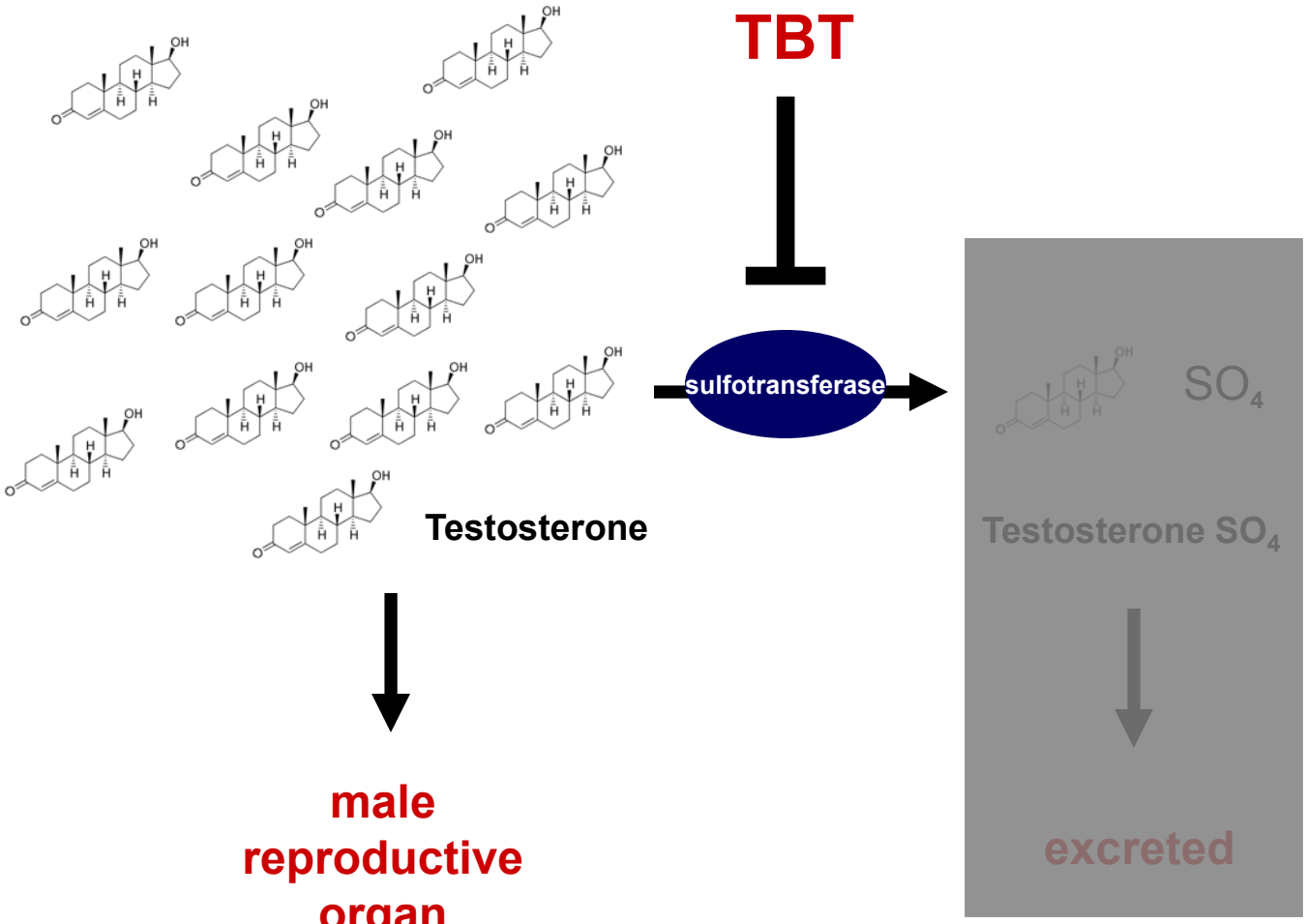
Hypothesis for TBT's MOA

- elevates free testosterone levels



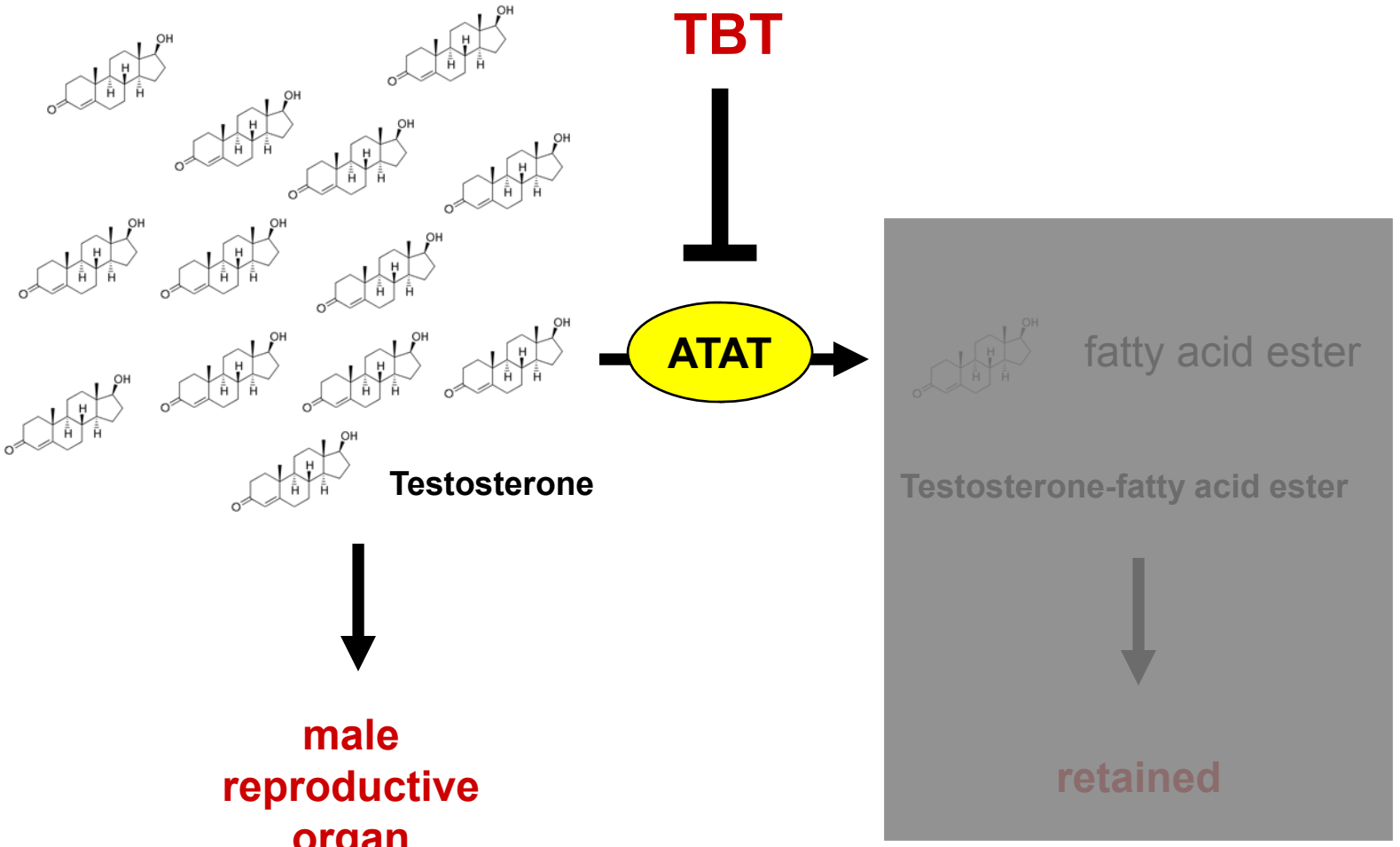
Hypothesis for TBT's MOA

- elevates free testosterone levels



Hypothesis for TBT's MOA

- elevates free testosterone levels



Hypotheses for TBT's MOA

- elevates free testosterone levels

① ②

TBT → ↑ free testosterone → male reproductive tract development

For this hypothesis to be accepted:

① the means by which TBT increases free testosterone must be confirmed

② the underlying assumption that testosterone functions in processes related to the development of the reproductive tract in neogastropods must be verified

