Amphibian Metamorphosis Assay for Thyroid Disruption: Status Report

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April 28, 2005

Outline of Presentation

- History, rationale, and background for amphibian metamorphosis-based thyroid assay
- OECD Phase I pre-validation results
- OECD Phase II pre-validation studies
- Supporting studies

EDSTAC Tier 1 Frog Metamorphosis Assay

- African Clawed Frog, *Xenopus laevis*
- Expose at Stage 60
  - Coincident with endogenous TH peak
- 14 Day Exposure Through Stage 66
- Tail Length
- Resorption faster than controls = agonist
- Resorption slower than controls = antagonist

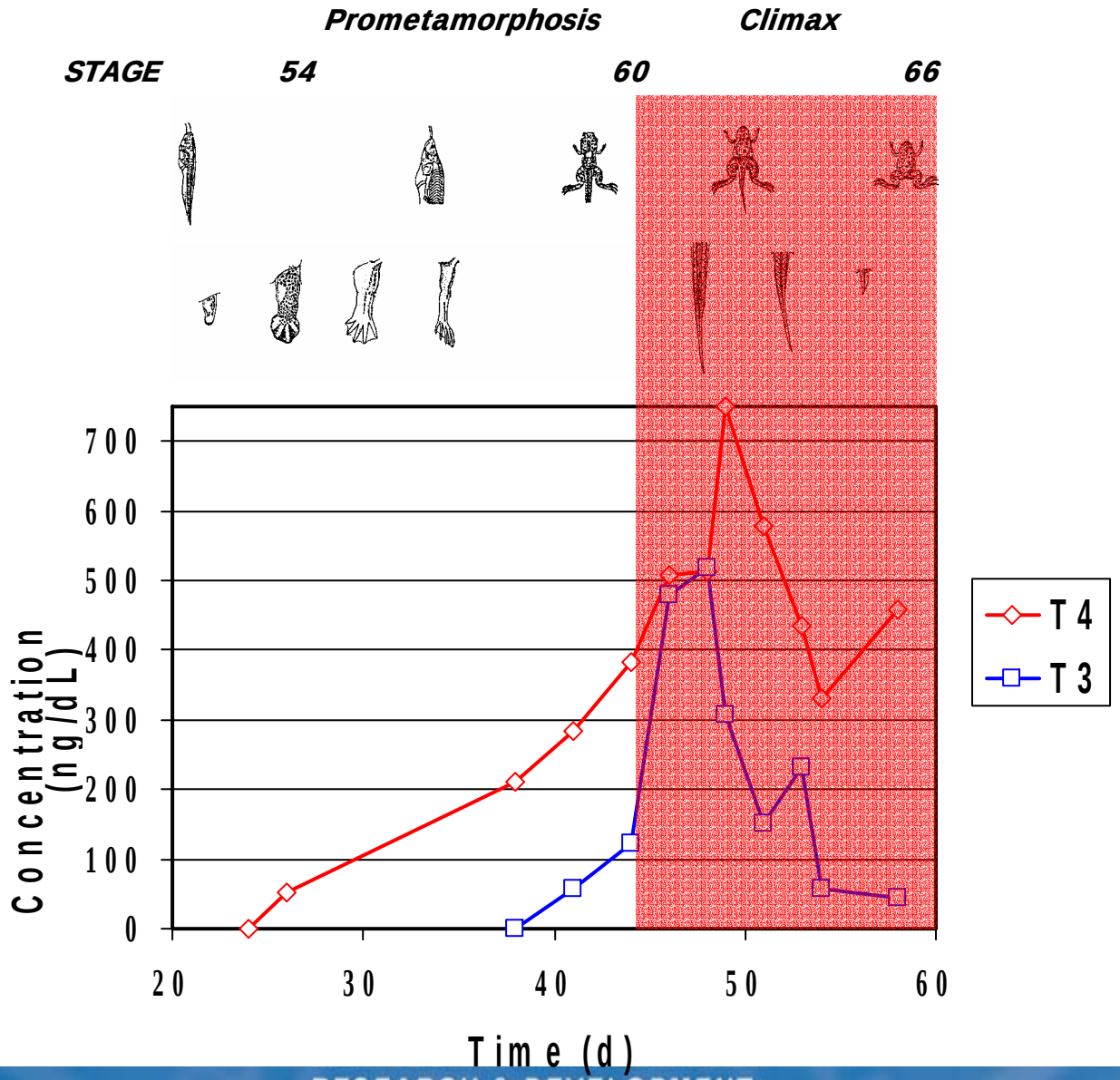
Conclusions From an Analysis of EDSTAC- Proposed Amphibian Metamorphosis Assay

- EDSTAC-Proposed Amphibian Metamorphosis Assay was not ready for use as a screening tool
  - 1) Developmental stage 60 is relatively insensitive
  - 2) Tail tissue is relatively insensitive
  - 3) Changes in tail resorption rates not diagnostic
  - 4) Insufficient data on known agonists/antagonists
  - 5) Interaction with other endocrine systems is source of uncertainty

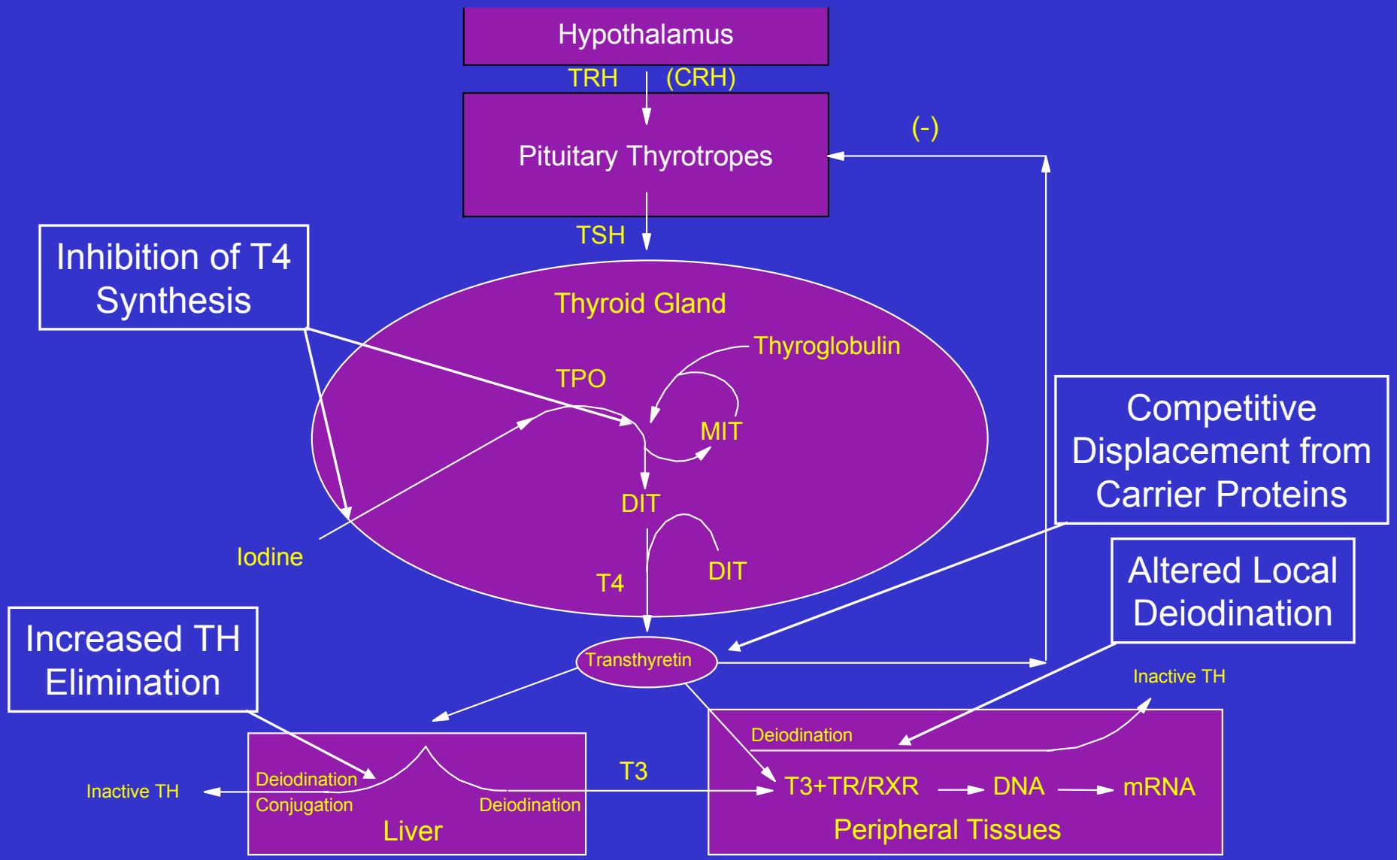
Early Research Needs

- Determine appropriate stage to maximize sensitivity yet minimize time on test
- Determine appropriate endpoints
- Establish diagnostic specificity of assay
- Conduct studies with known HPT disrupters
- Investigate interaction of other endocrine pathways on HPT

