TOXIC EFFECTS OF DISPERSED AND NON-DISPERSED OIL ON CHINOOK SALMON SMOLTS (*ONCORHYNCHUS TSHAWYTSCHA*) USING METABOLOMICS

Ronald S. Tjeerdema, Ching-Yu Lin, Brian S. Anderson
Dept of Environmental Toxicology
University of California, Davis

Mark R. Viant
School of Biosciences, University of Birmingham

Michael L. Sowby
Office of Spill Prevention and Response
California Department of Fish and Game

David B. Crane
Water Pollution Control Laboratory
California Department of Fish and Game

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INTRODUCTION

Due to the risk of coastal oil spills to migrating salmon, resource agencies need information on the influence of dispersant treatment on the relative toxicity of spills in near shore waters.

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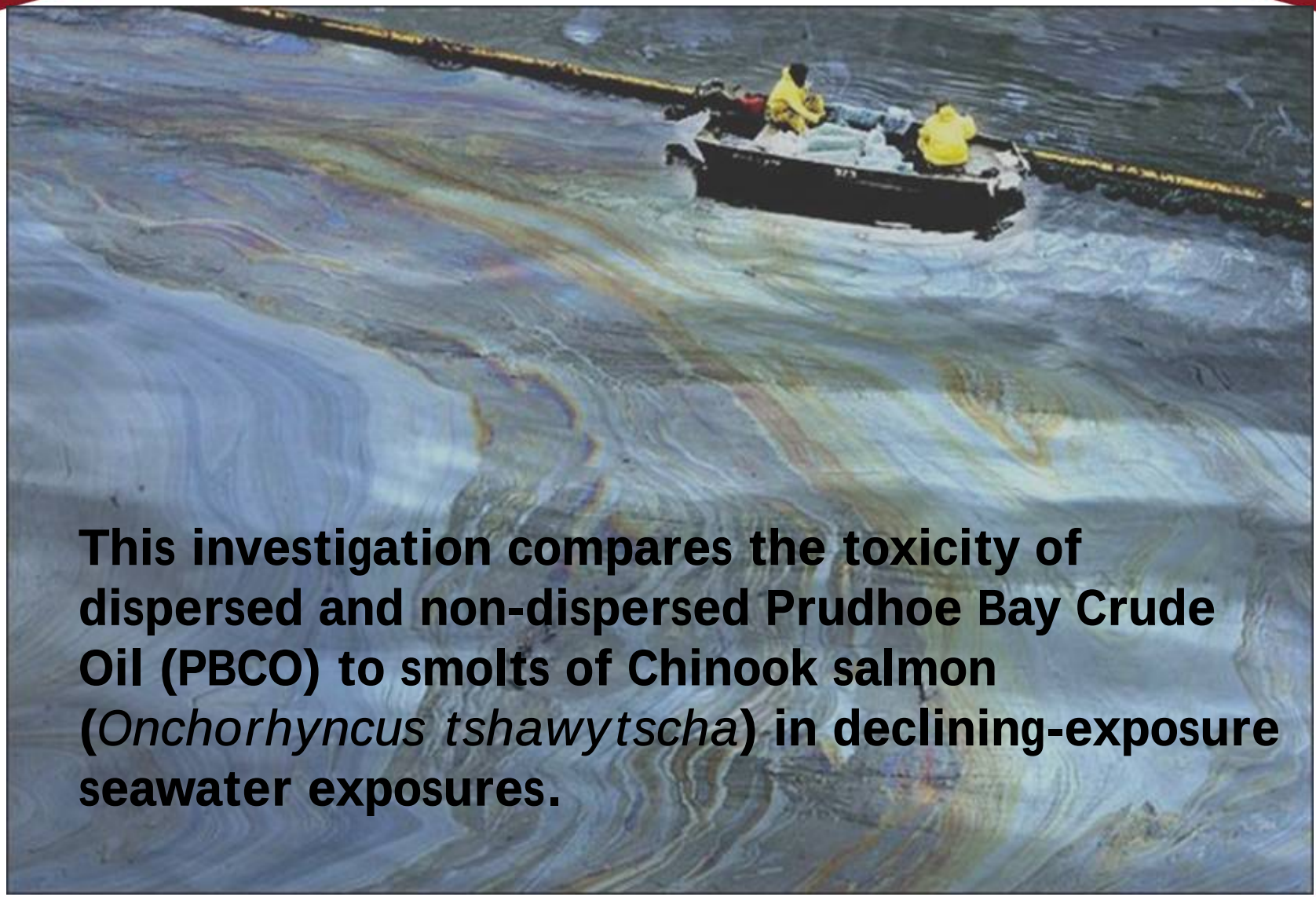

This investigation compares the toxicity of dispersed and non-dispersed Prudhoe Bay Crude Oil (PBCO) to smolts of Chinook salmon (*Onchorhyncus tshawytscha*) in declining-exposure seawater exposures.

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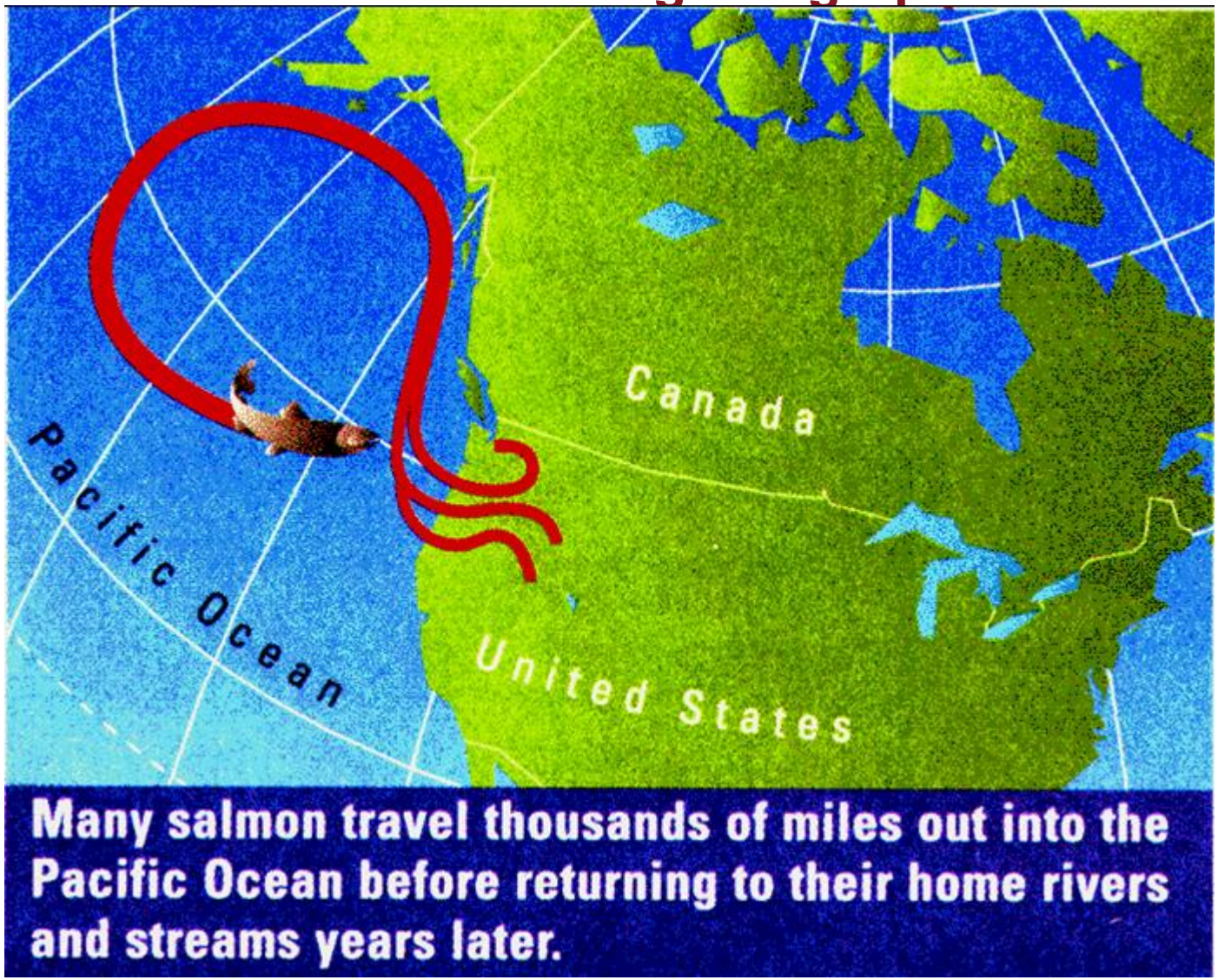
Chinook Salmon – A Migrating Species



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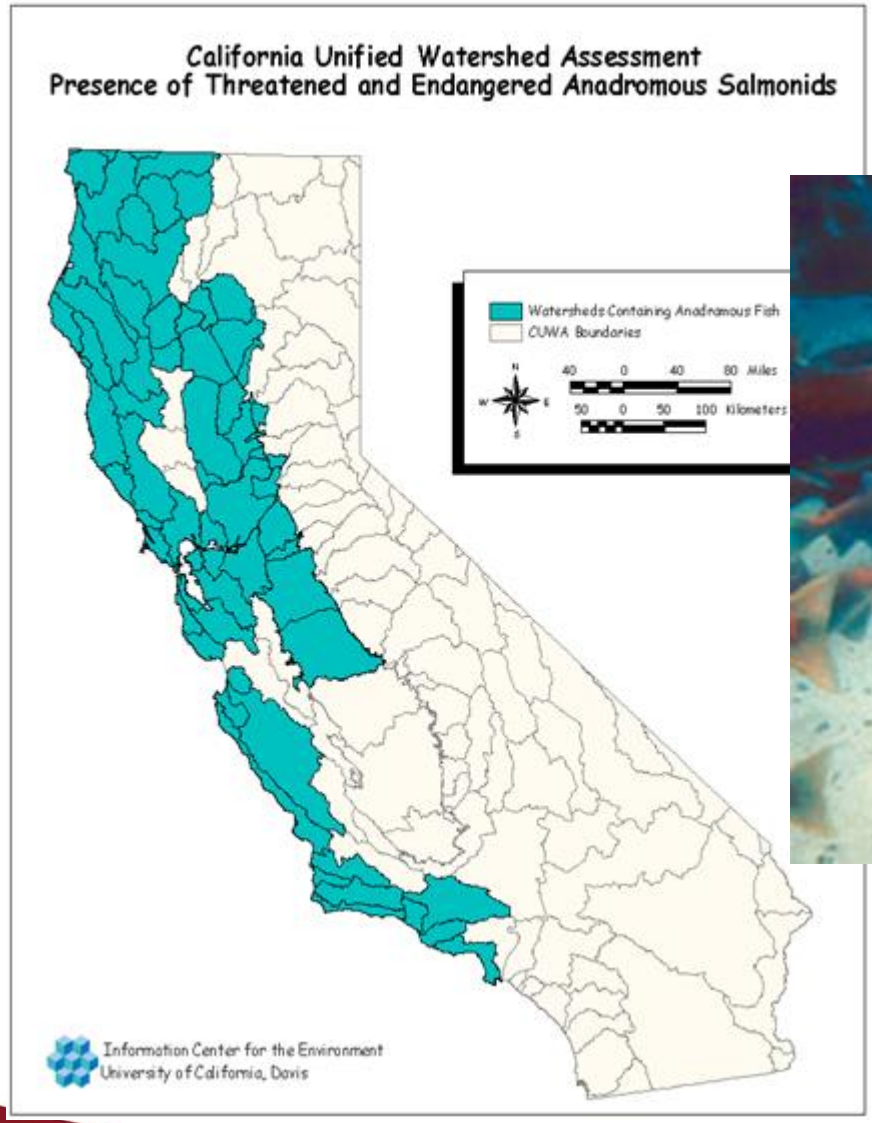
Chinook Salmon – An Anadromous Species