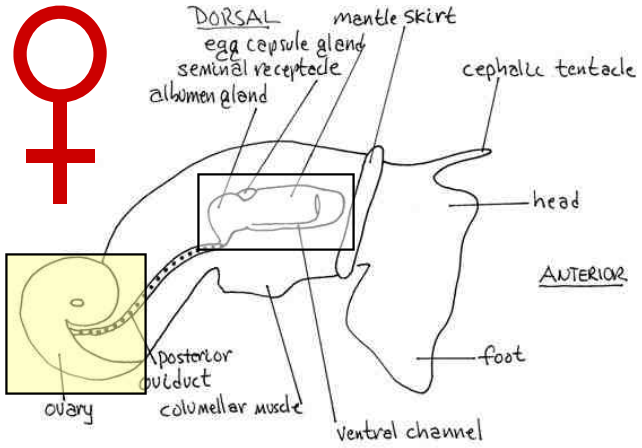
Model species: Eastern mud snail (*Ilyanassa obsoleta*)

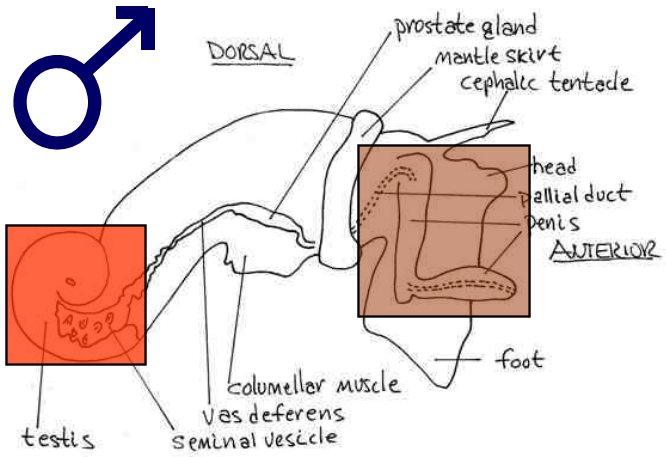
Phylum: Mollusca

Class: Gastropoda

Order: Caenogastropoda

Family: Nassariidae





Hypotheses for TBT's MOA

TBT → ↑ free testosterone → male reproductive tract development

Hypothesis for TBT's MOA

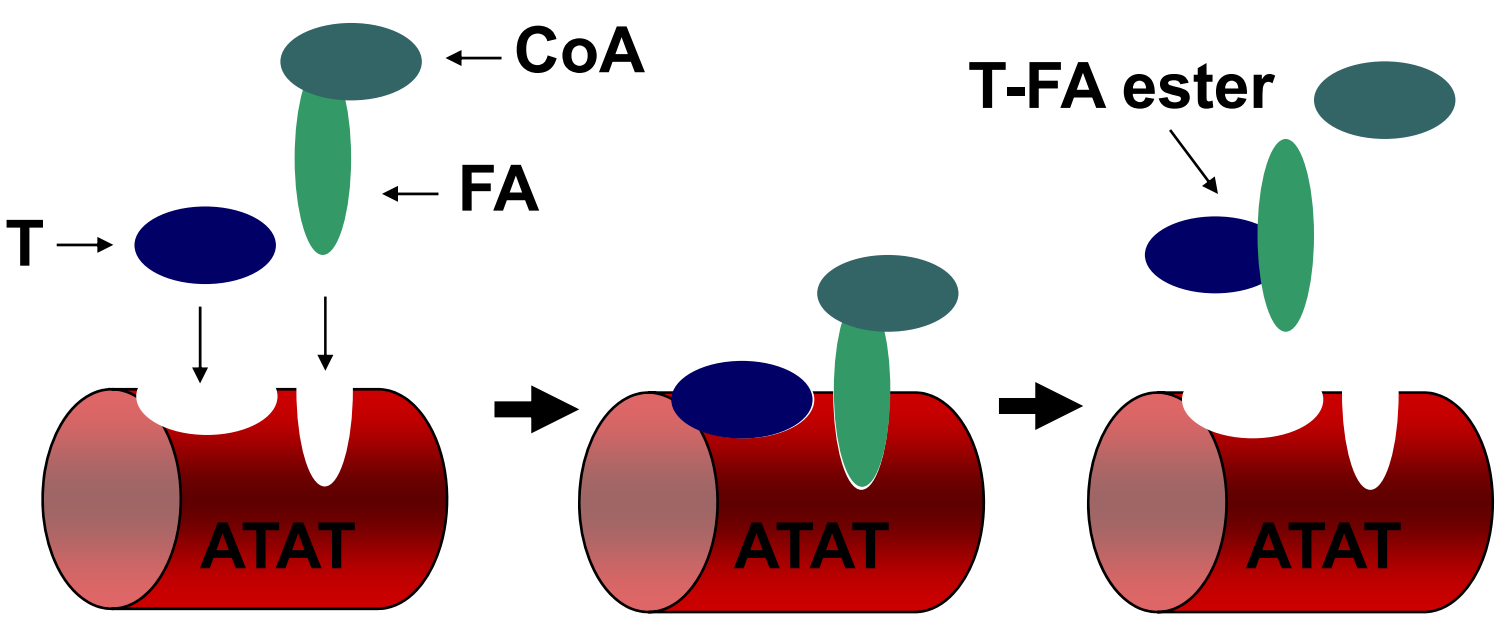
TBT $\xrightarrow{①}$ **↑ free testosterone** $\xrightarrow{②}$ **male reproductive tract development**

① How does TBT increase testosterone?

② Is the underlying assumption valid?
(i.e., testosterone as a functional androgen)

How does TBT increase testosterone?