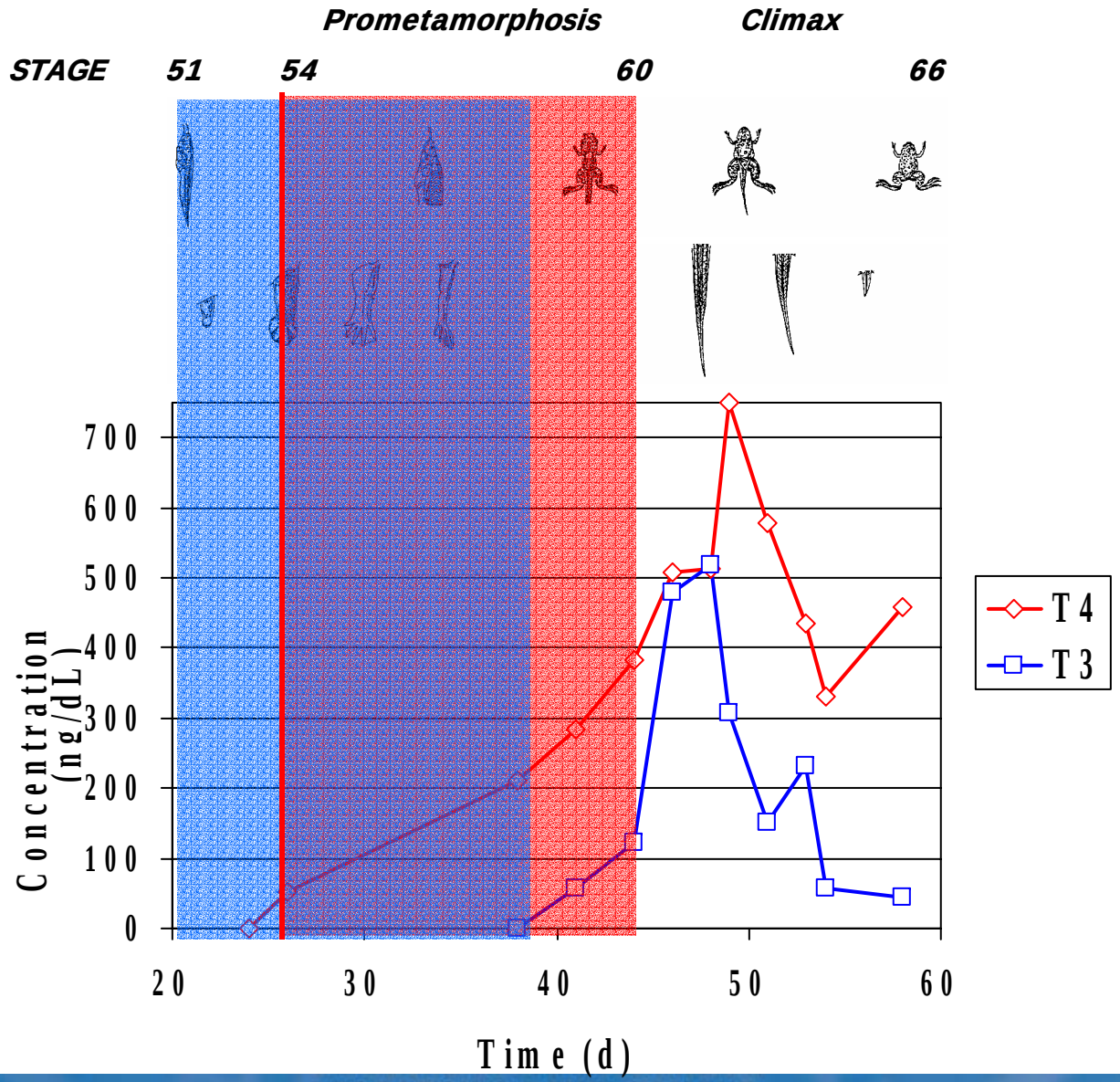
Xenopus Metamorphosis



Overview of Thyroid Hormone Pathway

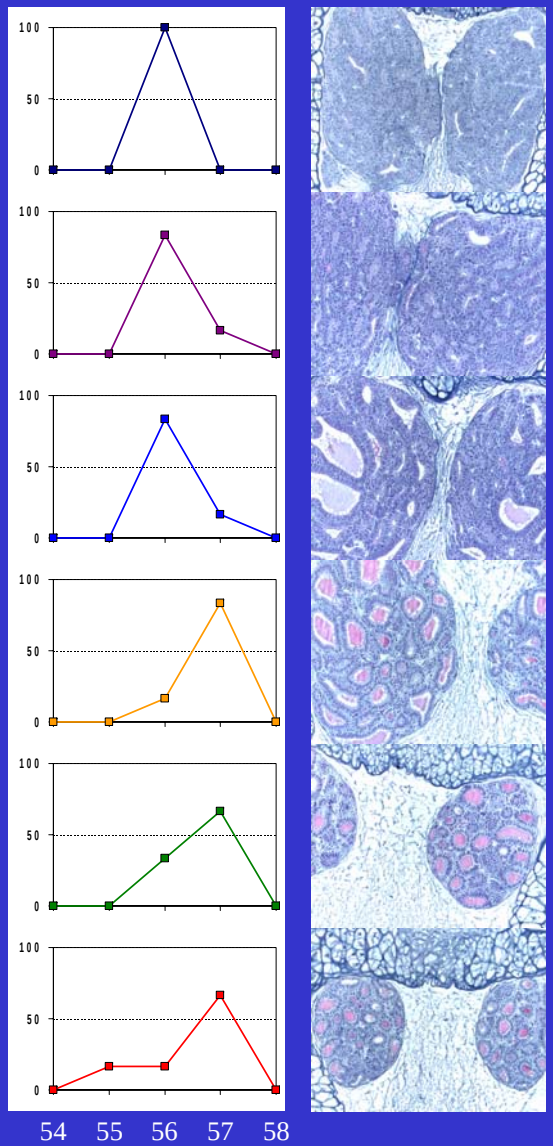


Initial EPA Studies



Effect of Methimazole on Stage 54 X. laevis

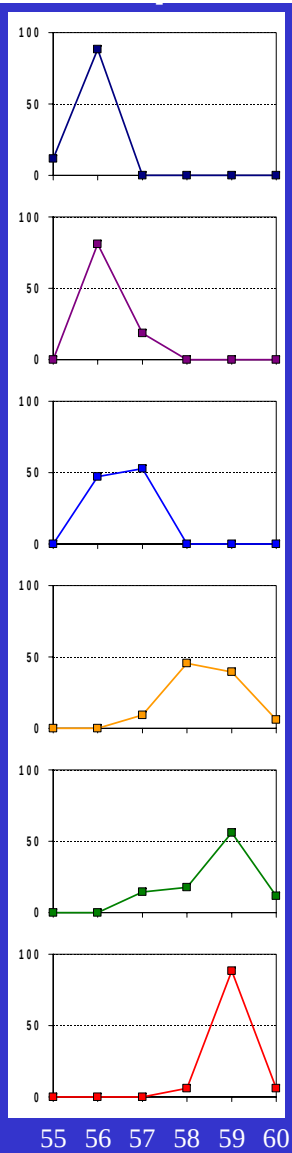
8 d Exposure



Proportion in Stage

Developmental Stage

14 d Exposure



Developmental Stage

100 mg/L

50 mg/L

25 mg/L

12.5 mg/L

6.25 mg/L

Control

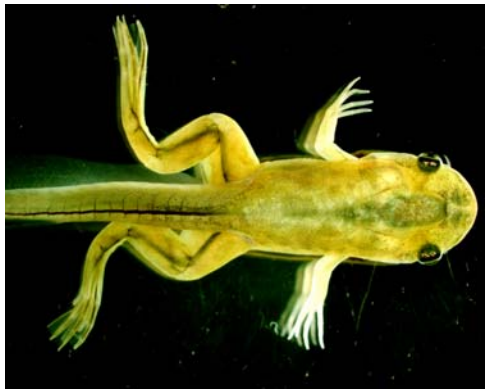
Progress through June 2003

- Several international laboratories were working on variations of the EDSTAC protocol
  - EPA completed studies with methimazole, PTU, T3,T4, IOP, and perchlorate
  - European and Japanese labs completed studies with T4 and ETU

Key Meetings June 2003

- MED Hosted Workshop on use of amphibians as toxicological model organism. Focused on HPT and HPG axis.
- OECD convened Amphibian Expert Group to plan inter-laboratory studies as an initial step in the validation process.

OECD Phase I Validation


Rationale for OECD Phase I Studies

- In June 2003 at Amphibian Expert Group Meeting
  - No standardized protocol existed
    - Therefore, use different methods to evaluate the extent of variation that might occur
  - Little history of comparable chemical testing among different laboratories
    - Therefore, we selected two well-defined model chemicals (6-PTU and T4) to perturb thyroid function

OECD Phase I Studies

- Review approach taken for these pre-validation studies
- Compare control performance among laboratories
  - How sensitive is *X. laevis* to the conditions in different labs?
- Compare chemical effects among laboratories
  - Are the effects of PTU and T4 on *X. laevis* development quantitatively similar among participating labs?

OECD Phase I Studies

- Evaluate the sensitivity of various endpoints
  - Which endpoint(s) are most sensitive to PTU and T4?
- General conclusions
  - What have we learned through this pre-validation exercise?

OECD Phase I: Participating Laboratories

• Werner Kloas	IGB
• Robert Opitz	IGB
• Osamu Tooi	Towa Kagaku
• Sigmund Degitz	USEPA, MED