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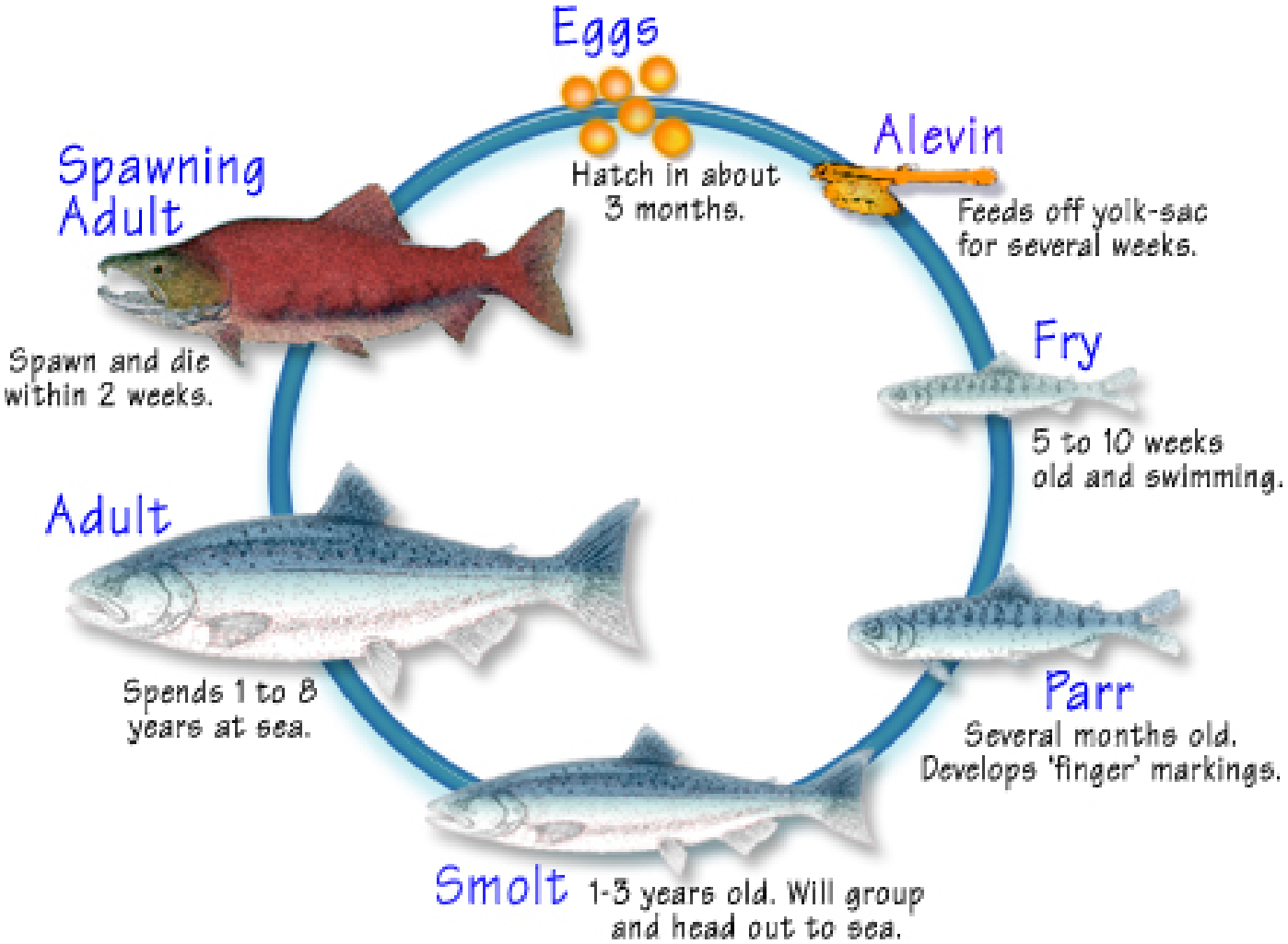
The Salmon Life cycle



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Objectives

- **Assess the relative acute toxicities of dispersed and non-dispersed water accommodated fractions (WAFs) of Prudhoe Bay Crude Oil (PBCO) to Chinook Salmon smolts (*ONCORHYNCHUS TSHAWYTSCHA*) under defined declining exposure conditions.**

- **Apply ^{1}H-Nuclear Magnetic Resonance (NMR)-based metabolomic analysis to investigate the sublethal effects of PBCO WAF and CEWAFs on salmon smolts.**

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Methods: System Development and WAF Exposures

- Methods followed CROSERF (Singer *et al.* 2000).
- 20-L polycarbonate carboys and 18-L aquaria.
- WAFs spun at low rate with minimal vortex (~150 rpm) for 24 h.

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Methods: WAF Tests (cont.)

- 33% of each of 3 carboys distributed to each of 3 replicate 18-L aquaria (2-cm headspace).
- Once full, each aquarium was sampled for TPH and THC chemistry, 8 fish were introduced, and clean seawater flushing initiated.
- Flush rate calibrated with flow meters @ 200 mL per minute; rates verified with THC.

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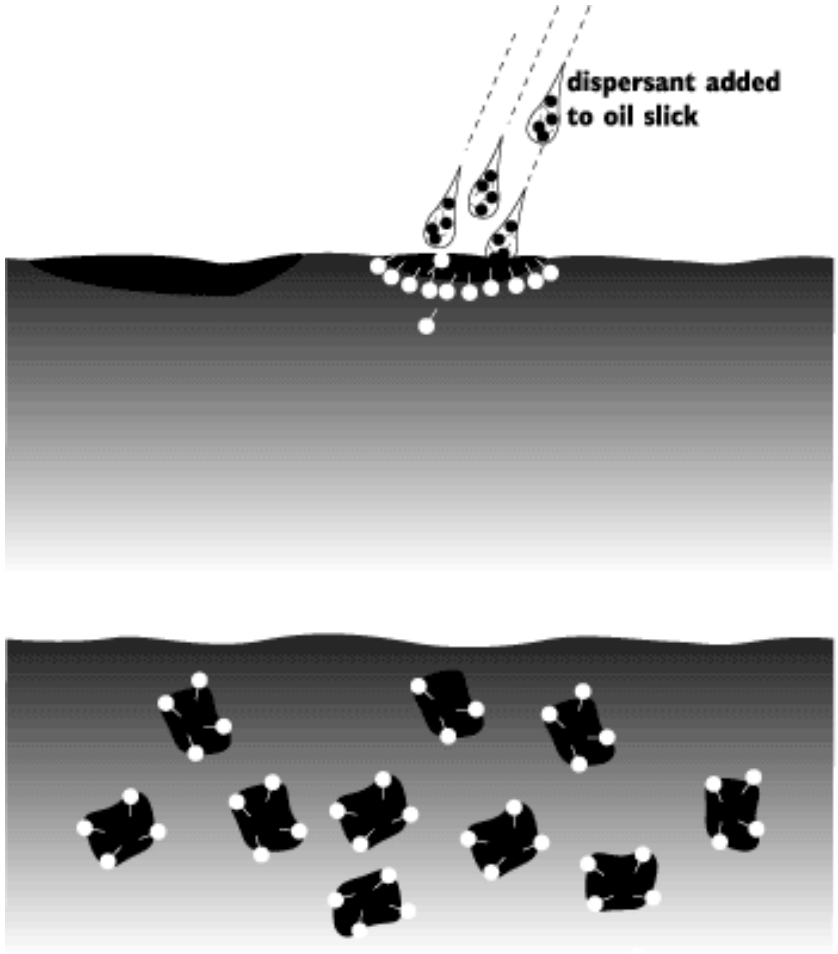
Formation of CEWAF



One end of each dispersant molecule 'chain' attaches to water molecules while the other end of the 'chain' attaches to the oil droplets.

A little energy from wind and waves breaks the oil slick into smaller oil droplets surrounded by dispersant molecules as shown.

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Methods: CEWAF Tests

•Add oil and create vortex size of 20 to 25%.

•Auto pipet 10% (by oil wt) Corexit 9500.

•Spin for 18 h, then settle for 6 h.
•Stagger the test start times.

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Analytical Chemistry

- Total Petroleum Hydrocarbons (TPH): C10 – C36 using GC/flame ionization detection.

- Volatiles hydrocarbons C6-C9: benzene, toluene, ethylbenzene and xylenes (BTEX) using GC/MS & purge-and-trap extraction.

- Total Hydrocarbon Content (THC) (C6–C36) = BTEX C6 to C9 compounds + TPH C10 to C36

- Declining exposures confirmed with THC during tests.

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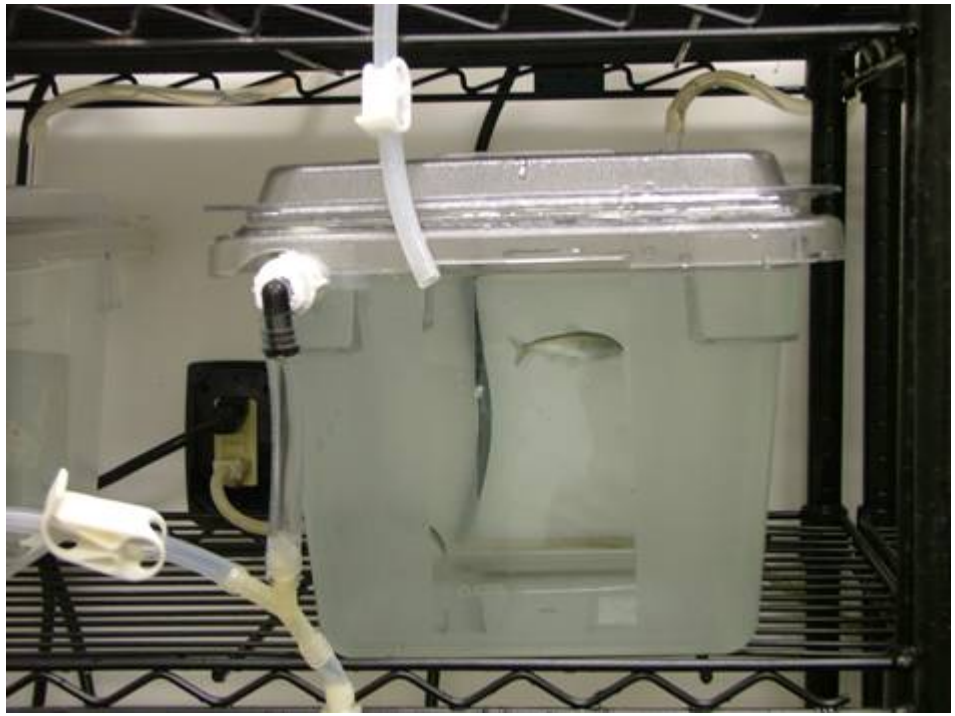
Methods: 96-h Exposures



- 8 salmon smolts per aquarium (~ 8cm).
- 96-h declining seawater exposures.
- 2 of the surviving fish were sacrificed for metabolomics
- Remaining survivors were cultured to assess long-term growth effects