In the eastern mud snail:



T = testosterone
FA = fatty acid
CoA = Coenzyme A
ATAT = acyl-Coenzyme A:testosterone acyltransferase

Gooding and LeBlanc (2001, 2004)

How does TBT increase testosterone?

Gooding et al. (2003):

TBT → ↑ free testosterone

↓ testosterone-fatty acid esters

↔ ATAT protein

Microsomes

TBT → ↔ ATAT activity

≤ 10 µg/L (0.03 µM)

How does TBT increase testosterone?

Gooding et al. (2003):

TBT → ↑ free testosterone

↓ testosterone-fatty acid esters

↔ ATAT protein

Microsomes

TBT → ↔ ATAT activity

≤ 10 µg/L (0.03 µM)

	Low-tin affected site	High-tin affected site
TBT (µM)	0.7	5.9
TBT (µg/kg)	80	700
% imposex	6	100

How does TBT increase testosterone?

HYPOTHESIS:

TBT increases free testosterone in neogastropods by inhibiting ATAT.

OBJECTIVE:

- determine if *in vitro* ATAT activity of *I. obsoleta* is directly inhibited by TBT at toxicologically-relevant *in vivo* concentrations, i.e., concentrations measured in field-collected neogastropods from TBT-contaminated areas

How does TBT increase testosterone?

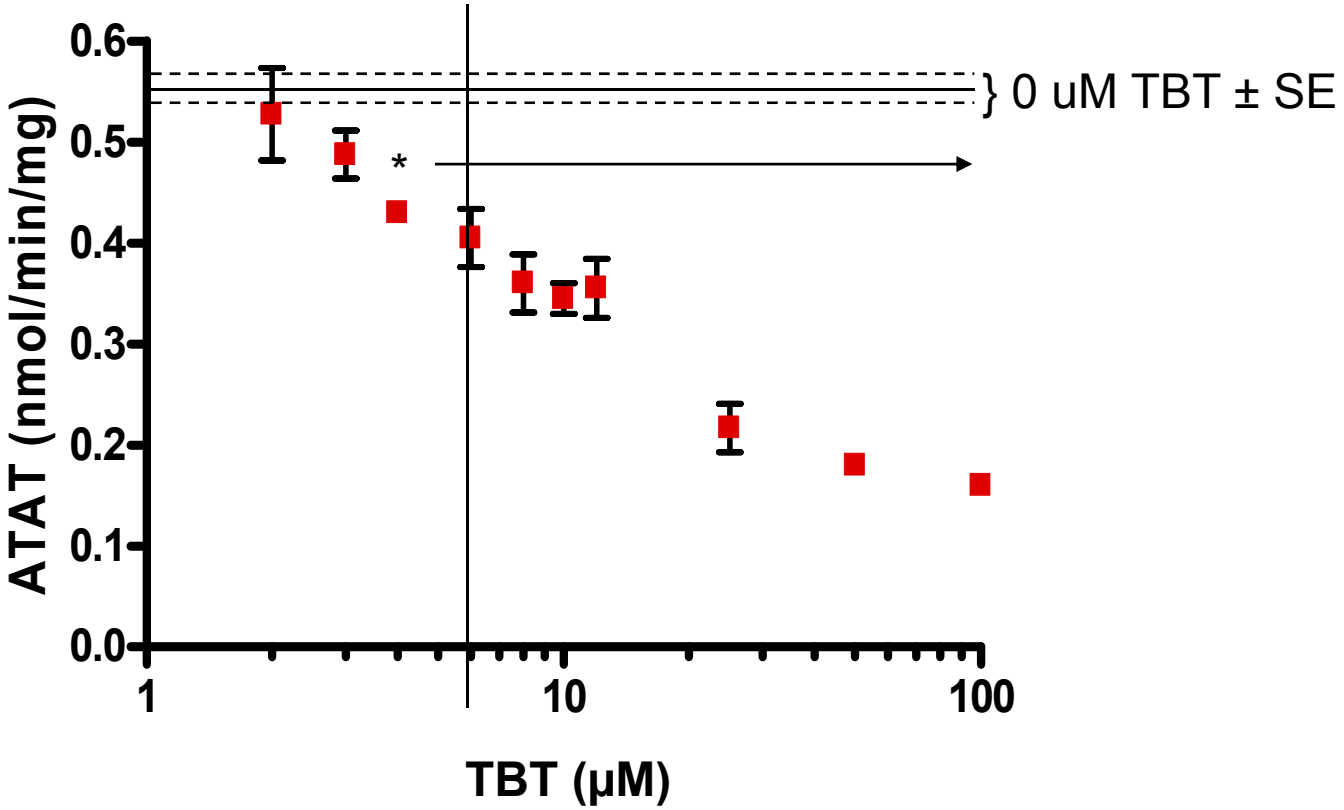
Microsomes
+
Testosterone
+
Fatty acid Coenzyme A
+

TBT

How does TBT increase testosterone?

Field-collected snails

5.9 µM TBT



How does TBT increase testosterone?

CONCLUSION:

TBT elevates free testosterone in neogastropods by inhibiting their major regulatory process for maintaining free testosterone homeostasis – the fatty acid esterification of testosterone.

Hypotheses for TBT's MOA

TBT $\xrightarrow{①}$ **↑ free testosterone** $\xrightarrow{②}$ **male reproductive tract development**

① How does TBT increase testosterone? ⊣ ATAT

➡ ② Is the underlying assumption valid?
(i.e., testosterone as a functional androgen)

Is the underlying assumption valid?

ASSUMPTION:

Testosterone functions in processes related to the development of the reproductive tract in neogastropods (i.e., recrudescence).

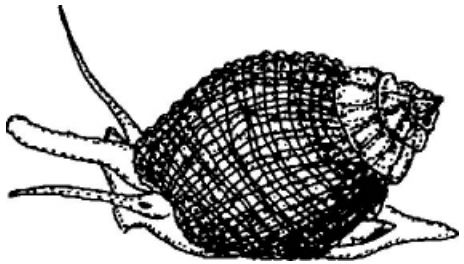
Word of the day:

recrudescence: a return of something after a period of abatement

(http://www.thefreedictionary.com/recrudescence)

Is the underlying assumption valid?

EMPIRICAL EVIDENCE:

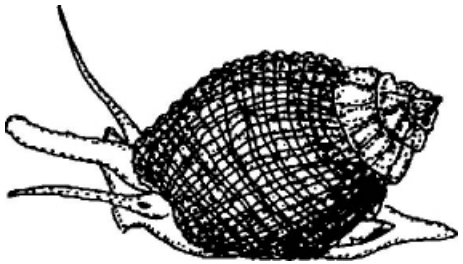
♀ 

testosterone →

↑ imposex

↑ vas deferens sequence index (VDSI)

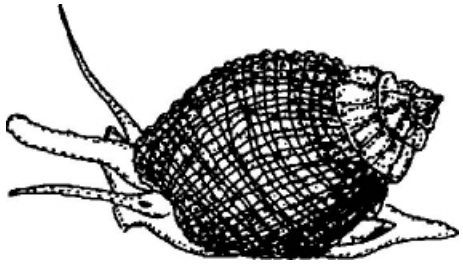
↑ penis length

♂ 

CPA →

↓ accessory sex organs

↓ penis length

♀ 

CPA + testosterone →

↓ incidence of imposex

Is the underlying assumption valid?