• Joesph Tiegte	USEPA, MED

Similarities in Approach

- Species: X. laevis
- Chemicals: PTU, T4
- Initial stage: 51 and 54
- Duration of exposure: 21 and 14 days
- Concentrations of chemicals
- Test specifics: larval density, replication, temperature, photoperiod
- Endpoints: developmental stage, weight, mortality, histology

Differences in Approach

- Exposure regime: static renewal vs. flow through
- Food type
- Handling on test
- Test medium
- Endpoints: whole body length, hind limb length, quantitative histopathology, gene expression
- Temporal sampling scheme
- Analytical chemistry

OECD Phase I: Comparison of Control Data

- How sensitive is *X. laevis* to the conditions in different labs?

Development of Stage 51 Controls for 21 d Among Laboratories

		Developmental Stage							
		56	**57**	**58**	**59**	**60**	**61**	**62**	**63**
PTU	US		2	13	18	4	7	5	1
	JPN		16	13	8	2	1		
	GER	2	17	4	14	2	1		
T4	US		6	4	15	8	4	3	
	JPN	2	3	16	12	4	3		
	GER			3	15	11	4	7	

Development of Stage 54 Controls for 14 d Among Laboratories

		Developmental Stage				
		56	**57**	**58**	**59**	**60**
PTU	US		11	19	18	2
	JPN	6	32	2		
	GER	1	14	14	9	2
T4	US	1	14	11	13	1
	JPN		25	6	7	2
	GER		6	15	17	2

Mean Weight of Controls Among Laboratories

		Japan	Germany	US
21 d	PTU	1065 (153)	919 (162)	1047 (230)
	T4	761 (127)	824 (169)	1042 (202)
14 d	PTU	895 (113)	957 (139)	1069 (161)
	T4	882 (131)	861 (117)	943 (157)

Mean Body Length of Controls Among Laboratories

		Japan	Germany	US
21 d	PTU	64.2 (3.9)	59.8 (2.6)	19.5 (1.9)
	T4	56.1 (3.4)	58.9 (4.2)	19.5 (1.7)
14 d	PTU	60.4 (2.5)	59.7 (2.9)	19.9 (1.2)
	T4	59.6 (3.2)	59.6 (2.4)	19.2 (0.9)

OECD Phase I:
PTU Results

PTU; Stage 51; 21 days

Japan

PTU Conc. mg/L	Stage at 7 days					Stage at 14 days						Stage at 21 days										
	51	52	53	54	55	52	53	54	55	56	57	53	54	55	56	57	58	59	60	61	62	63
0.0			6	32	2				2	18	20					16	13	8	2	1		
2.5		1	2	37					4	22	13				2	7	17	5	3	4	1	
5		1	8	30	1			3	10	23	3			2	2	17	14	3	0	1		
10		1	2	35	2			2	9	18	11			1	4	7	11	12	5			
20	2	3	8	27		1	4	21	10	2	1		14	10	5	6	2					