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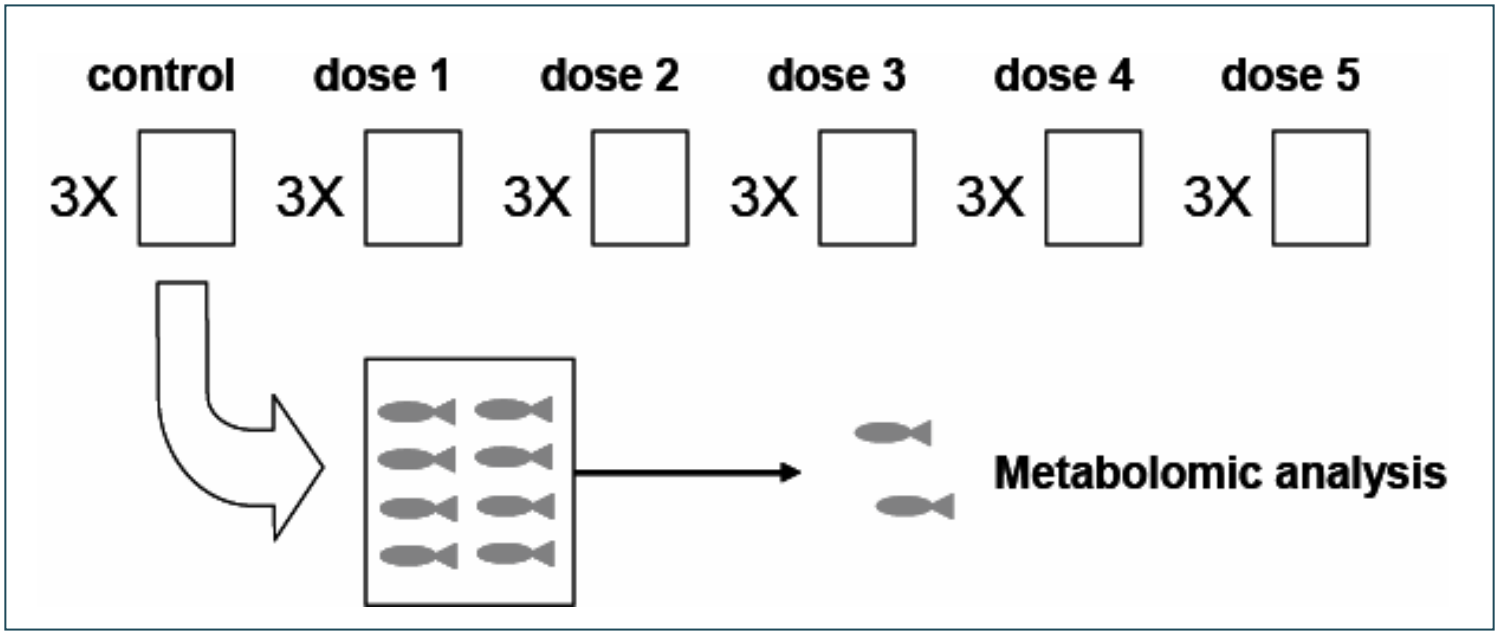
Experimental design



4 times WAF/CEWAF

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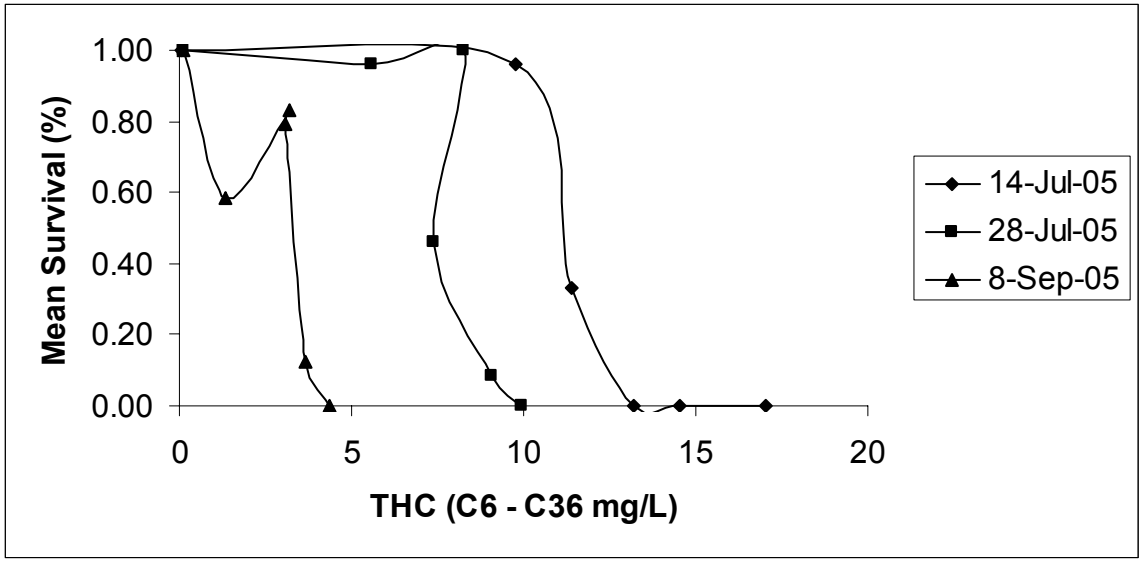
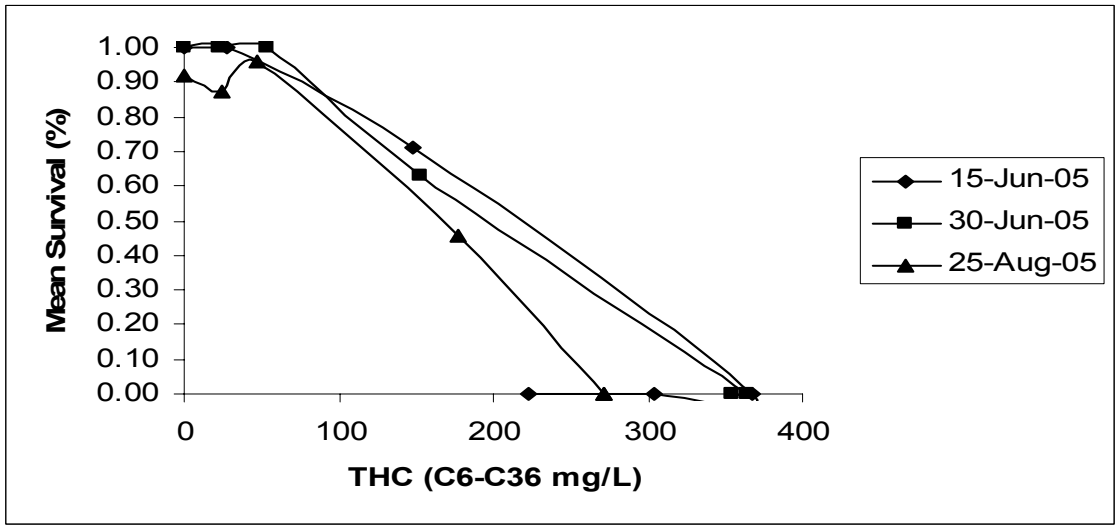
WAF



Mean WAF
LC50 = 7.46
mg/L THC

CEWAF



Mean CEWAF
LC50 = 155.93
mg/L THC

Salmon smolt survival in three WAF and CEWAF tests after 96-h.
THC = Total Hydrocarbon Content.

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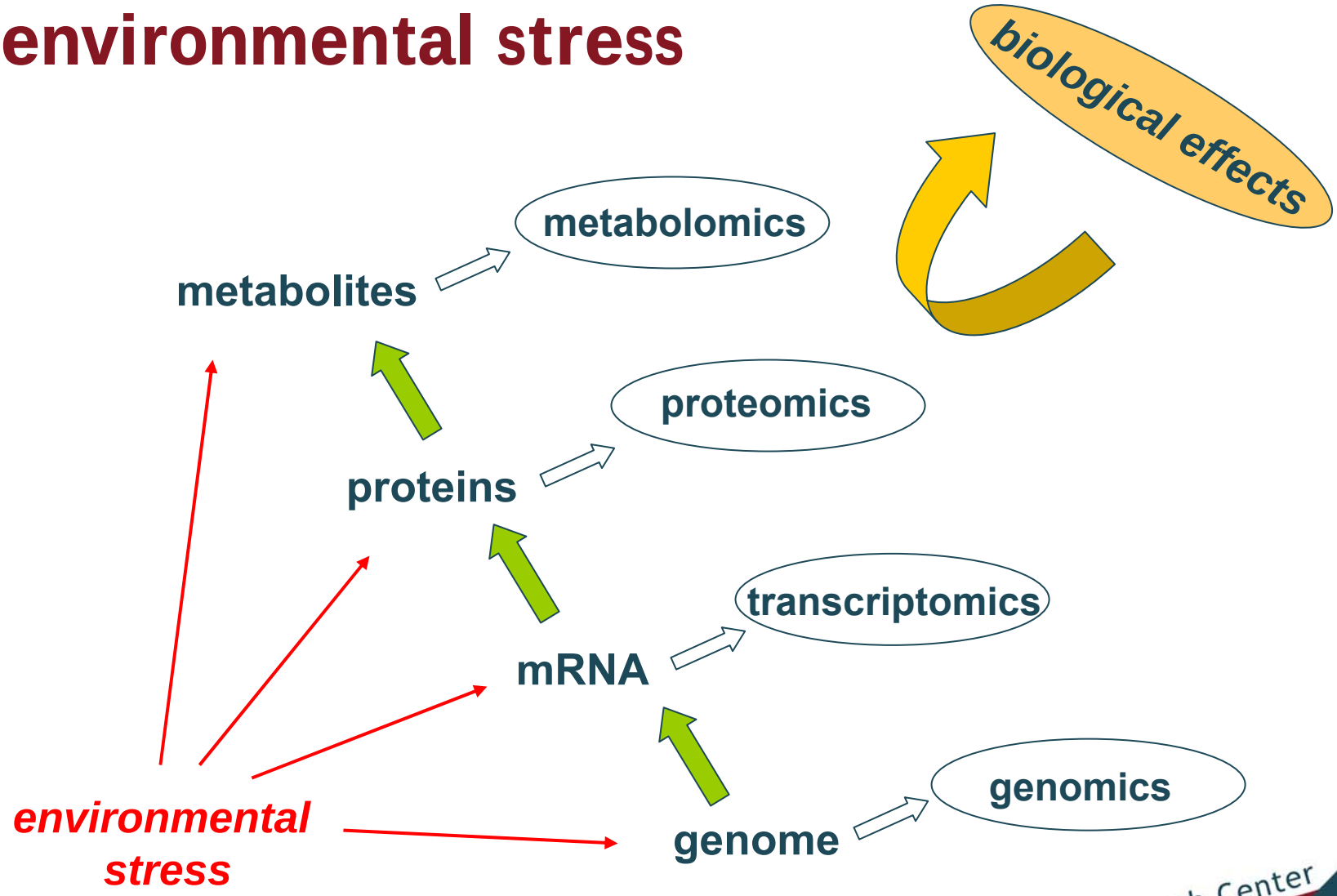
Metabolomics to examine effects of environmental stress



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Metabolomics in environmental science

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NUCLEAR MAGNETIC RESONANCE PROFILING AND CHEMOMETRICS APPLIED TO THE DISCRIMINATION OF ORIGIN OF WILD AND FARMED SALMONS

Rezzi S.*,•, Reniero F.*, Heberger K.*, Guillou C.*

*European Commission, JRC, IHCP, Physical and Chemical Exposure Unit, Ispra (VA), Italy; •present address: BioAnalytical Science, Metabonomics & Biomarkers, Nestle' Research Center, Lausanne (CH).