- investigate putative roles for steroidal androgens in reproductive recrudescence

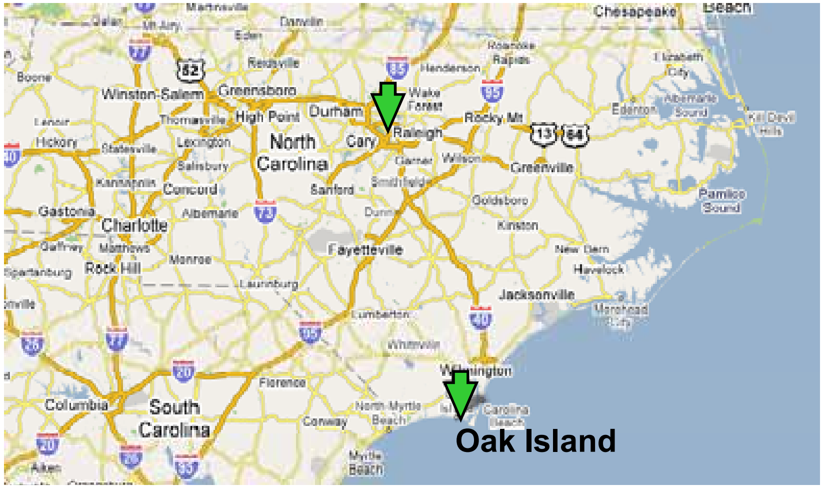
OBJECTIVES

Hormones:

- identify any associations among concentrations of testosterone, sex, and reproductive status in mud snails that suggest these hormones are involved in recrudescence

Receptors:

- determine whether mud snails express NR3C4-like androgen receptor (AR) mRNAs in a manner indicative of a role in recrudescence


Oak Island
North
Carolina
South
Carolina
Winston-Salem
Greensboro
Durham
Raleigh
Cary
Charlotte
Fayetteville
Jacksonville
Wilmington
Greenville
Chesapeake


OAK ISLAND
CITY LIMIT


BEACH STRAND
LAST EXIT

OBJECTIVE

Hormones:

- identify any associations among concentrations of testosterone, sex, and reproductive status in mud snails that suggest these hormones are involved in recrudescence

Collection
↓
Sex
↓
Wet weight
↓
Homogenization
↓
Extraction (free)
or
Extraction + saponification (total*)
↓
Evaporation
↓
RIA

*total = free + fatty acid esterified

OBJECTIVE

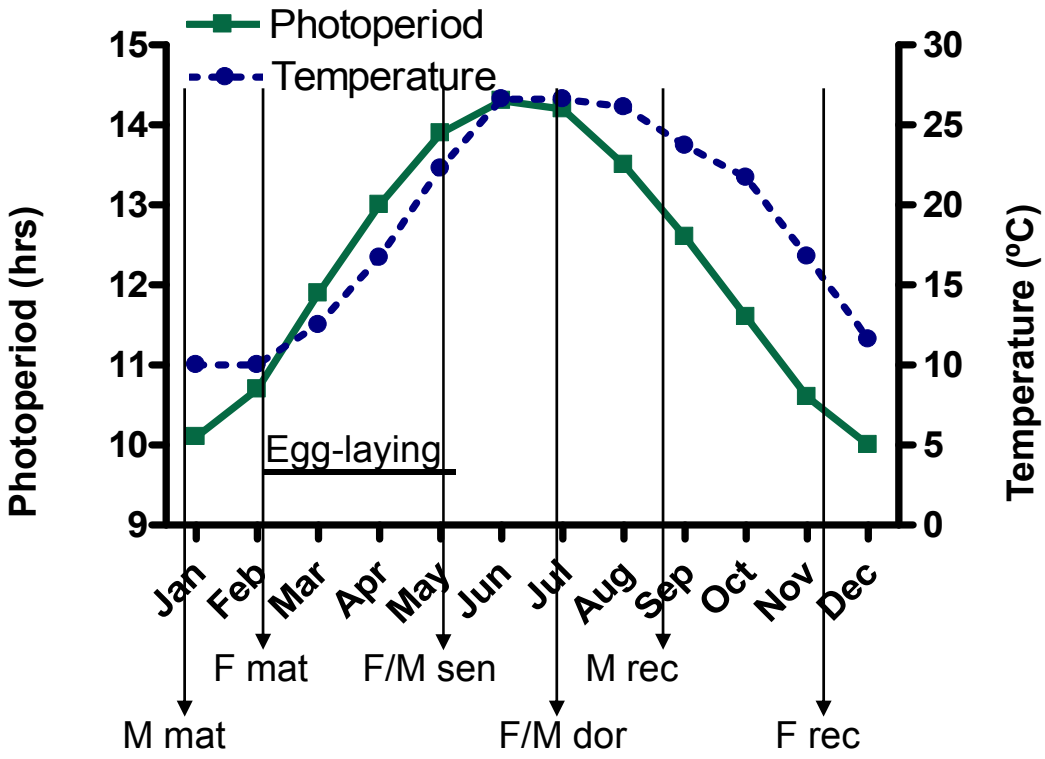
Receptors:

- determine whether mud snails express NR3C4-like androgen receptor (AR) mRNAs in a manner indicative of a role in recrudescence

RNA
↓
cDNA
↓
Targeted, degenerate PCR
↓
RACE

Collection
↓
Sex
↓
RNA
↓
cDNA
↓
Quantitative, real-time PCR (qPCR)