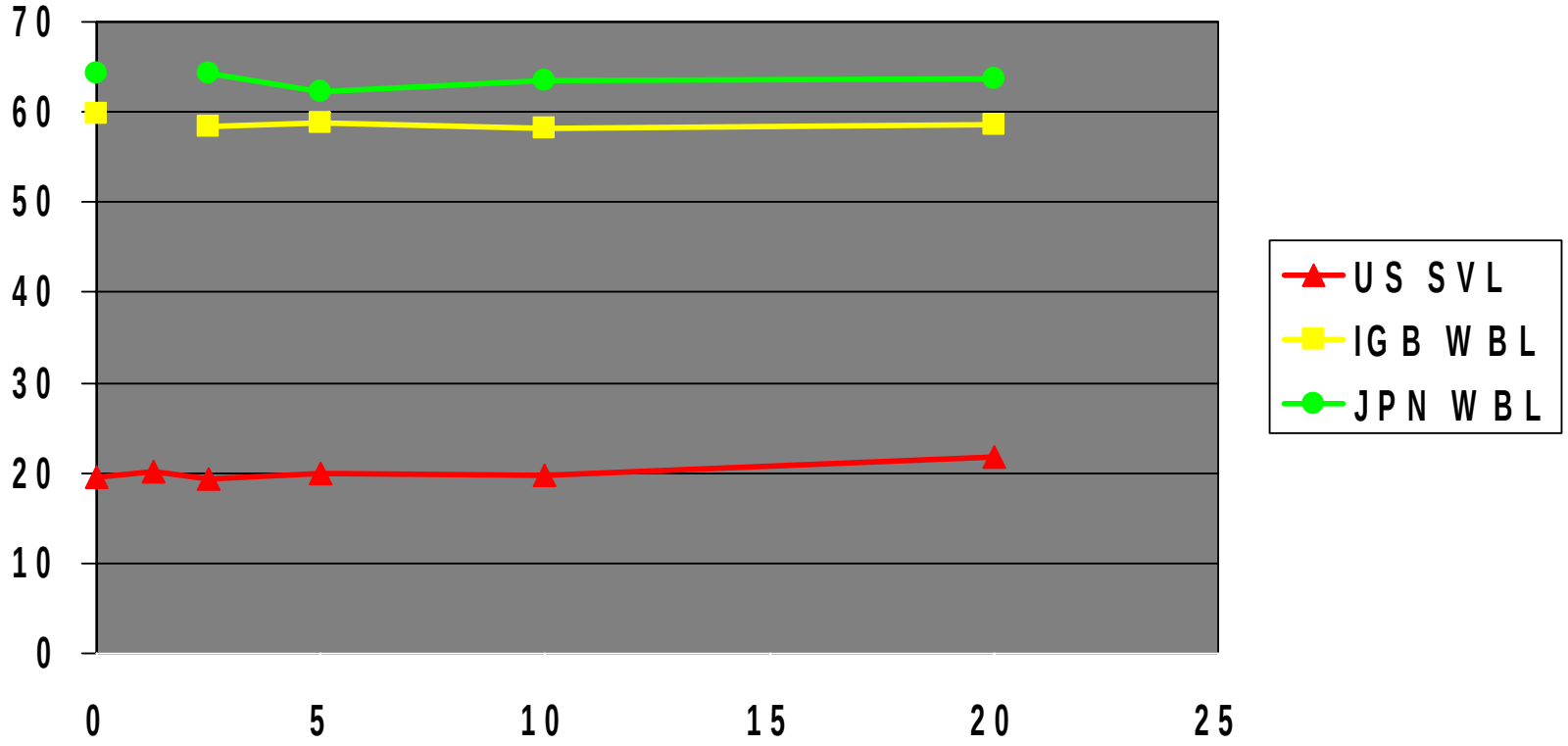
Germany

PTU Conc. mg/L	51	52	53	54	55	52	53	54	55	56	57	53	54	55	56	57	58	59	60	61	62	63
0.0			2	32	6				4	34	2				2	17	4	14	2	1		
2.5			3	37					9	30	1				3	20	9	7	0	1		
5			1	34	5				5	32	3					24	9	7				
10			1	38	1				9	31					3	25	9	3				
20			10	29	1			1	13	26				1	4	24	8	3				

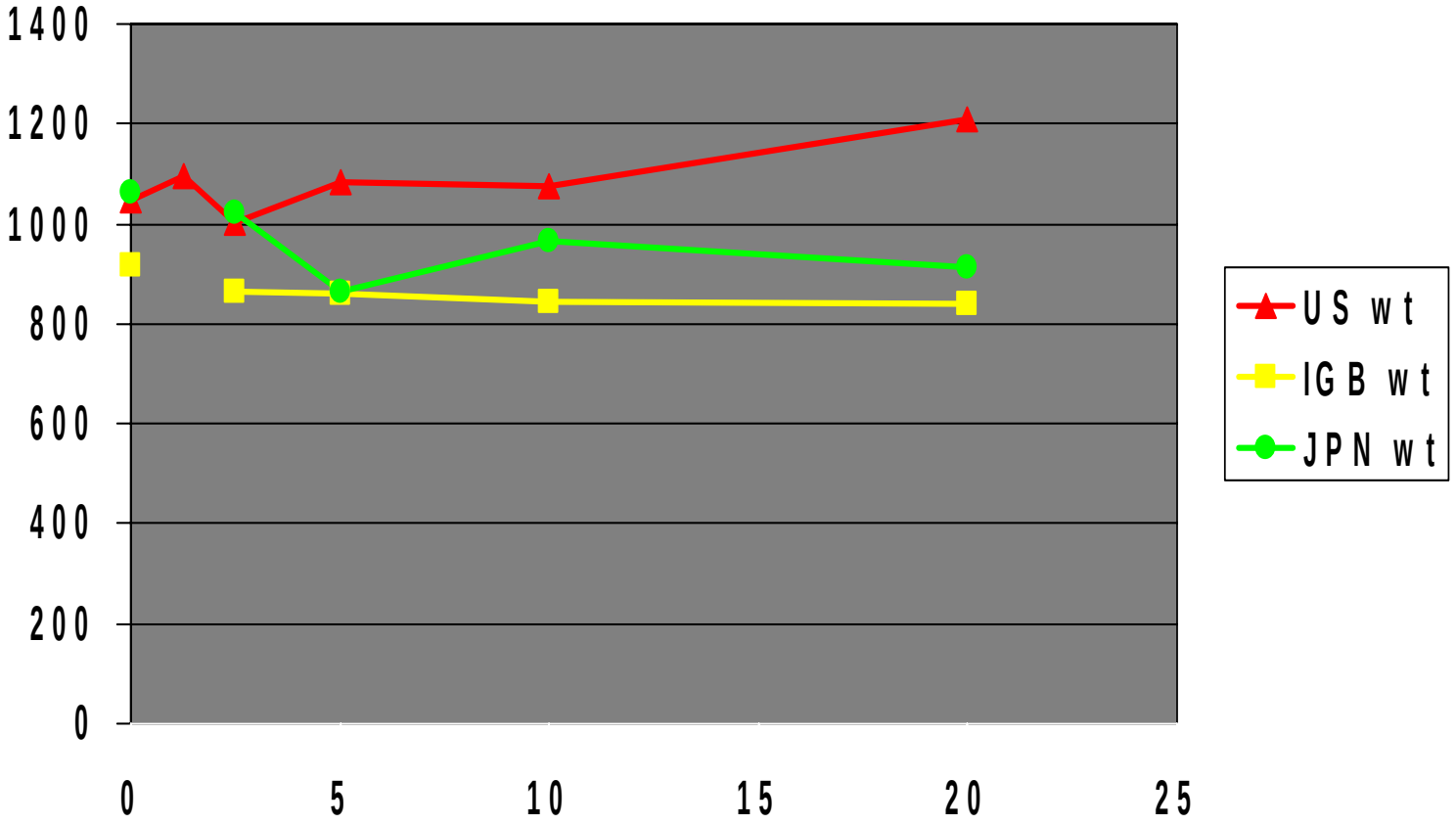
US

PTU Conc. mg/L	53	54	55	56	57	58	59	60	61	62	63
0.0					2	13	18	4	7	5	1
1.25					4	21	14	6	2	3	
2.5					9	5	23	8	1	3	1
5					10	12	14	7	2	5	
10				1	7	12	22	2	4	2	
20	2	20	13	4	5	3	2				

Effect of 6-PTU on Body Length
Stage 51 at 21 d



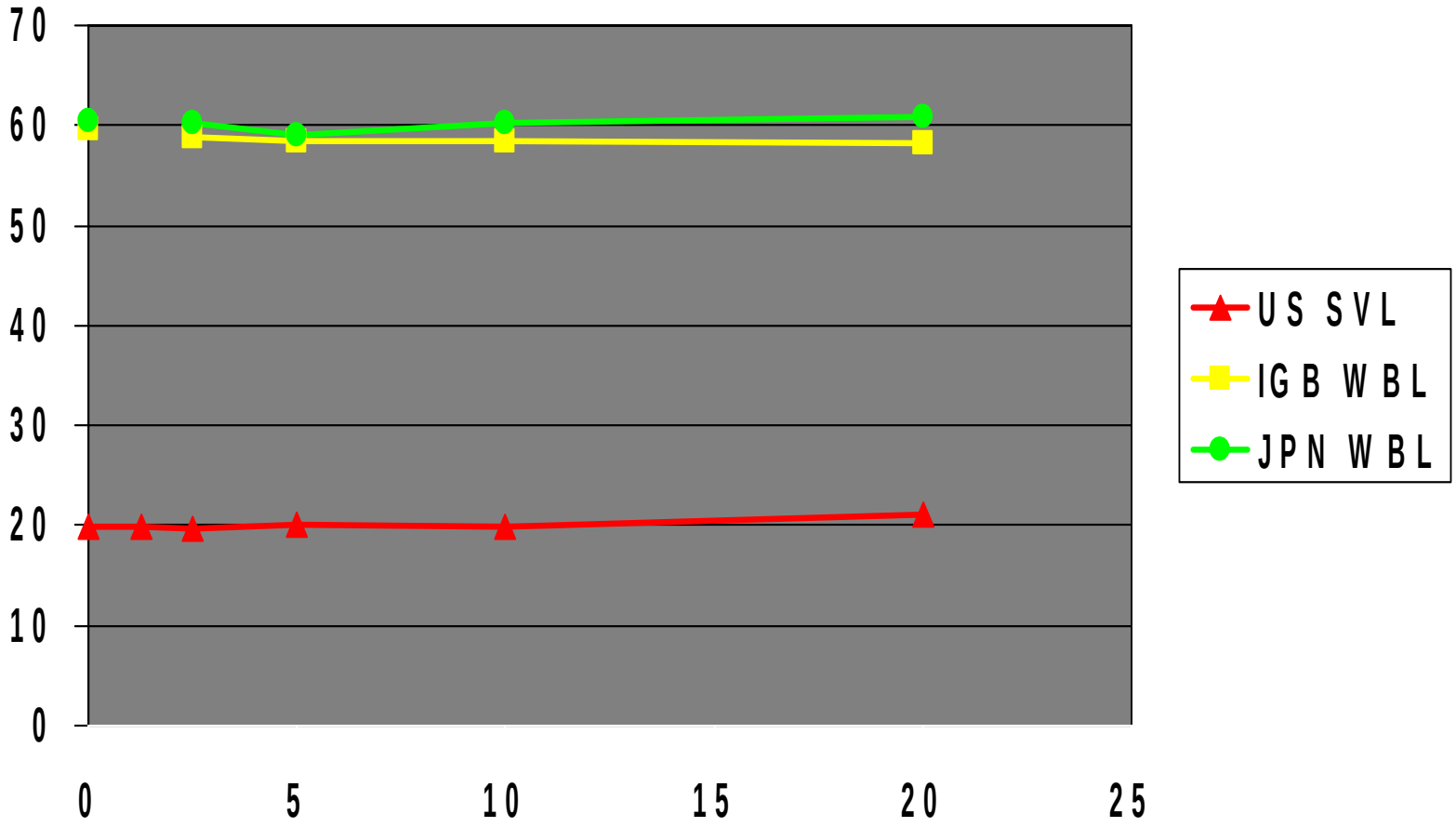
Effect of 6-PTU on Body Weight Stage 51 at 21 d