INTRODUCTION

Fishing and aquaculture are important providers of food and employment in coastal and rural areas of the EU. At present, there is no reliable method either to distinguish wild from farmed fish, or to verify geographical origin. This leads to possible problems with mislabelling and dumping of certain fish, from non-approved sources. A labelling regulation for fishery and aquaculture products requires identification of the official commercial and scientific name, the origin of the fish and its production method (farmed or wild). In the frame of the project COFAWS (Confirmation of the Origin of Farmed and Wild Salmon), we have applied ^{1}H-NMR spectroscopy fingerprint of fish oil and multivariate data analysis techniques.

METHODS

Over 130 specimen of wild and farmed salmons from different geographical origins (Scotland, Ireland, Norway, Iceland, Faroe Islands, Canada, Alaska, and Tasmania) have been considered (Figure 1). Wild and farmed fish were collected by the partners of the COFAWS project. SINTEF and NAFC carried out the extraction of fish oils from tissues of freshly frozen fish using the classical "Bligh & Dyer" method (Figure 2). ^{1}H-NMR spectra were acquired on a Bruker GmbH Advance spectrometer operating at the frequency of 500 MHz using an inverse broadband 5 mm probe. The NMR sample preparation was simply the dilution of 120µL of fish oil in 700 µL of deuterated chloroform. Quantitative spectra were obtained in 25 minutes experimental time under a fully automated way (automatic sample tube insertion, lock, shimming and acquisition). An example of the ^{1}H-NMR spectrum of a salmon (*Salmo salar*) oil is shown in Figure 3. ^{1}H-NMR spectra present many signals, several of them being correlated together. The application of multivariate statistical analysis methods requires a prior step of data reduction performed by reducing each raw spectrum into a series of standardized variables so-called buckets. Also some signals (eg. Phosphatidylcholine PC) may show variations of chemical shifts related to matrix effects. Such signals were excluded for statistical evaluation as shown in Figure 4.

Figure 1: salmon from different geographical origins

Figure 2: Sample preparation

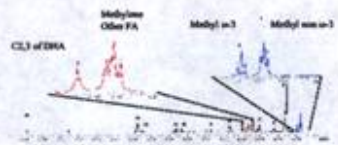

Figure 3 1H NMR spectrum of a salmon (*Salmo salar*) oil

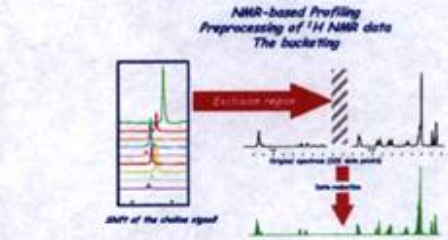

Figure 4

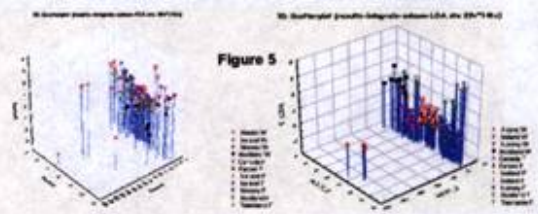

Figure 5

CONCLUSION

The ^{1}H-NMR profiling is a very attractive analytical technique for the amount of information that it provides on complex mixtures while requiring only simple sample preparation. Moreover, using modern NMR spectrometer, this information is accessible in a very reproducible way within a short time of acquisition of the order of a few minutes per sample. The present work shows the possibilities for differentiation between wild and farmed salmon: The application of LDA without taking into account information about the declared geographical origin also allows the discrimination between wild and farmed salmon although an overlap between the two groups is observed (Figure 5). Additional analytical data (e.g. isotopic data) could be included to refine the prediction model. The recognition of the geographical origin is also possible, however it is then necessary to carefully cross validate the models for prediction purpose. The representativeness of the training set is also certainly to be further extended building up a more comprehensive database.

Reference
Bligh, E.G. & Dyer, W.J. (1959) A rapid method of total lipid extraction and purification. Can. J. Biochem. Physiol. 37: 911-917.

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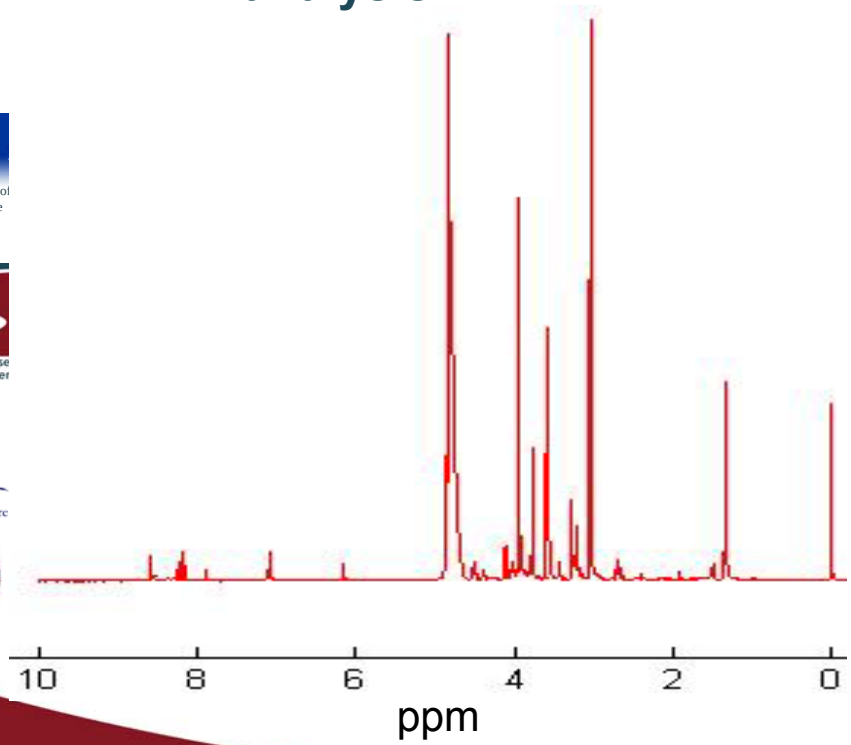
The COFAWS Consortium

Contact
Claude Guillou
European Commission • DG Joint Research Centre
Institute for Health and Consumer Protection (IHCP)
Tel. +39 0332 789878 • Fax +39 0332 789303
E-mail: claude.guillou@jrc.it

NMR-Based Metabolomics Approach

Sample prep (tissue or biofluid)

1-D NMR analysis



Peak assignment

1-D (^{1}H NMR)

2-D (^{1}H-^{1}H COSY & ^{1}H-^{13}C HSQC)

Multivariate statistical analysis

$$ab = \left\{ \begin{array}{l} a_1b_1,\ a_1b_2,\ a_1b_3 \text{ -------- } a_1b_n \\ a_2b_1,\ a_2b_2,\ a_2b_3 \text{ -------- } a_2b_n \\ a_3b_1,\ a_3b_2,\ a_3b_3 \text{ -------- } a_3b_n \\ \vdots \quad \vdots \quad \vdots \qquad \vdots \\ a_mb_1,\ a_mb_2,\ a_mb_3 \text{ --------- } a_mb_n \end{array} \right\}$$

Metabolite profiling
(metabolomic classification/
biochemical mechanism)

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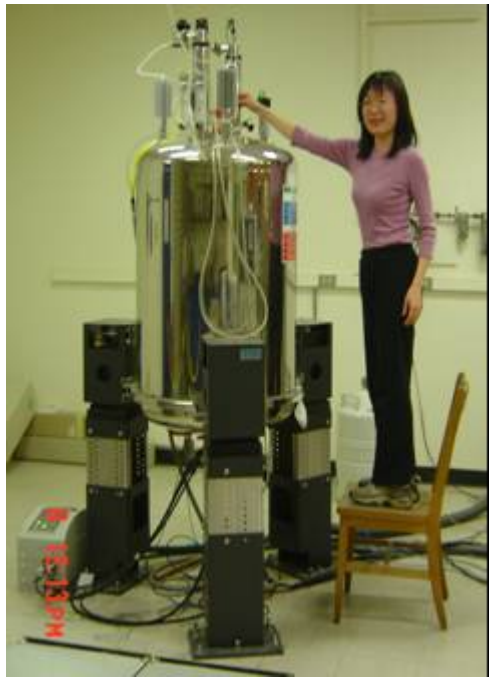
Metabolomics Method Overview

Dorsal muscles and liver from 2 surviving fish from each replicate exposure were flash frozen for metabolomic analysis.



- Small molecular mass metabolites were extracted with MeOH/H_20.
- ^{1}H-NMR analysis provides metabolite profiles.
- Metabolite profiles are then subjected to multivariate analyses (PCA).

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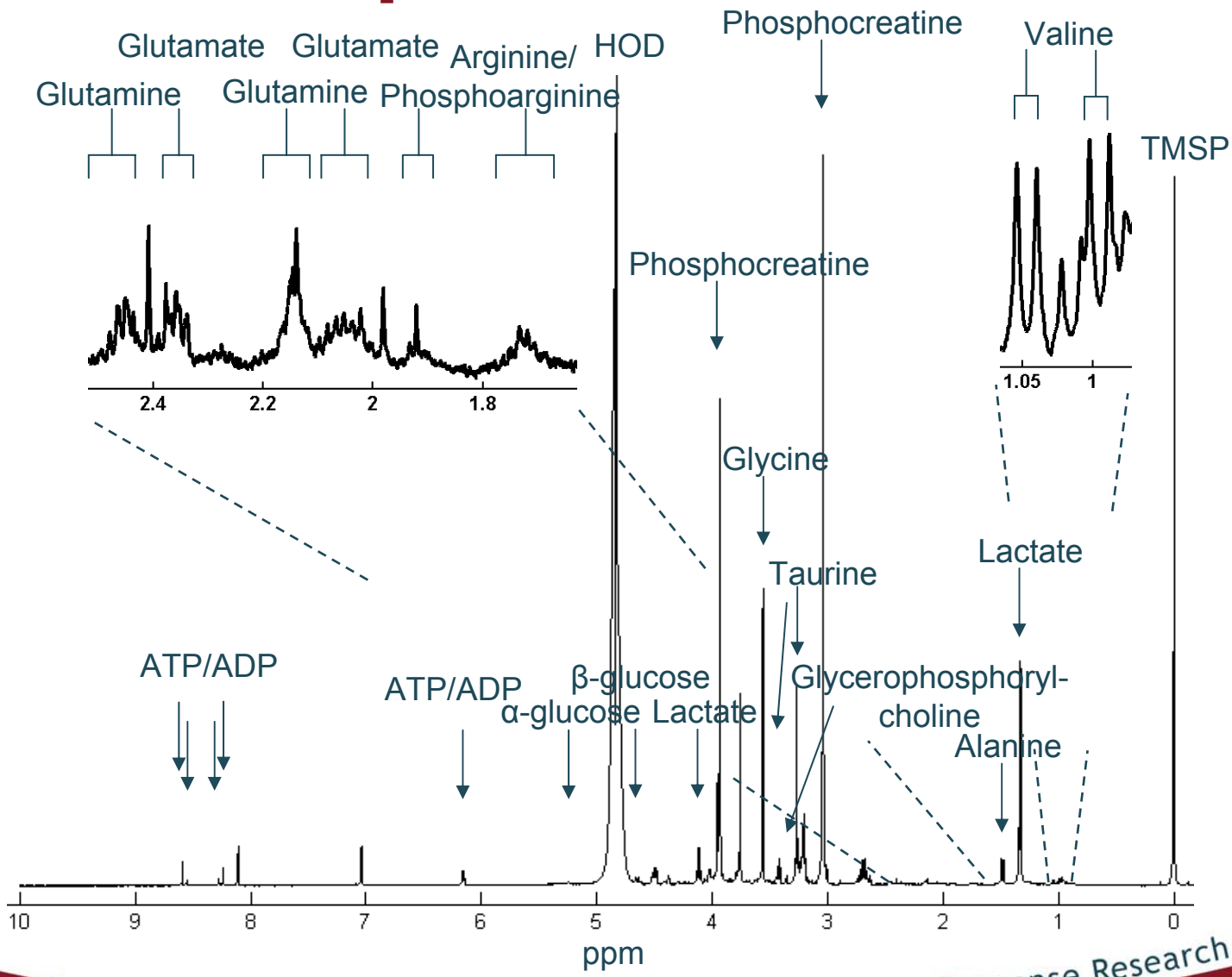
NMR Spectrum of Muscle