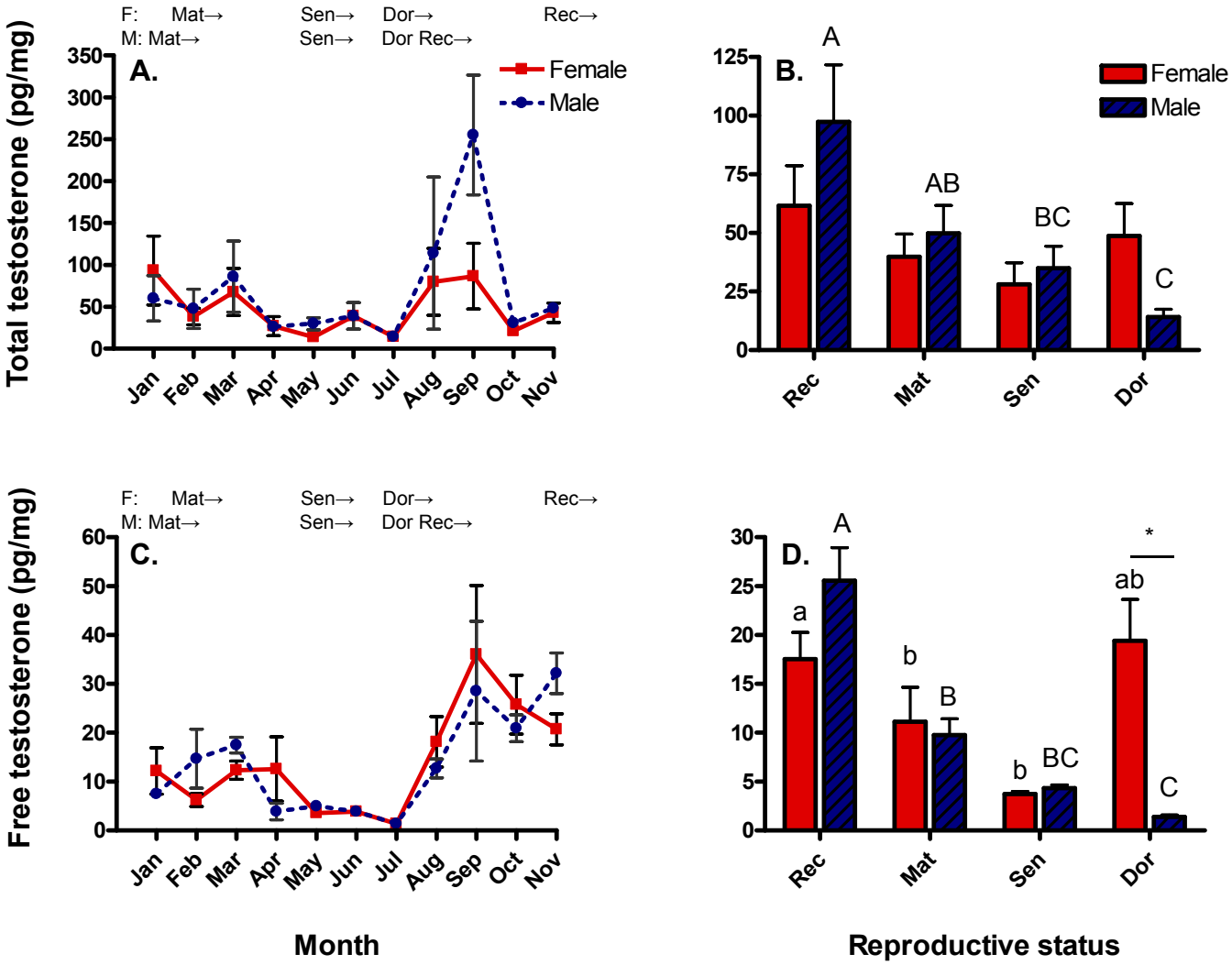
Annual reproductive cycle of eastern mud snails (Oak Island field site)



F = female
M = male

mat = mature
sen = senescent
rec = recrudescent
dor = dormant

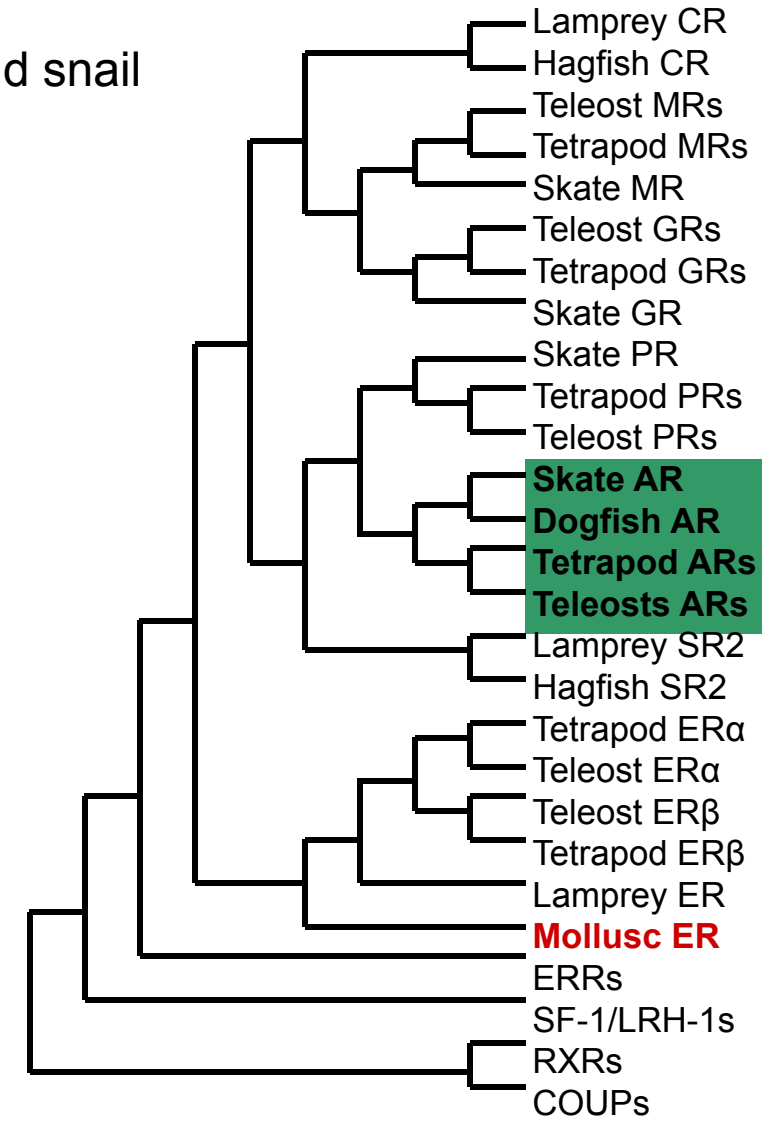
Are androgens involved in the regulation of recrudescence in gastropods?



Temporal changes in testosterone levels in males were consistent with a positive role in recrudescence. Such a trend was not evident in females.

Are androgens involved in the regulation of recrudescence in gastropods?

- no evidence for an NR3C4-like AR in the mud snail
- phylogenetic analyses indicate that the AR evolved after the emergence of jawless fish



modified from Thornton (2001), Thornton et al. (2003), and Bridgham et al., (2006)

Are androgens involved in the regulation of recrudescence in gastropods?

CONCLUSIONS:

- Testosterone may have a role in male reproductive tract recrudescence.
- This putative activity is independent of a NR3C4-type androgen receptor.

∴ The evidence does not support a role for testosterone in the regulation of male recrudescence via androgen receptor signaling.