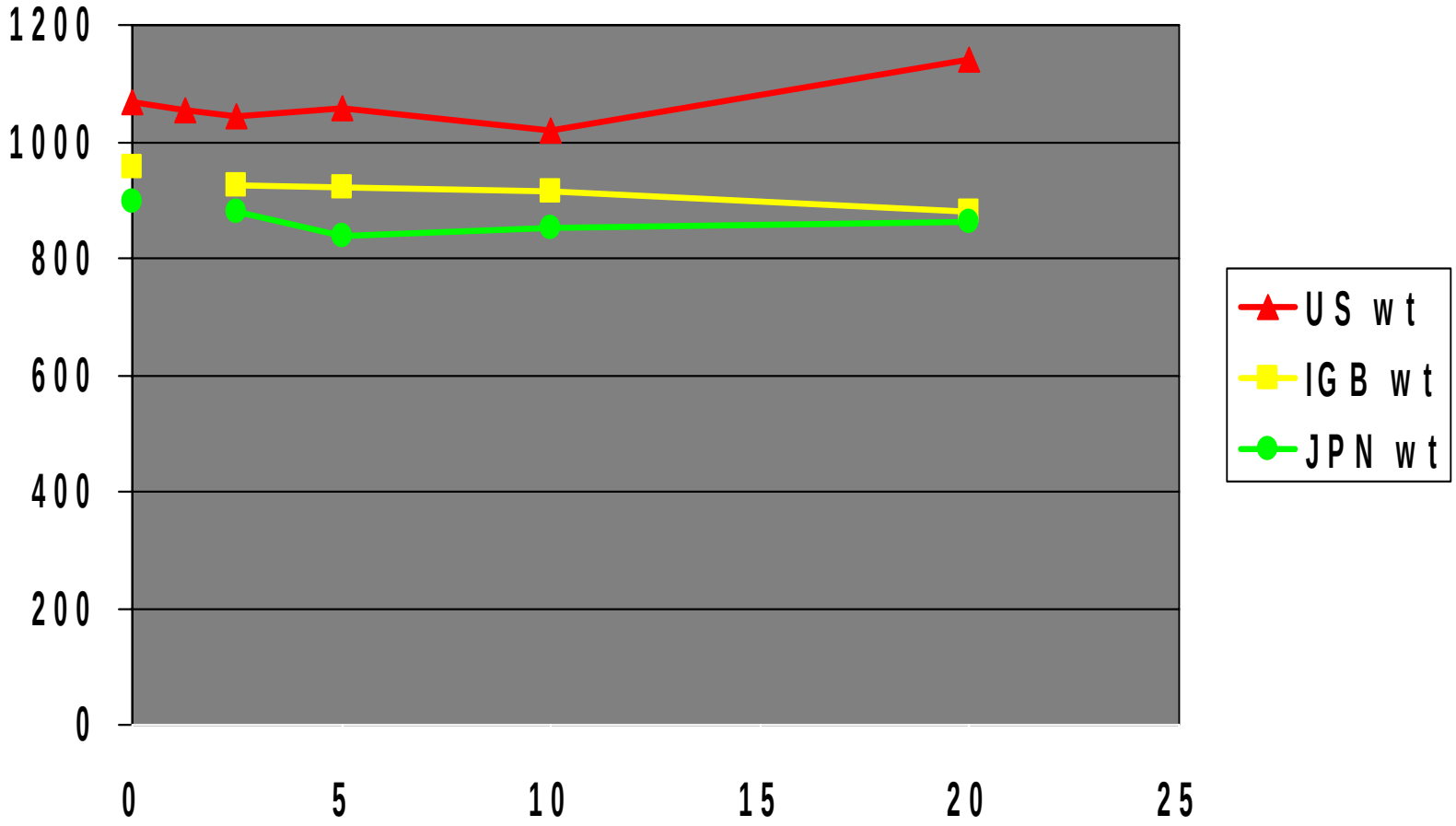
PTU; Stage 54; 14 days

	PTU Conc. mg/L	Stage at 7 days				Stage at 14 days					
		54	55	56	57	55	56	57	58	59	60
Japan	0.0		26	14			6	32	2		
	2.5	3	26	11			12	25	1	2	
	5	1	33	6			7	32	1		
	10	1	29	10			5	32	1	2	
	20	1	34	5			19	20	0	1	
		54	55	56	57	55	56	57	58	59	60
Germany	0.0		1	29	10		1	14	14	9	2
	2.5			34	6	1	0	21	11	6	1
	5		3	31	6		1	25	6	8	
	10			34	6			25	12	2	1
	20		3	33	4		2	24	11	3	
						55	56	57	58	59	60
US	0.0							11	19	18	2
	1.25							16	17	17	
	2.5							16	13	21	
	5							22	14	14	
	10							28	13	9	
	20					11	30	7	0	2	