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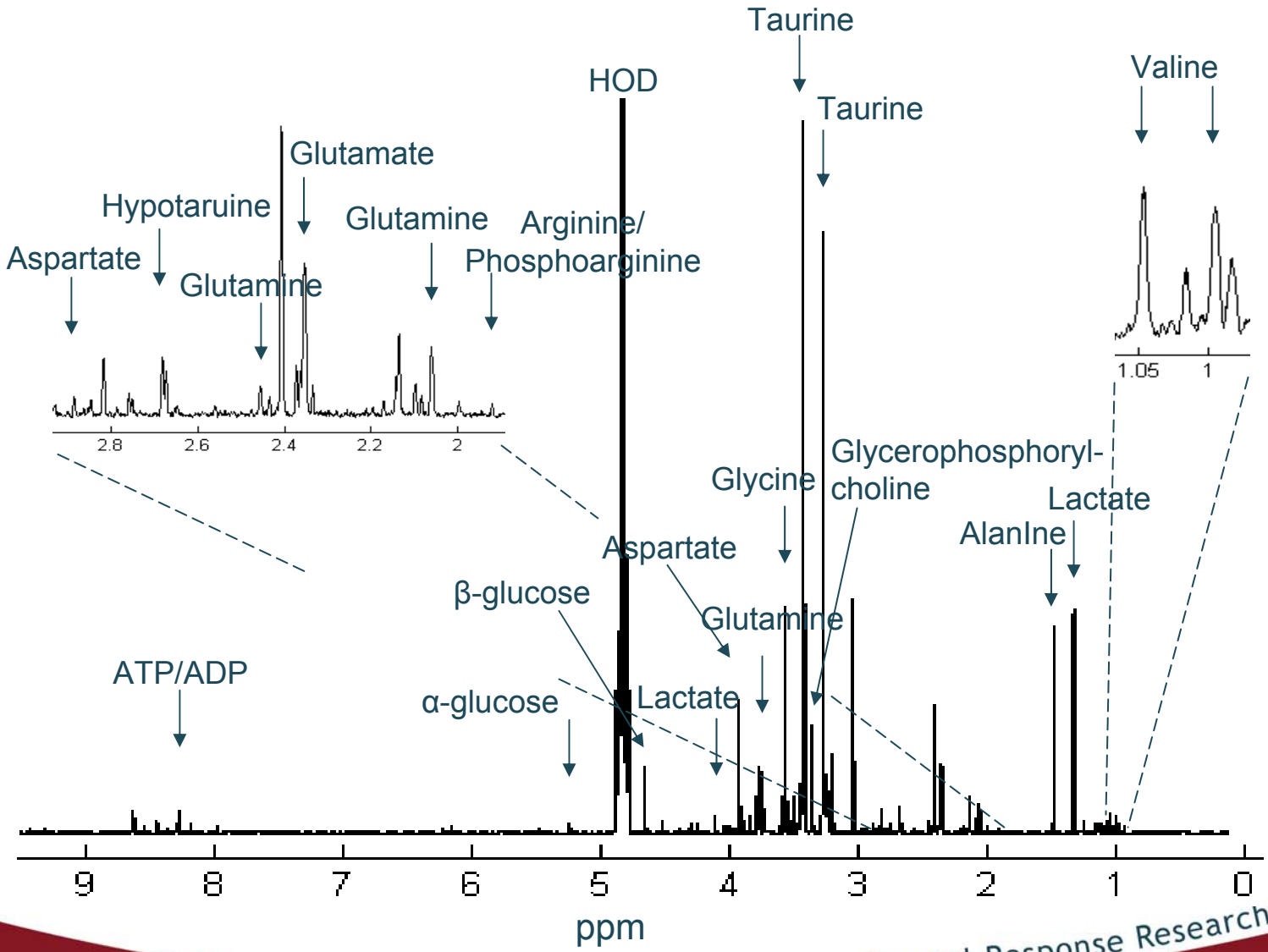
p-JRES NMR of Liver



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Multivariate Statistical Analysis

$$ab = \left\{ \begin{array}{l} a_1b_1,\ a_1b_2,\ a_1b_3 \text{ -------- } a_1b_n \\ a_2b_1,\ a_2b_2,\ a_2b_3 \text{ -------- } a_2b_n \\ a_3b_1,\ a_3b_2,\ a_3b_3 \text{ -------- } a_3b_n \\ \vdots \qquad \vdots \qquad \vdots \qquad\qquad \vdots \\ a_mb_1,a_mb_2,\ a_mb_3 \text{ --------- } a_mb_n \end{array} \right\}$$

Principal component analysis (PCA), an unsupervised clustering method requiring no knowledge of the data set and used to reduce the dimensionality of multivariate data while preserving most of the variance within it.

Scores plots provide the scores on the principal components (PCs) from each data set. The first PC contains the largest part of the variance of the data set with subsequent PCs containing correspondingly smaller amounts of variance.

Loadings plots show how much the variable has in common with that component. Thus, for NMR data, loading plots can be used to detect the metabolites responsible for the separation in the data.

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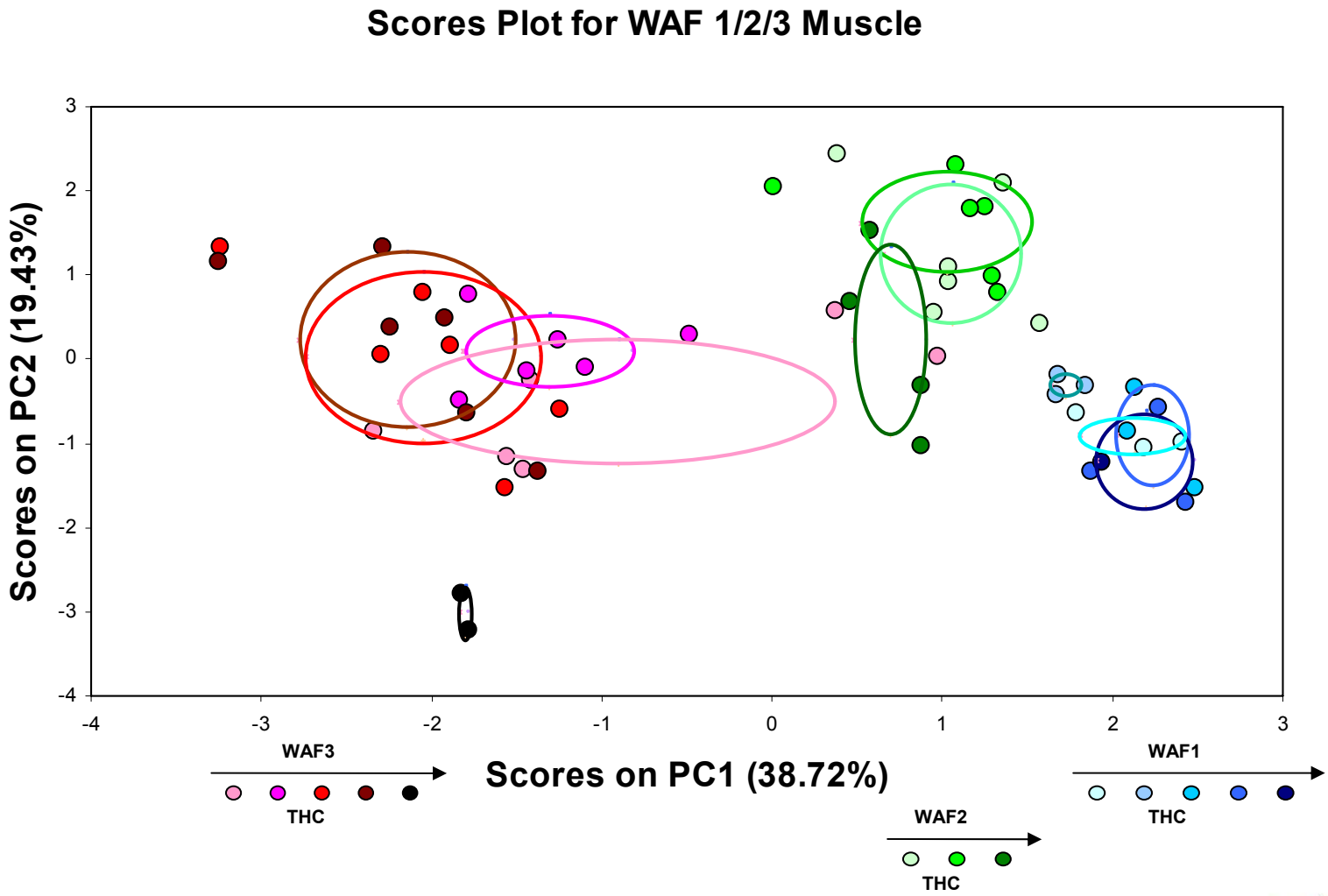
PCA Scores Plot from WAF Exposures