Hypotheses for TBT's MOA

TBT $\xrightarrow{①}$ **↑ free testosterone** $\xrightarrow{②}$ **male reproductive tract development**

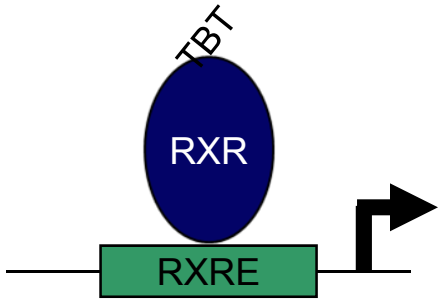
① How does TBT increase testosterone? ⊣ ATAT

➡ ② Is the underlying assumption valid? **NO**
(i.e., testosterone as a functional androgen)

∴ some other mechanism must be considered to explain the observed effect of TBT on neogastropods

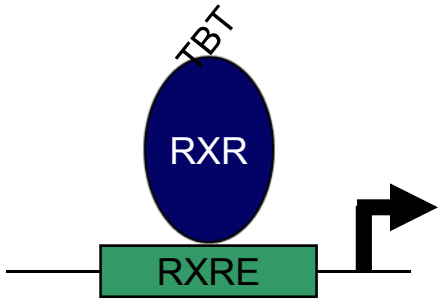
What other mechanism?

TBT as an RXR agonist

TBT → 

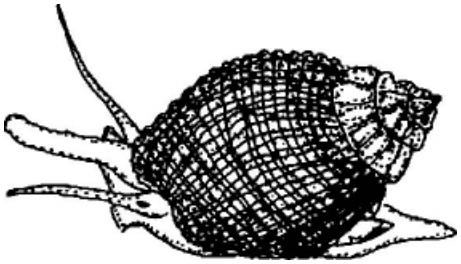
→ **male reproductive tract development**

TBT as an RXR agonist

TBT ① → 

② → **male reproductive tract development**

①
- TBT is a high-affinity ligand for human RXRs
- TBT is capable of transactivating human RXRα
- RXR of the rock shell (*Thais clavigera*) binds both 9-cis retinoic acid and TBT with high affinity

② 

9-cis retinoic acid → imposex

Underlying hypothesis:

RXR contributes to the seasonal development of the male reproductive tract in neogastropods.

TBT as an RXR agonist

Hypothesis:

RXR contributes to the seasonal development of the male reproductive tract in neogastropods.

OBJECTIVE:

- determine whether mud snails express NR2B-like (RXR) mRNA in a manner indicative of a role in male recrudescence

TBT as an RXR agonist

Clone and sequence RXR in *I. obsoleta*

↓

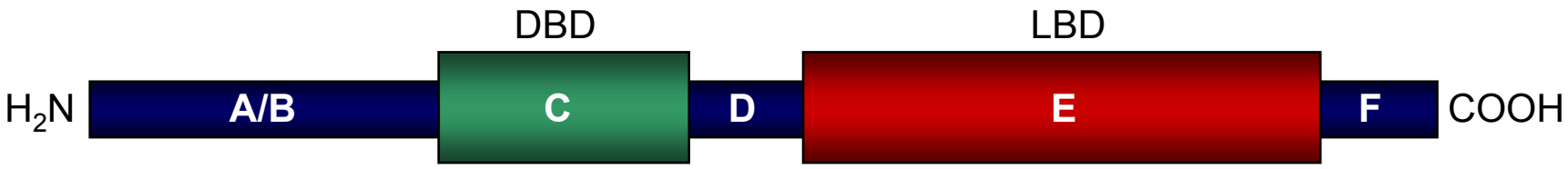
Confirm phylogenetic identity of *I. obsoleta* RXR

↓

Determine expression of RXR mRNA levels
in males and females through the annual
reproductive cycle