Effect of 6-PTU on Body Length
Stage 54 at 14 d



Effect of 6-PTU on Body Weight Stage 54 at 14 d

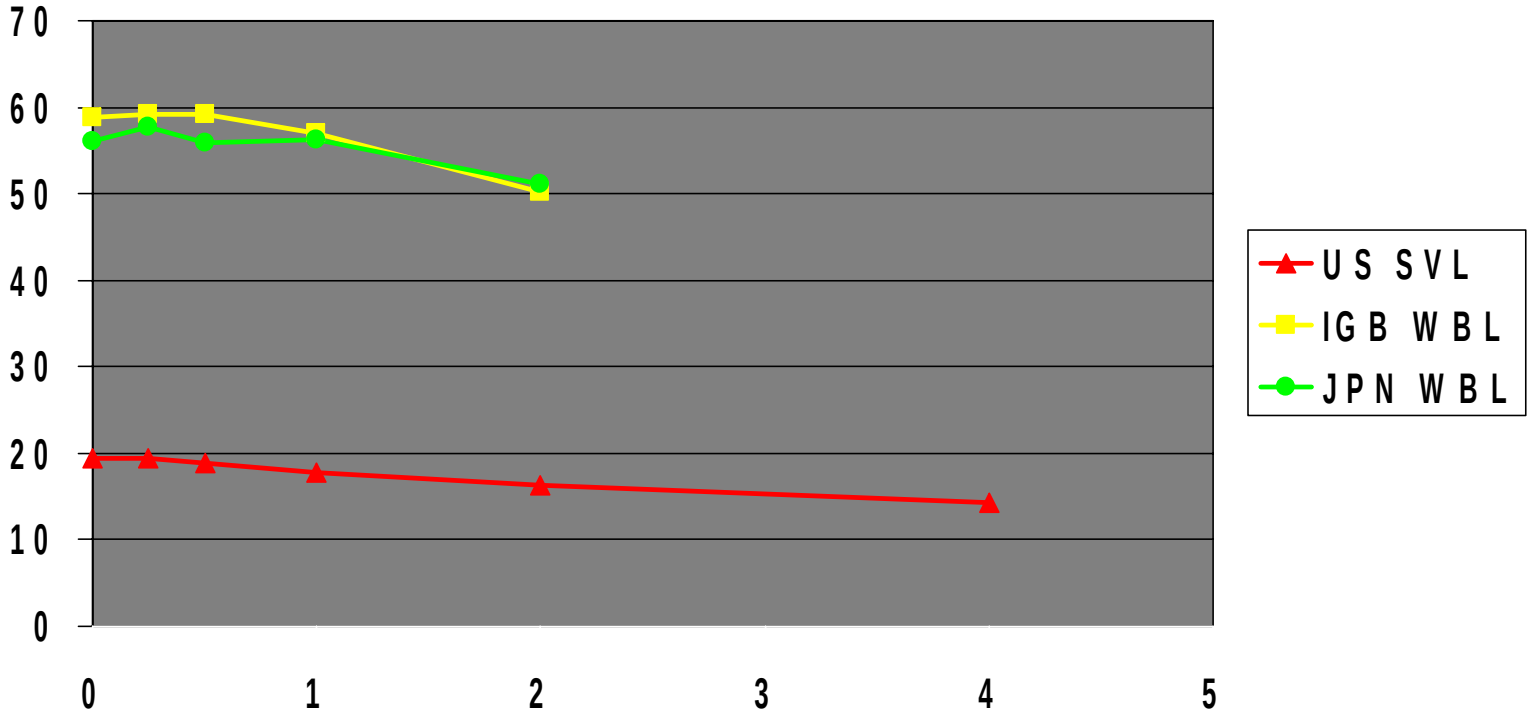


OECD Phase I:
T4 Results

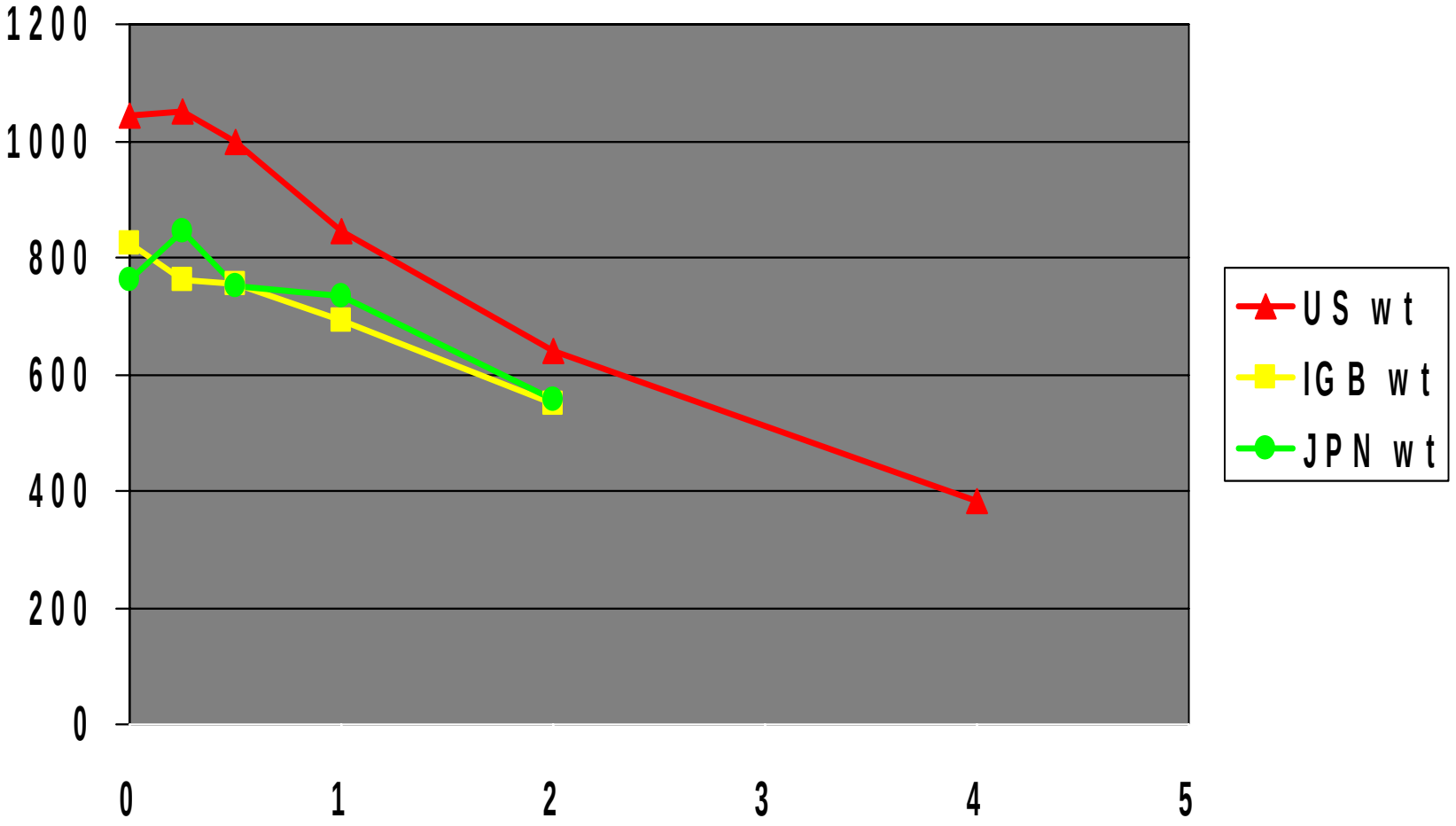
T4; Stage 51; 21 days

	T4 Conc. (µg/L)	Stage at 7 days				Stage at 14 days					Stage at 21 days									
		53	54	55	56	54	55	56	57	58	56	57	58	59	60	61	62	63	64	65
Japan	0.0	11	29			3	6	31			2	3	16	12	4	3				
	0.25	2	38				11	21	8			7	14	13	4	0	2			
	0.5		34	6			1	29	10			3	15	13	2	5	2			
	1.0		14	26				29	11				6	19	9	1	5			
	2.0		2	38						40			1	8	15	6	10			
		53	54	55	56	54	55	56	57	58	56	57	58	59	60	61	62	63	64	65
Germany	0.0		12	28				7	33				3	15	11	4	7			
	0.25		6	34				6	34			1	1	8	13	15	2			
	0.5		2	38				3	37				2	11	13	7	7			
	1.0			40					38	2				3	18	7	12			
	2.0			38	2				7	33						13	25	1	0	1
											56	57	58	59	60	61	62	63	64	65
US	0.0											6	4	15	8	4	3			
	0.25											3	3	22	7	1	4			
	0.5											1	2	20	7	5	5			
	1.0													18	10	5	7			
	2.0													3	8	11	16	2		
	4.0																	11	15	8