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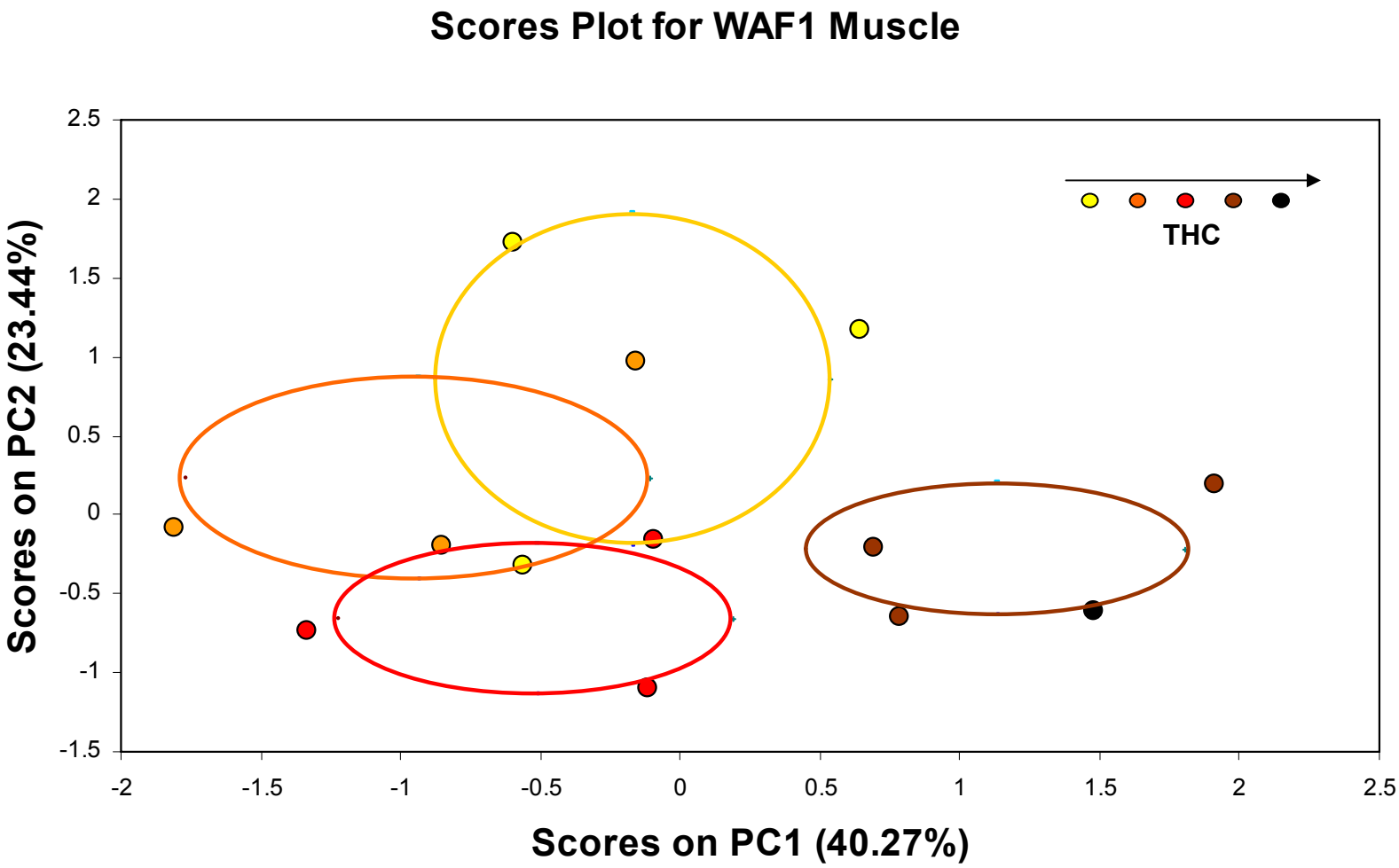
PCA Scores Plot from Each WAF Exposure



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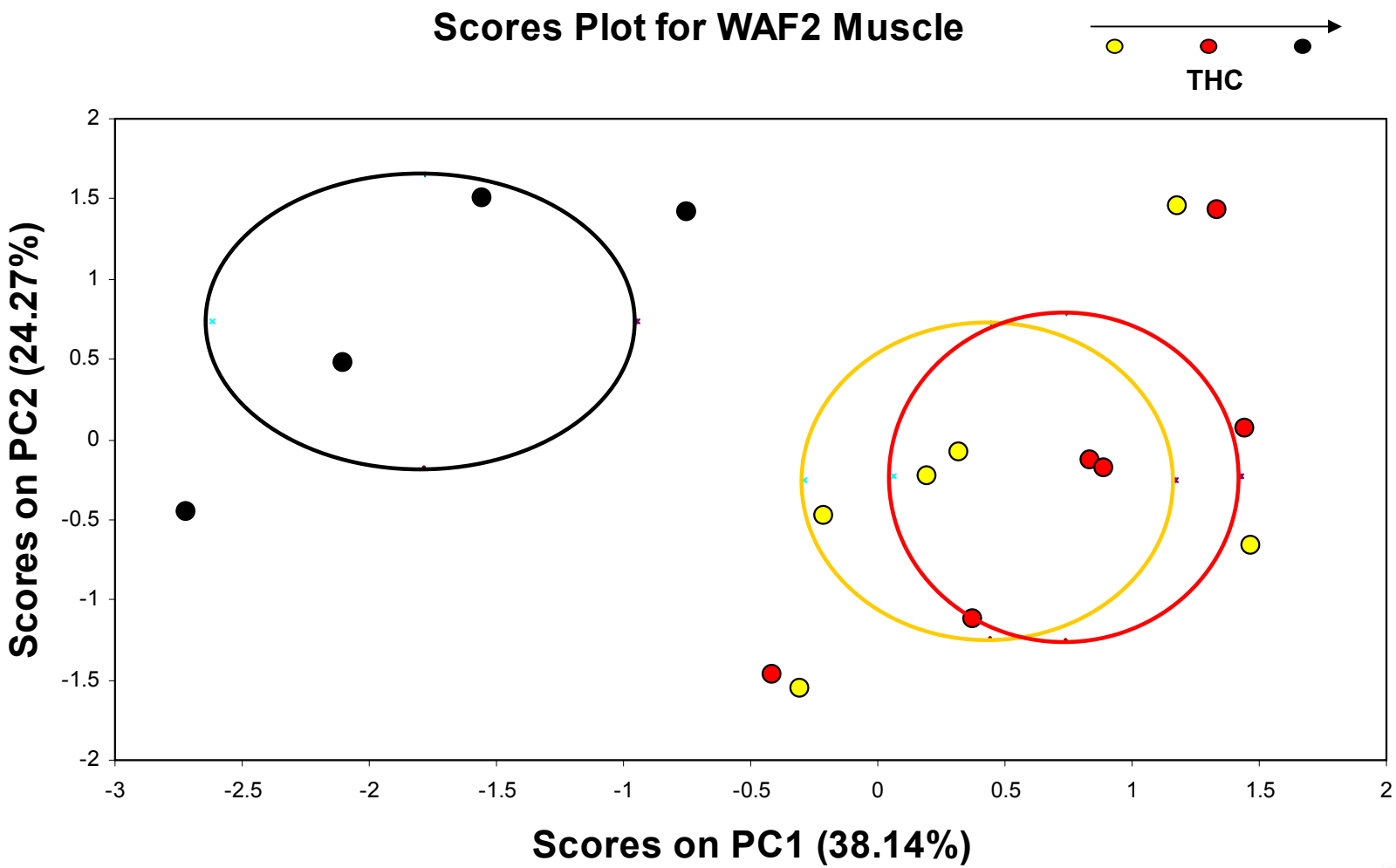
PCA Scores Plot from Each WAF Exposure



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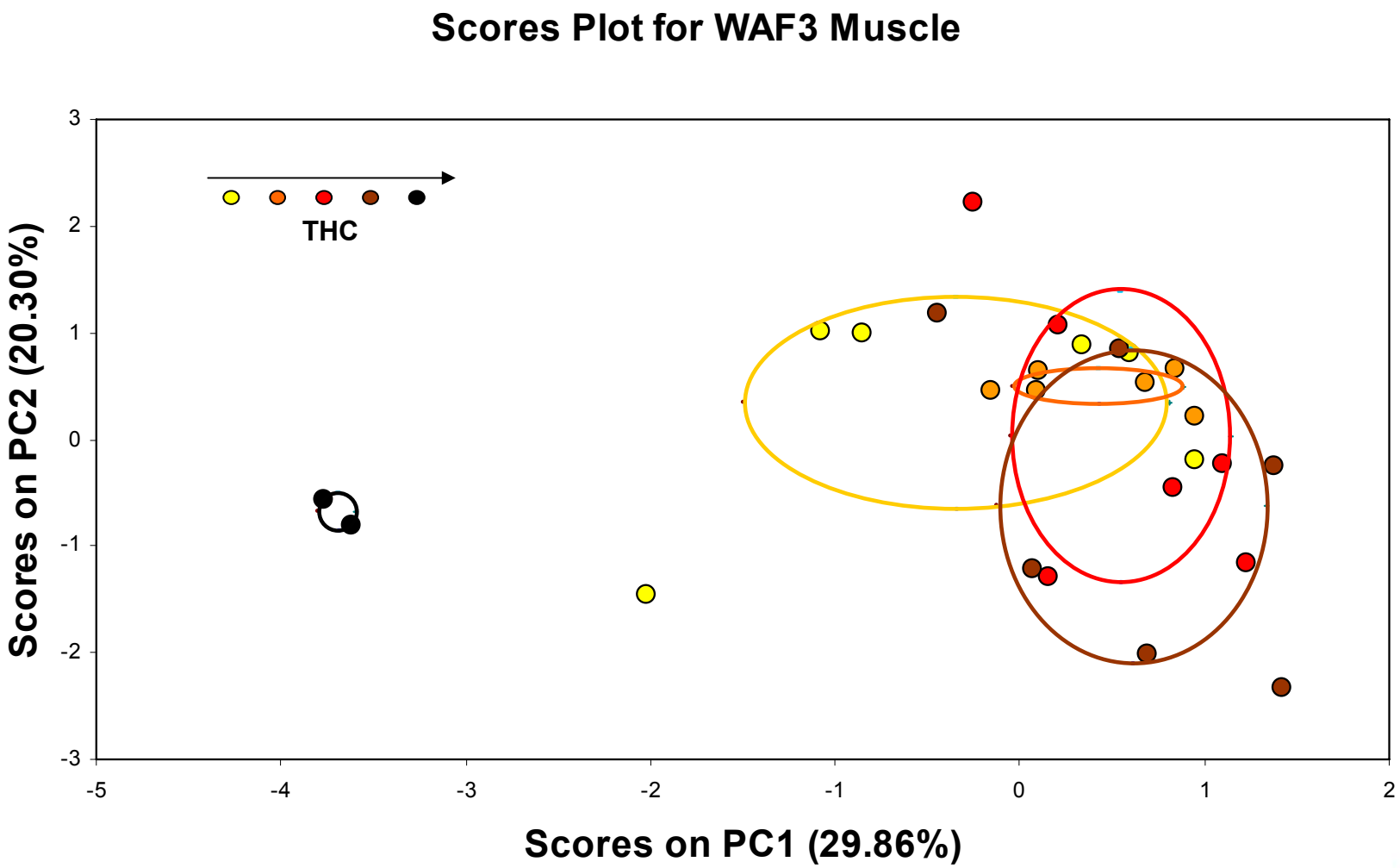
PCA Scores Plot from Each WAF Exposure