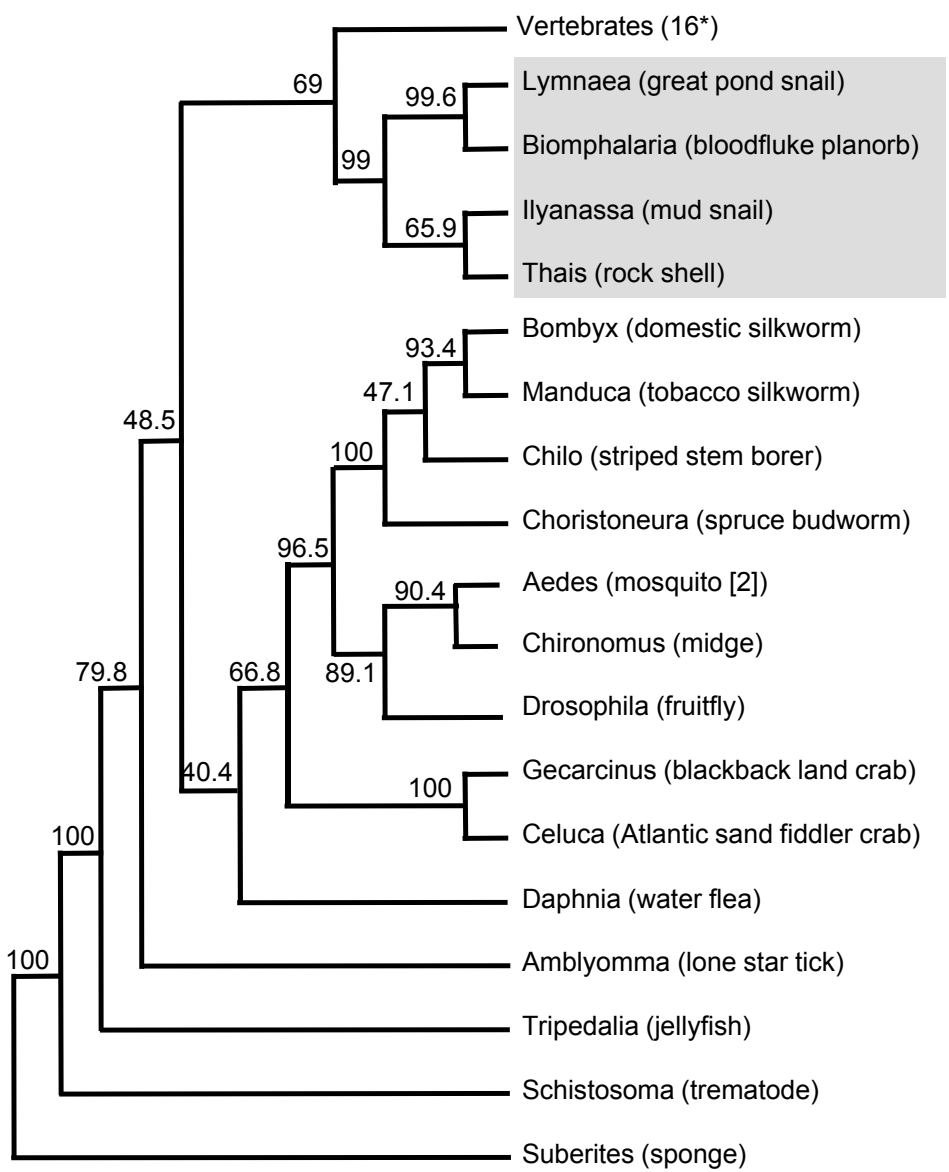
TBT as an RXR agonist

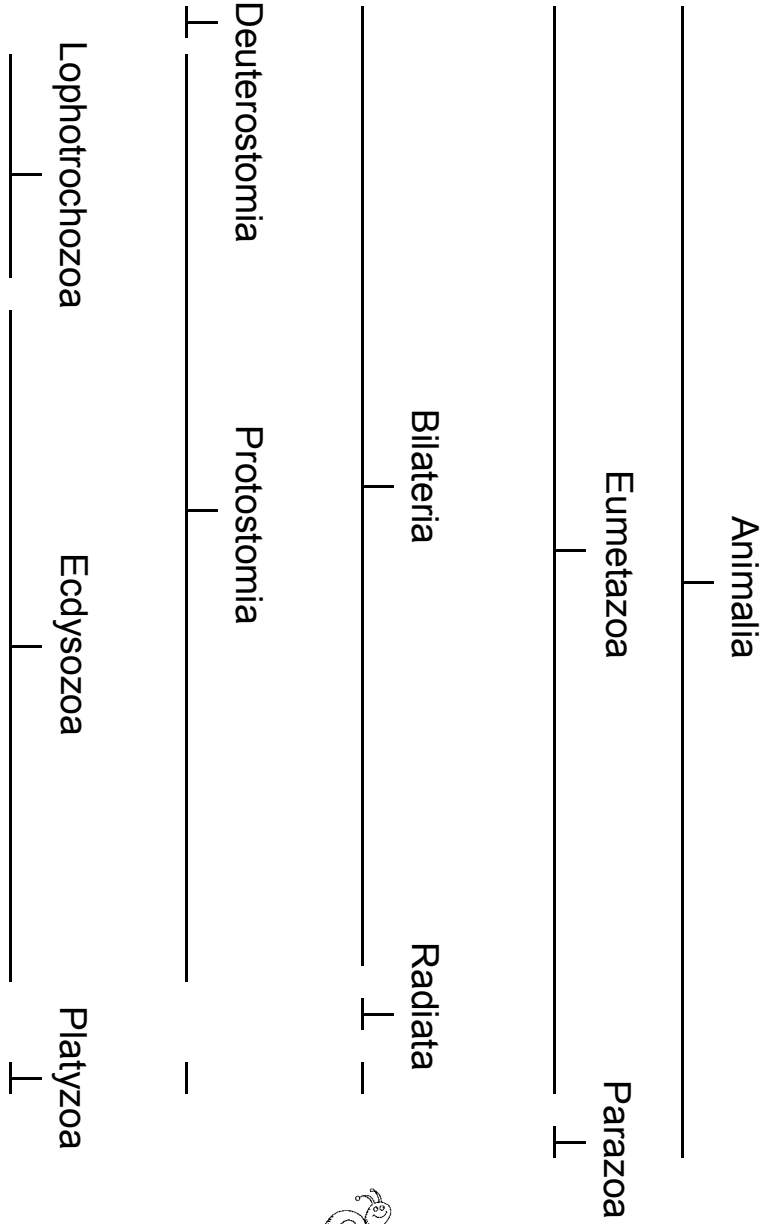
- identified a partial RXR cDNA in the mud snail, consisting of the LBD, the HD, and the DBD



Species (common name)	**DBD (% similar)**	**P-box**	**LBD (% similar)**	**AF-2**
Ilyanassa obsoleta (eastern mud snail)	-	CEGCKG	-	FLMEML
Thais clavigera (rock shell)	100	CEGCKG	93	FLMEML
Biomphalaria glabrata (bloodfluke planorb)	97	CEGCKG	92	FLMEML
Human (RXRα)	90	CEGCKG	87	FLMEML
Tripedalia cystophora (box jellyfish)	60	CEGCKG	59	FLLDML
Drosophila melanogaster (fruitfly)	87	CEACKG	51	LFLEQL

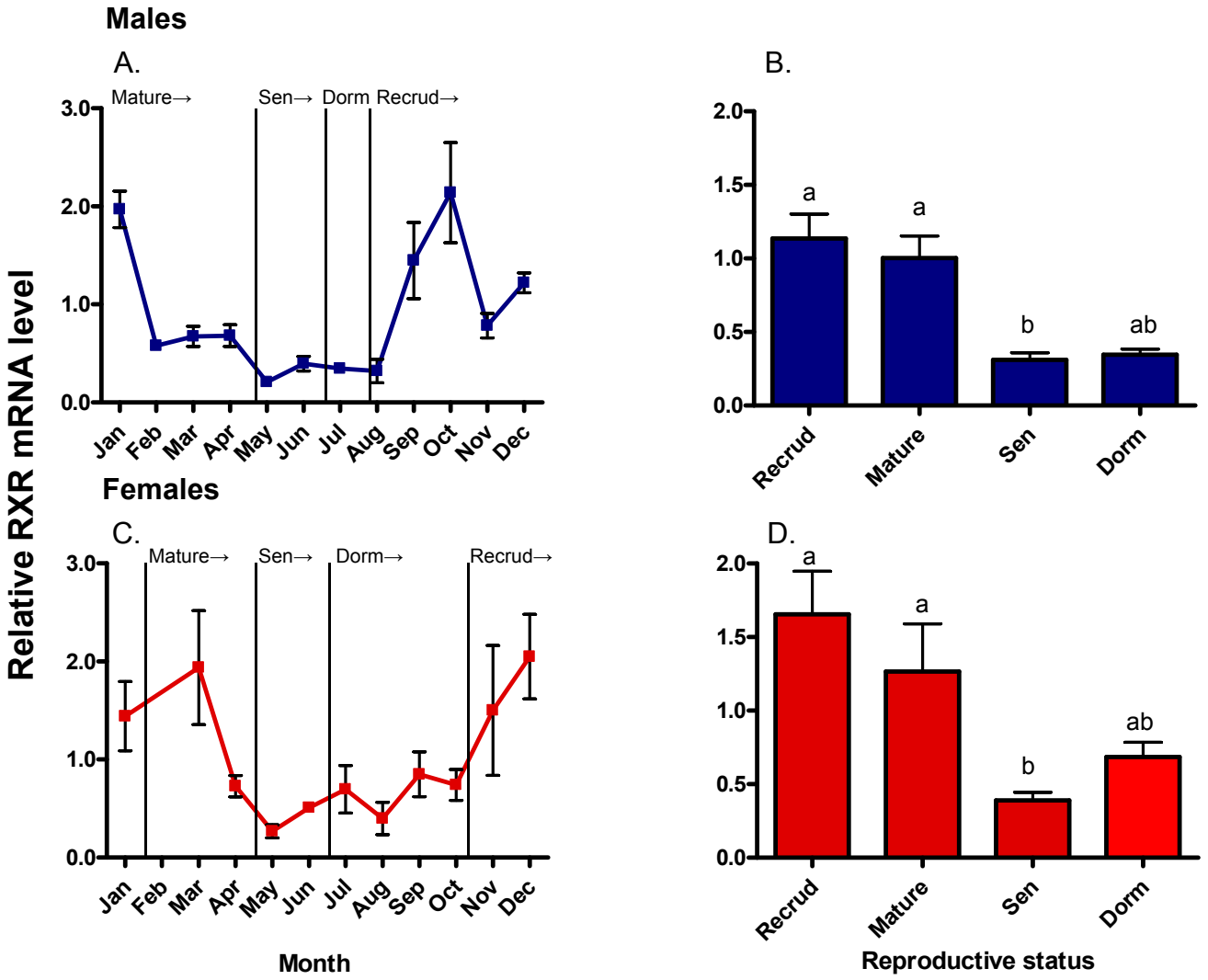
TBT as an RXR agonist





* Gallus, chicken (1); Danio, zebrafish (4); Sus, pig (4); Petromyzon, sea lamprey (3); Xenopus, African clawed frog (1); Homo, human (3)

TBT as an RXR agonist



The development of sex-specific characteristics during recrudescence may reflect RXR-mediated signaling during sex-specific windows of recrudescence.