Effect of T4 on Body Length
Stage 51 at 21 d



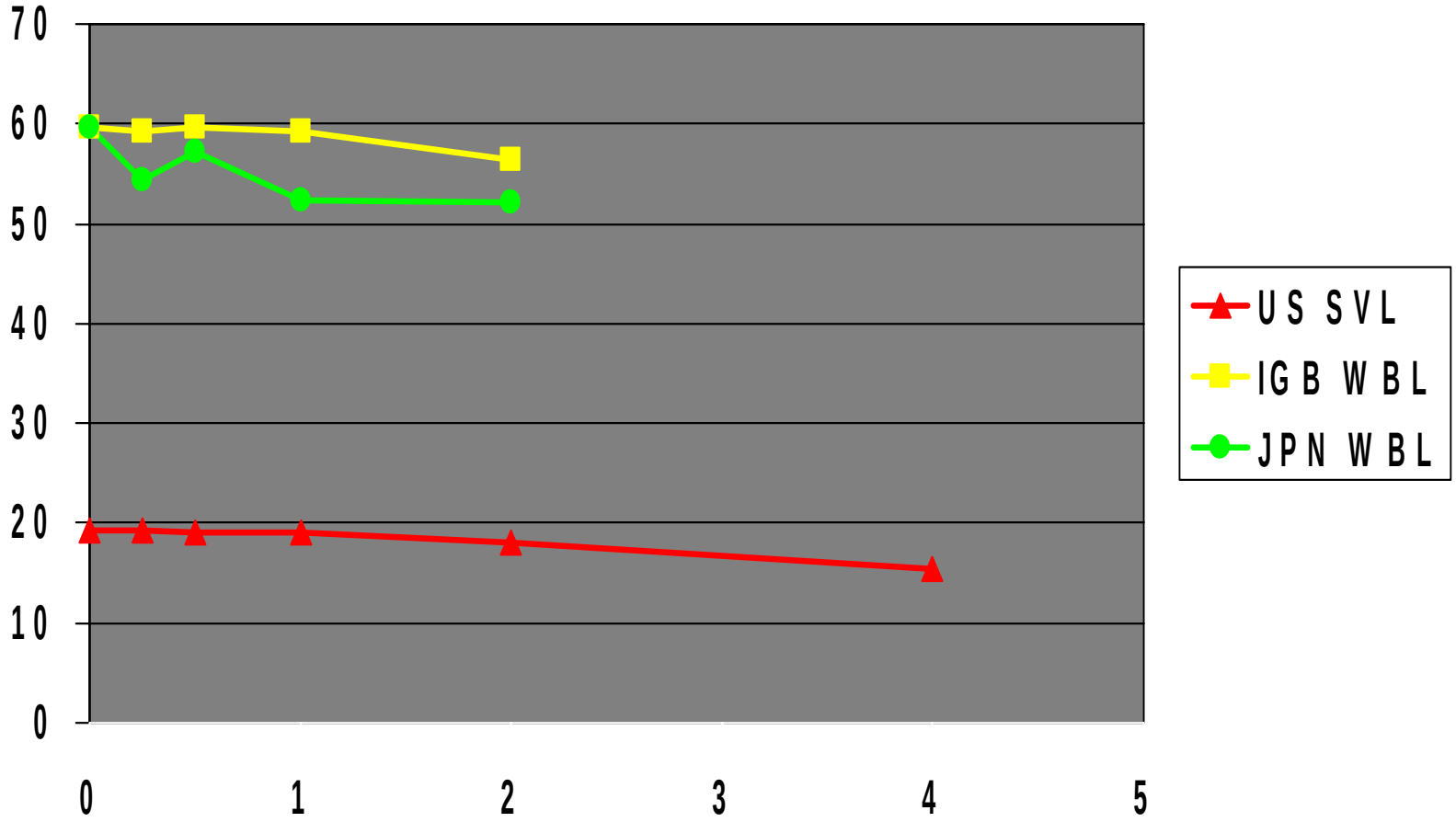
Effect of T4 on Body Weight
Stage 51 at 21 d



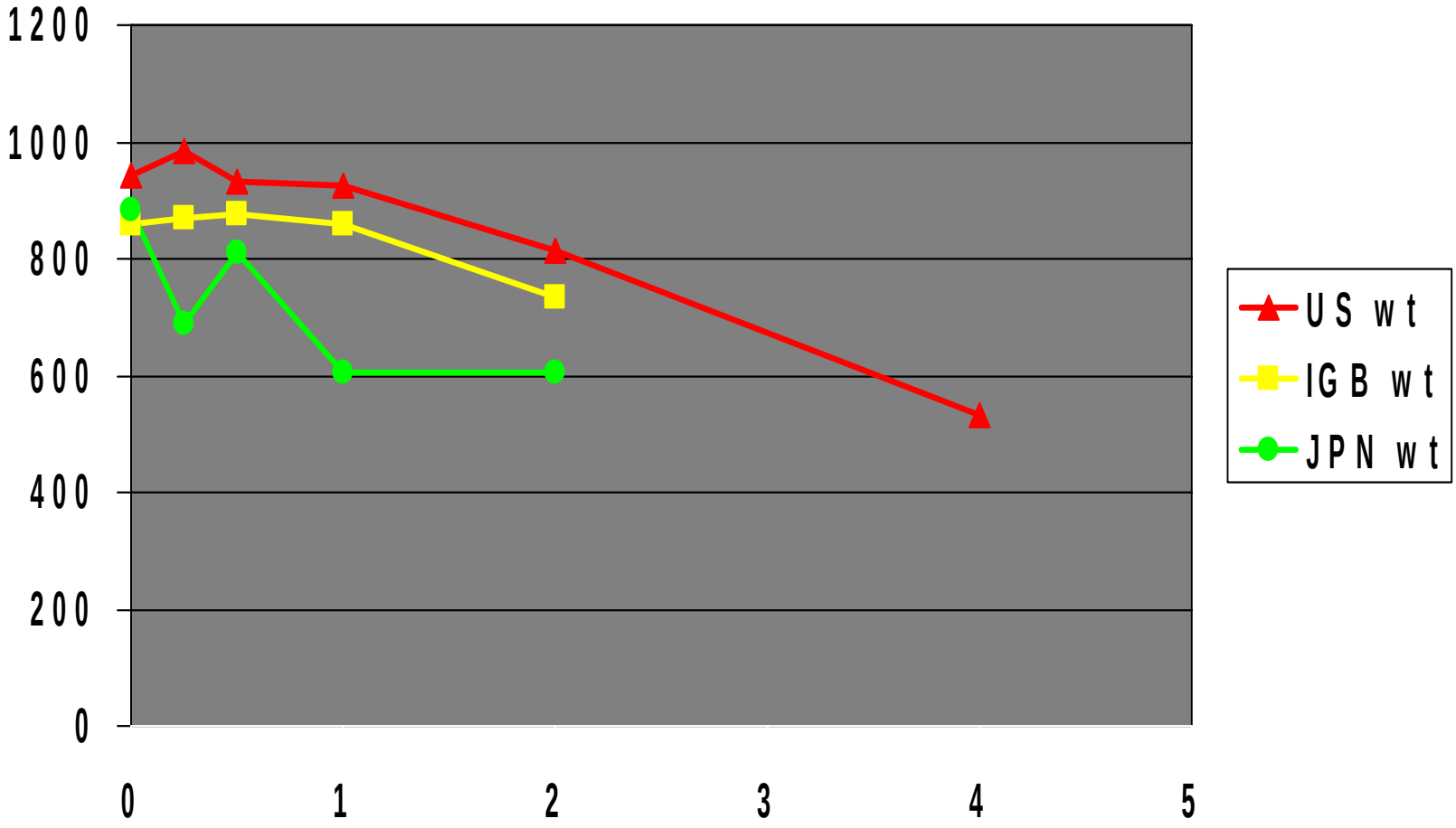
T4; Stage 54; 1 days

	T4 Conc. (µg/L)	Stage at 7 days				Stage at 14 days						
		55	56	57	58	56	57	58	59	60	61	62
Japan	0.0	21	17	2			25	6	7	2		
	0.25	25	12	3		1	25	6	5	3		
	0.5	22	17	1			22	14	4			
	1.0	21	19				27	10	3			
	2.0		26	14				12	24	4		
		55	56	57	58	56	57	58	59	60	61	62
Germany	0.0	4	35	1			6	15	17	2		
	0.25	3	31	6			3	12	18	4	2	1
	0.5	2	36	2			4	18	16	1		
	1.0		35	4			2	15	17	4	0	1
	2.0		12	27	1			2	20	12	6	
						56	57	58	59	60	61	62
US	0.0					1	14	11	13	1		
	0.25						16	6	15	3		
	0.5					1	10	10	15	3	1	
	1.0							23	17			
	2.0							1	32	5	0	1
	4.0									13	13	14