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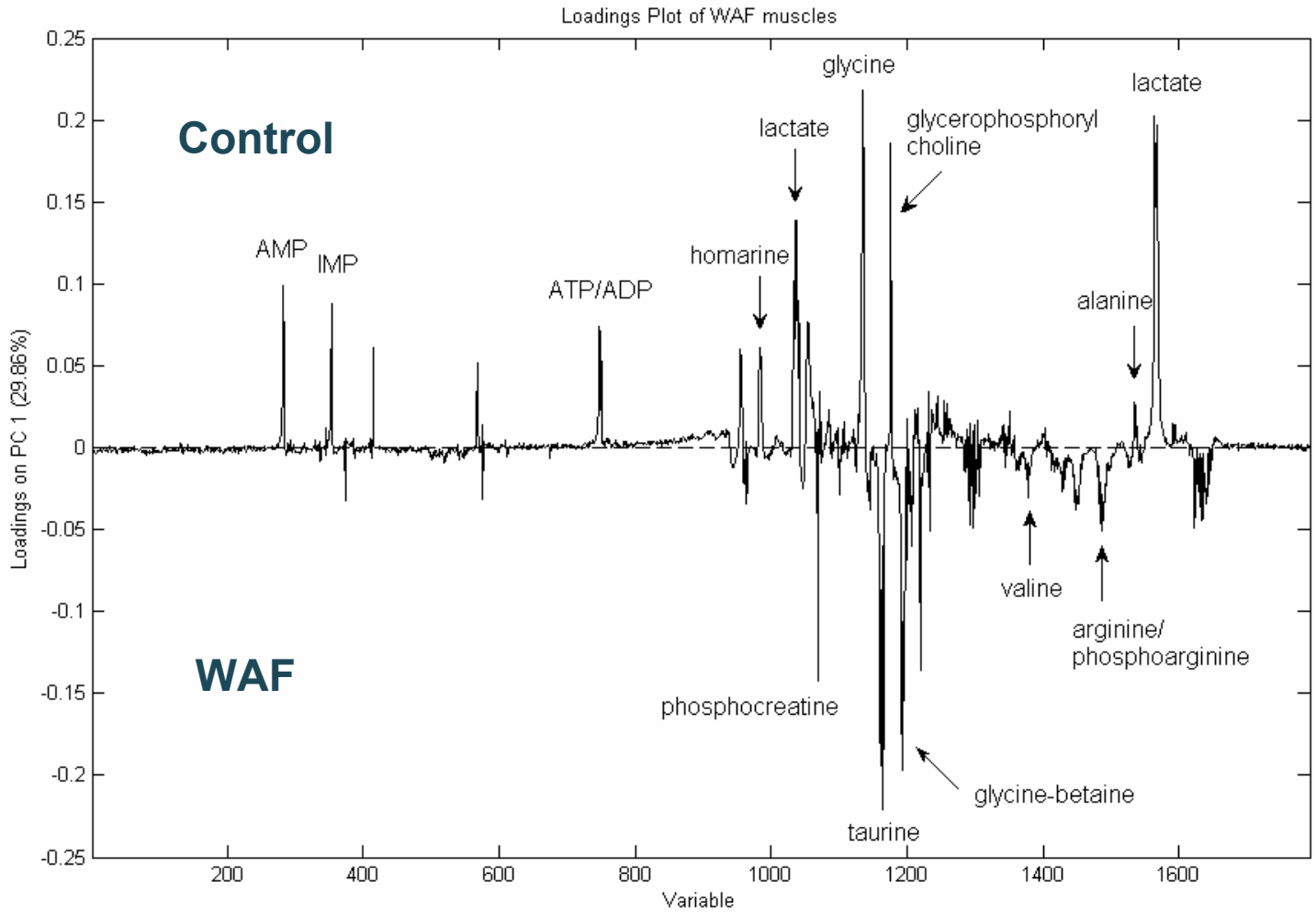
Muscle Loadings Plot from WAF Exposure



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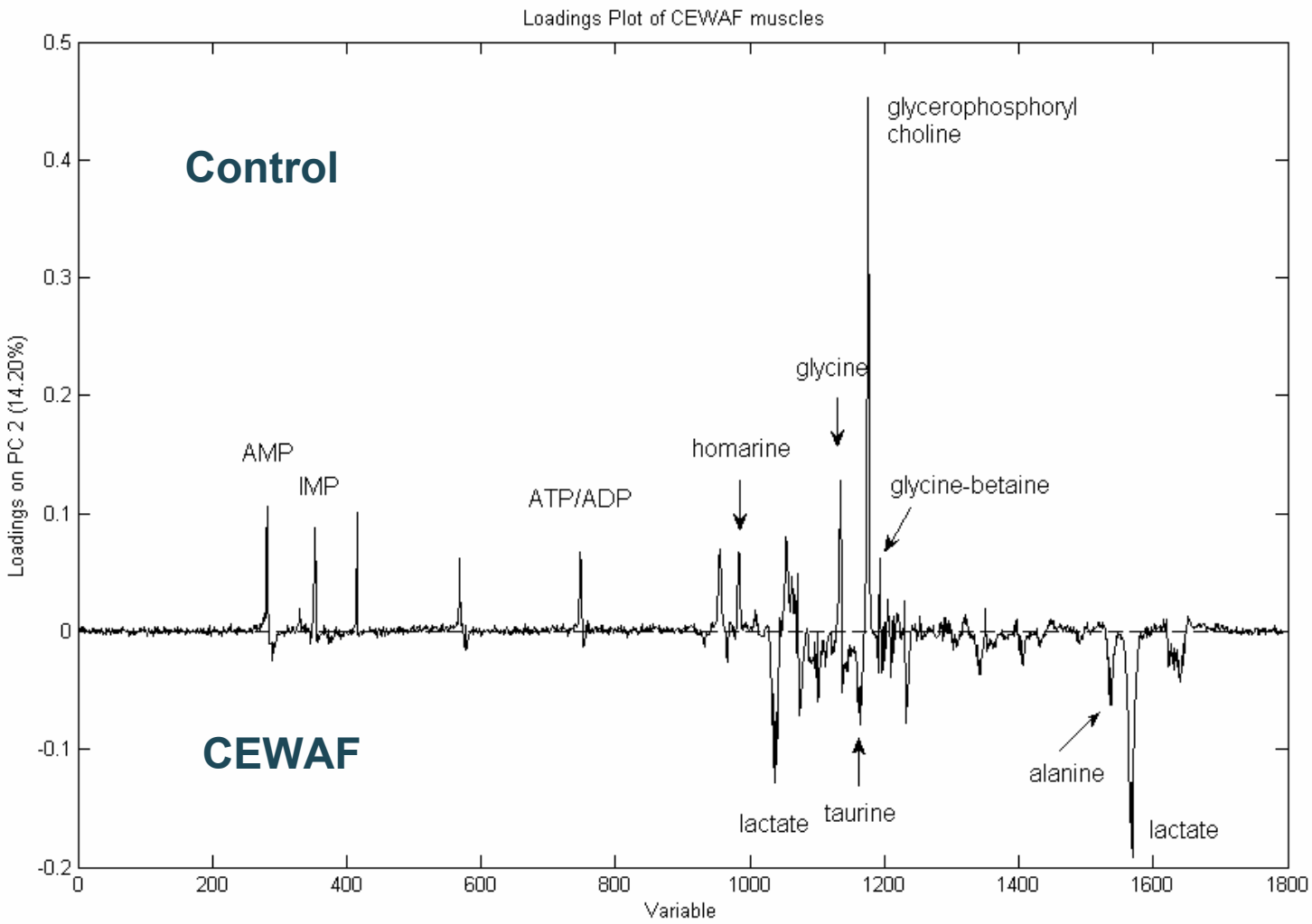
Muscle Loadings Plot from CEWAF Exposure



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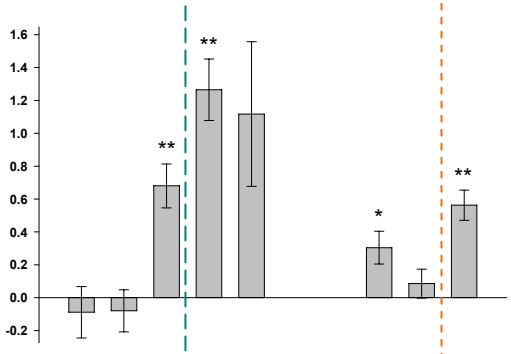
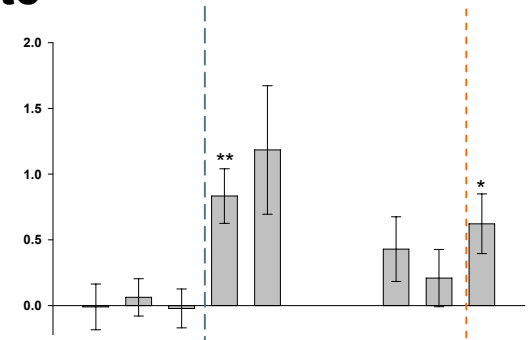
Metabolic Effects in Muscle

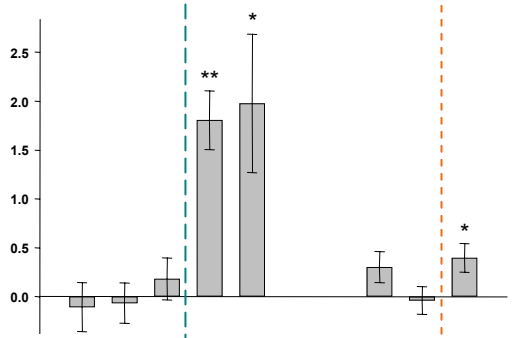
valine



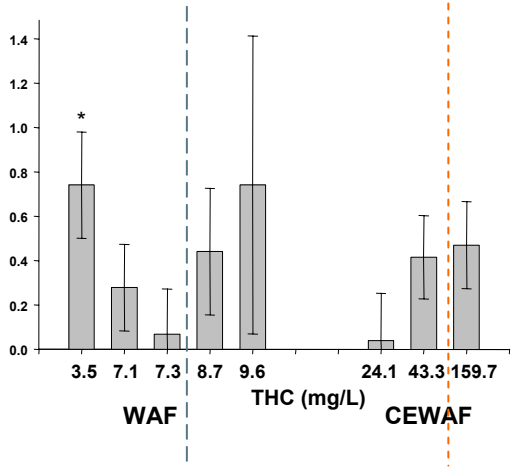
glutamate



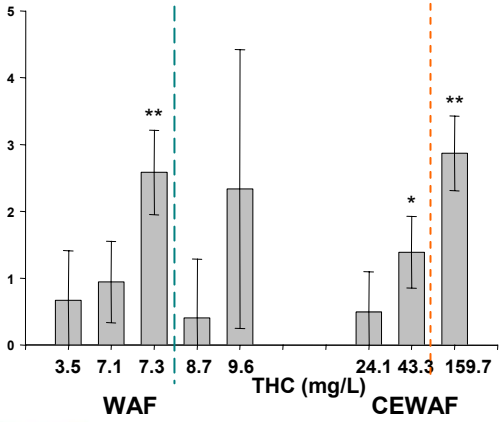
arginine/ phosphoarginine



glutamine



alanine



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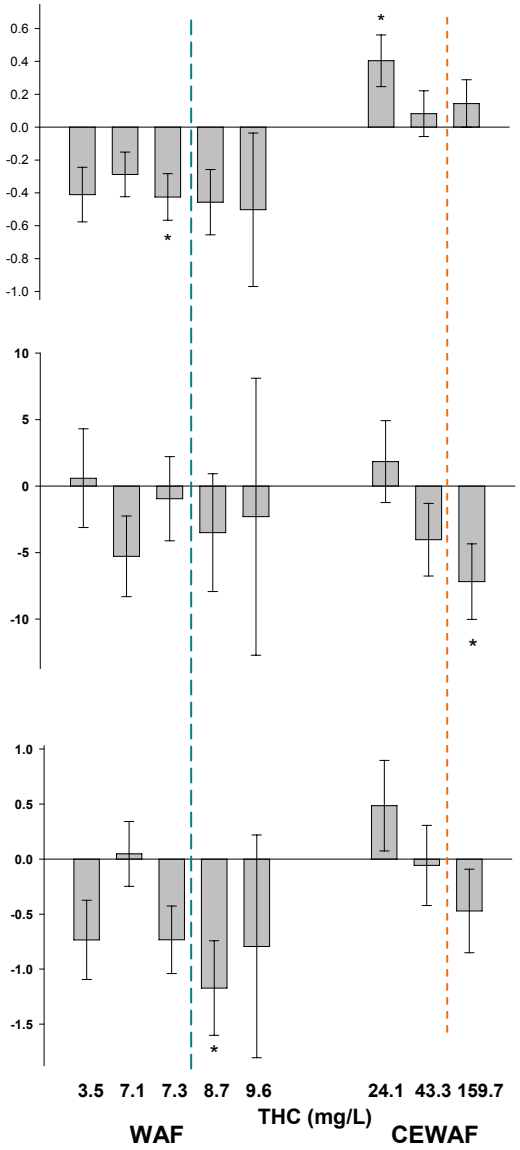
succinate