TBT as an RXR agonist

If:

The development of sex-specific characteristics during recrudescence reflects RXR-mediated signaling during sex-specific windows of recrudescence,

Then:

TBT may induce the development of male sex characteristics in females (imposex) by stimulating RXR signaling in females during the time of normal male recrudescence.

So:

<u>0 or 10 ng/L TBT</u>

♀

senescence/dormancy
(May – Jul)

→

male recrudescence
(Aug – Oct)

→

female recrudescence
(Nov – Dec)

→

imposex

TBT as an RXR agonist

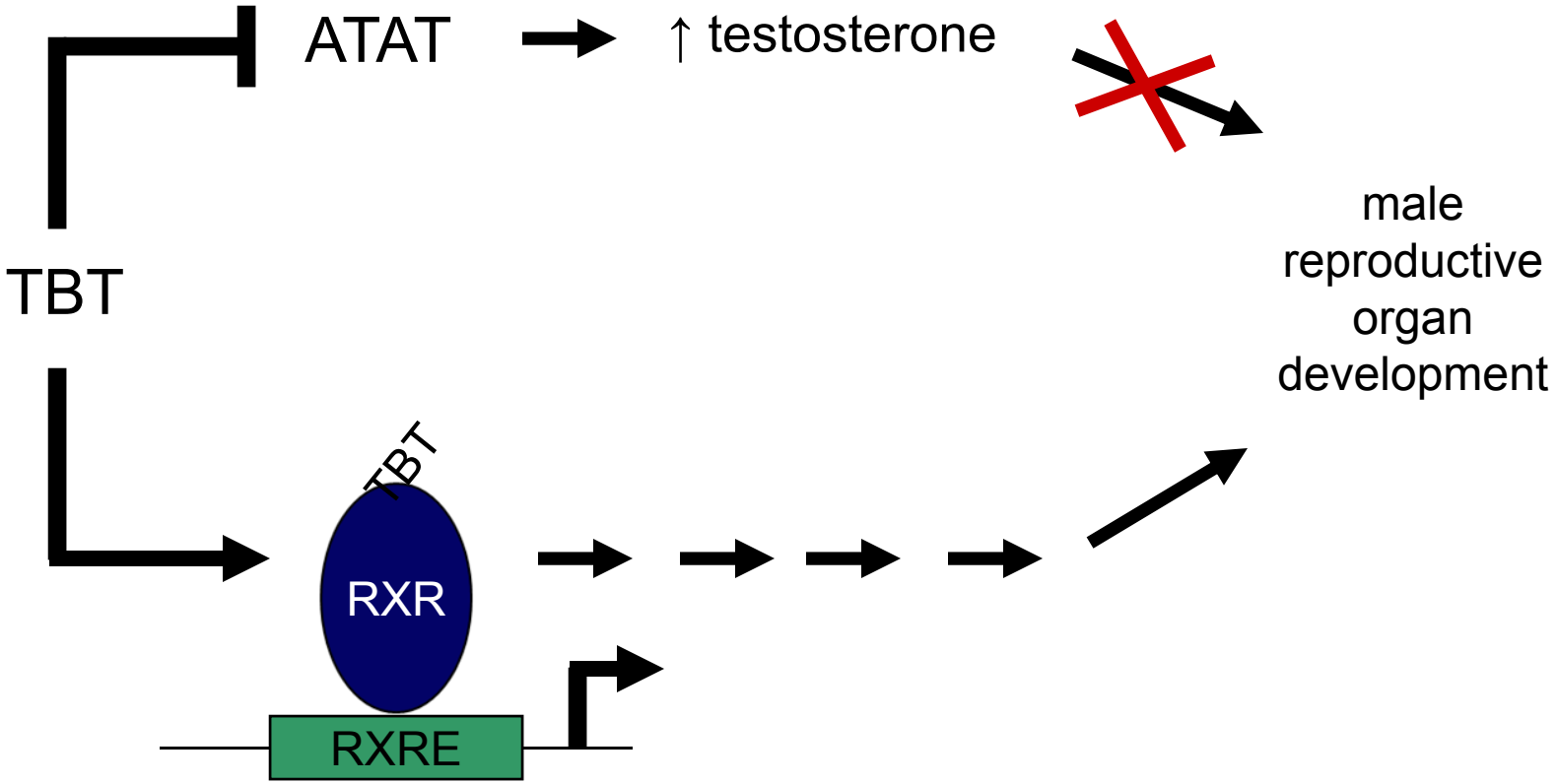
Treatment	% Imposex (n)		
	Senescence/ dormancy (May – Jul)	Male recrudescence (Aug – Oct)	Female recrudescence (Nov – Dec)
Control	0 (9)	21 (24)	0 (12)
TBT	0 (10)	56*(25)	10 (10)

* = significantly different ($p < 0.05$) from control

CONCLUSION:

TBT may be initiating retinoid signaling prematurely in females resulting in the development of the male sex phenotype.

SUMMARY


ATAT
↑ testosterone
TBT
male
reproductive
organ
development
TBT
RXR
RXRE

ACKNOWLEDGEMENTS

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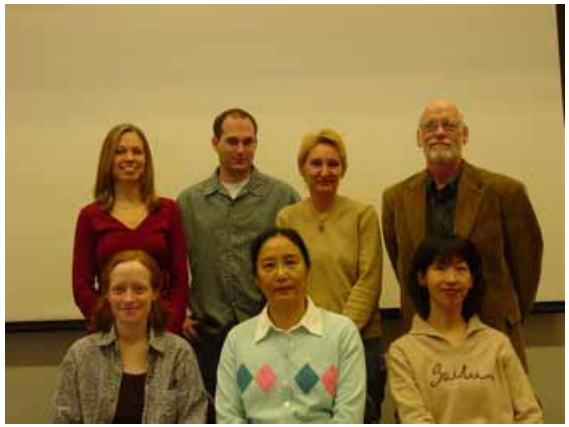
Guirong Wang
Heather Hoy



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Proposed mechanism of TBT-induced imposex