Effect of T4 on Body Length
Stage 54 at 14 d



Effect of T4 on Body Weight
Stage 54 at 14 d



Conclusions Based on PTU and T4 Studies

- Both stages are affected similarly by each chemical
- Either approach is effective at detecting agonism and antagonism
- Interlaboratory differences were minor

OECD Phase I:
Endpoint Sensitivity

Endpoint Sensitivity: PTU Stage 51 for 21 d LOEC in mg/L

		Germany			Japan			US
		7	**14**	**21**	**7**	**14**	**21**	**21**
Final Stage		ns	ns	10	ns	20*	20	20
Hind Limb Length		20*	10	10	20	20*	20*	--
Whole Body Length		10*	10	ns	10	20*	ns	20↑
Thyroid Histology		--	--	5	--	--		5
Follicular Cell Height		--	--	20	--	--	--	--
Whole Body Wt.		--	--	10				20

* transient effects at lower concentrations but no dose response

Endpoint Sensitivity: PTU Stage 54 for 14 d LOEC in mg/L

		Germany		Japan		US
		7	**14**	**7**	**14**	**14**
Final Stage		ns	20	ns	20	10
Hind Limb Length		ns	ns	ns	20	--
Whole Body Length		20*	ns	ns	ns	20 ↑
Thyroid Histology		--	5	--		5
Follicular Cell Height		--	10	--	--	--
Whole Body Wt.		--		--		ns

* transient effects at lower concentrations but no dose response

Endpoint Sensitivity: T4 Stage 51 for 21 d LOEC in µg/L

		Germany			Japan			US
		7	**14**	**21**	**7**	**14**	**21**	**21**
Final Stage		2	2	1	1	1	1	2
Hind Limb Length		0.5	1	2	1	1	1	--
Whole Body Length		ns	ns	2	ns	2	2	1
Thyroid Histology		--	--	2	--	--		1
Follicular Cell Height		--	--	2	--	--	--	--
Whole Body Wt.		--	--		--	--		1

* transient effects at lower concentrations but no dose response

Endpoint Sensitivity: T4 Stage 54 for 14 d LOEC in µg/L

		Germany		Japan		US
		7	**14**	**7**	**14**	**14**
Final Stage		2	2	2	2	2
Hind Limb Length		2*↑	2↑	2↑	1*↓	--
Whole Body Length		ns	2	1*	0.25	2
Thyroid Histology		--	2	--		1
Follicular Cell Height		--	1	--	--	--
Whole Body Wt.		--		--		2

* transient effects at lower concentrations but no dose response

OECD Phase I: Endpoint Sensitivity Conclusions

- Most of the endpoints are sensitive within a factor of two
- Thyroid histology tends to be most sensitive endpoint for antagonism (German and US labs)
- Hind limb length tends to be most sensitive to agonism (at 7 d) (German lab)

What did we learn in Phase I?

- *X. laevis* can be successfully used in different laboratories as a toxicological model for thyroid axis disruption.

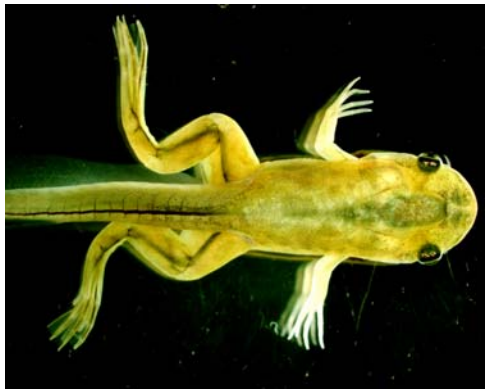
- *X. laevis* is relatively insensitive to different culture and testing conditions.
  - When conditions are standardized, we should expect variation to decrease.

What did we learn in Phase I?

- Results of the PTU and T4 studies were remarkably similar among laboratories, even while using different methods.

- The various endpoints used in these studies were similarly sensitive and consistent with the modes of action of each chemical.

OECD Phase II Validation


OECD Phase II Studies

- Objectives
  - Use more standardized protocol
  - Increase number of participating laboratories
  - Increase number of chemicals tested
    - Sodium perchlorate (NIS inhibitor)
    - T4 (TR agonist)
    - Iopanoic acid (deiodinase inhibitor)

OECD Phase II Studies

- Participating laboratories
  - IGB
  - Towa Kagaku
  - US EPA
  - Springborn
  - Bettelle (Fort Environmental)

Phase II Protocol

- Standards derived from Phase I
  - Flow through methods
  - Use same diet
  - Initial stage 51
  - 21 d Duration
  - Analytical verification of exposures
  - Use same histopathology protocol
- Major changes from Phase I
  - Increase replication to 4/treatment

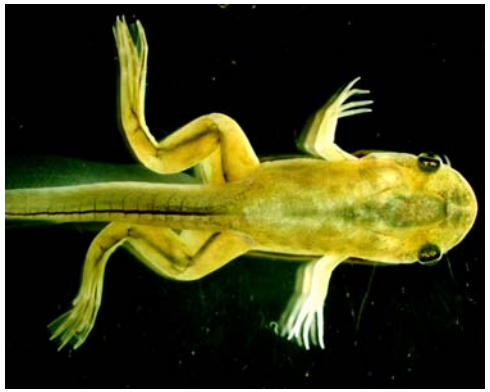
Phase II Protocol: Endpoints

Endpoint	Days
Developmental Stage	7, 21
Body Length	7, 21
Hindlimb Length	7, 21
Wet Weight	7, 21
Mortality	Daily
Thyroid Histology	21

Phase II Status

- Statistical review completed
- Histopathology guidance in development
- Tests on-going in several labs
- Results expected in Fall 2005

Additional Supporting Studies



Supporting Studies

- Numerous supporting studies have been completed or are on-going
- Define responsiveness of model to
  - Chemicals with various MOAs
  - Protocol variations
- Develop new endpoints
  - RIA and ELISA measurements of T3 and T4
  - ELISA for TSH

Supporting Studies

Chemical	Lab	Results	MOA
Methimazole	EPA, Fort Envir	+	TPO inhibition
PTU	EPA, IGB, Towa Kagaku	+	TPO inhibition
Perchlorate	EPA	+	NIS inhibition
Phenobarbital	EPA, Fort Envir	+	UDPGT inducer
Pregnenolone-16α – carbonitrile	EPA, Fort Envir	+?	UDPGT inducer
Dexamethasone	EPA, Fort Envir	-	GR agonist
Corticosterone	EPA, Fort Envir	-	GR agonist
17β-Estradiol	EPA	-	ER agonist
17β-Trenbolone	EPA	-	AR agonist
T4	EPA , IGB, Towa Kagaku, Fort Envir	+	TR agonist
T3	EPA	+	TR agonist

Supporting Studies

Chemical	Lab	Results	MOA
Iopanoic Acid	EPA, IGB	+	Deiodinase inhibitor
ETU	IGB (multi)	+	TOP inhibitor

Supporting Studies

- Demonstrate that the assay is sensitive to different MOAs relevant to HPT disruption
- Improves confidence in the specificity of the assay

Amphibian Metamorphosis Assay for Thyroid Disruption: Contributors

• **Werner Kloas**	**IGB**
• **Robert Opitz**	**IGB**
• **Thomas Braunbeck**	**University of Heidelberg**
• **Sigmund Degitz**	**USEPA, MED**
• **Joseph Tiegte**	**USEPA, MED**
• **Osamu Tooi**	**Towa Kagaku**
• **Douglas Fort**	**Fort Environmental**
• **Tom Gries**	**Springborn**
• **Leslie Touart**	**USEPA, OSCP**
• **Christiana Grim**	**USEPA, OSCP**