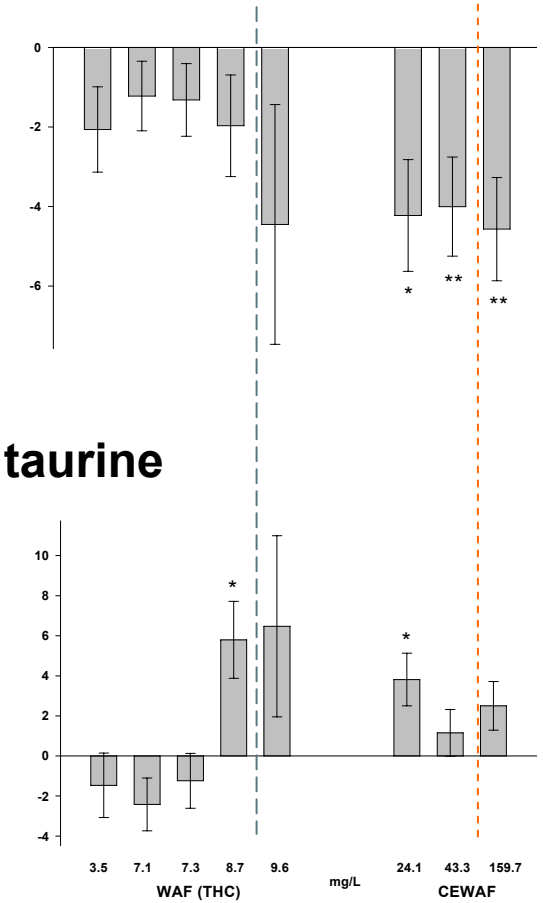
phosphocreatine

ATP/ADP



glycerophosphorylcholine

taurine



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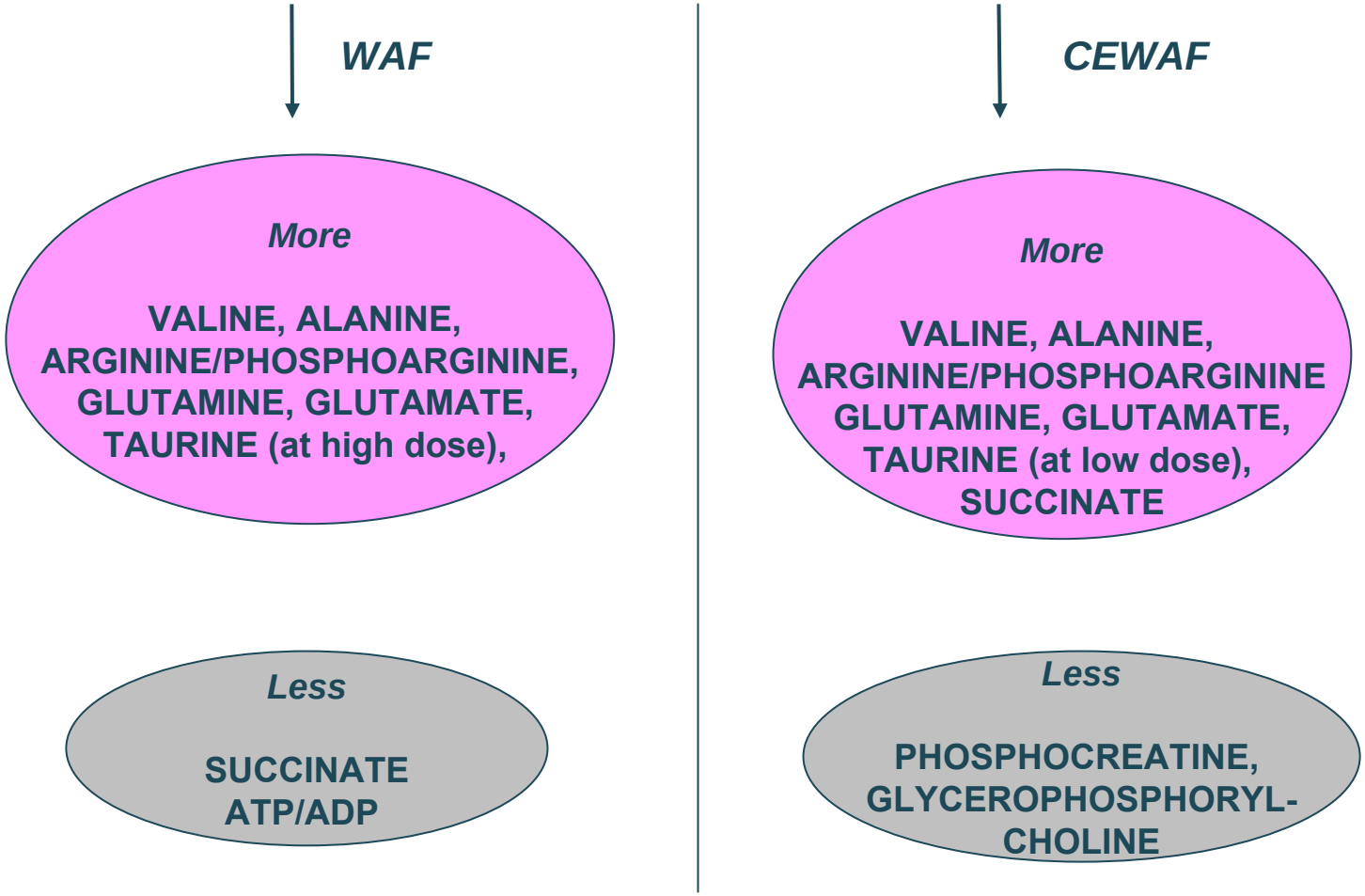
Summary of Metabolic Results in Muscle



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Metabolites Detected from Liver

Metabolites	^{1}H NMR assignment (ppm)[a]	p-JRES assignment (ppm)
Valine	1.047 (d)	0.995*, 1.047*
Lactate[b]	1.328 (d), 4.115 (q)	1.328*, 4.115*
Alanine[b]	1.488 (d), 3.785 (m)	1.488*
Arginine/Phosphoarginine	1.725 (m), 1.918 (m), 3.833 (t)	1.918*
Glutamate[b]	2.070 (m), 2.355 (t)	2.355*
Glutamine[b]	2.138 (m), 2.455 (m), 3.763 (m)	2.138*, 2.455*, 3.763*
Hypotaurine	2.658 (t)	2.658*
Aspartate	2.817 (dd), 3.900 (dd)	2.817*, 3.900*
Unknown	3.039 (s)	3.039*
Taurine[b]	3.268 (t), 3.423 (t)	3.268*, 3.423*
Glycerophosphoryl-choline	3.358 (s)	3.358*
Glycine[b]	3.563 (s)	3.563*
Glycine-betaine[b]	3.933 (s)	3.933*
β-glucose[b]	4.650 (d)	4.650*
α-glucose	5.238 (d)	5.238*
NAD+ / NADP+	6.042 (d), 6.093 (d), 8.174 (s), 8.833* (d), 9.147* (d), 9.342* (s)	
ATP/ADP[b]	6.158 (d), 8.273 (s), 8.546 (s)	8.273*
Unknown	7.093* (s)	
Unknown	7.865* (s)	
Unknown	7.965 (d)	7.965*
Formate	8.458* (s)	
AMP	8.593* (s)	
Unknown	8.618 (s)	8.618*

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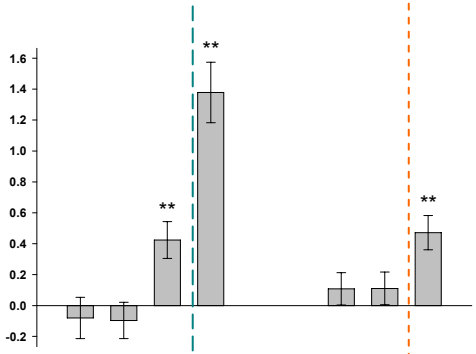
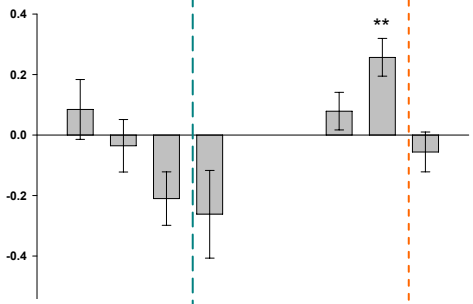
Metabolic Effects in Liver

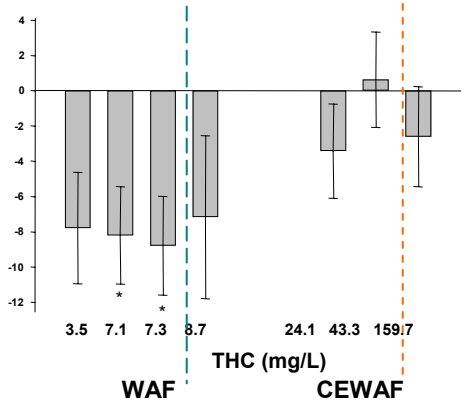
valine



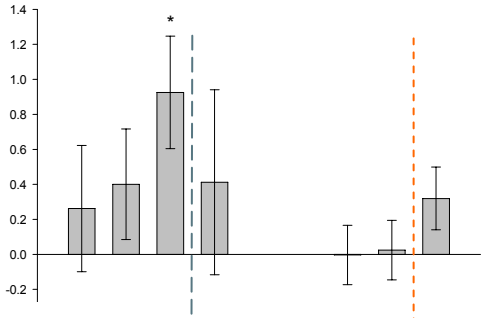
glutamine



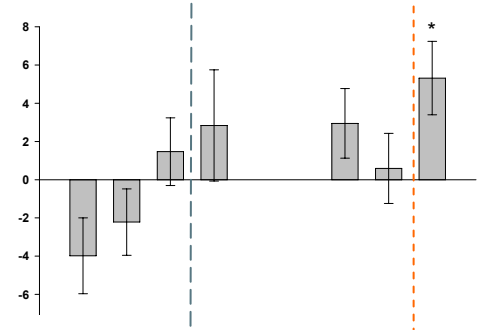
glycerophosphorylcholine



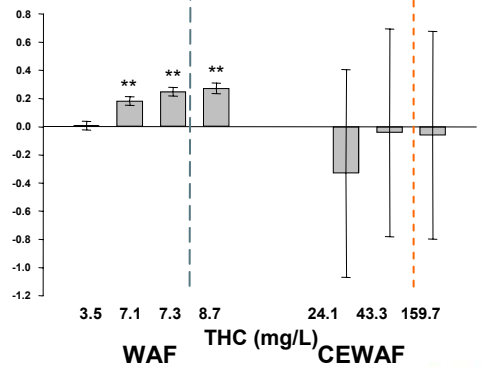
aspartate



glycine



formate



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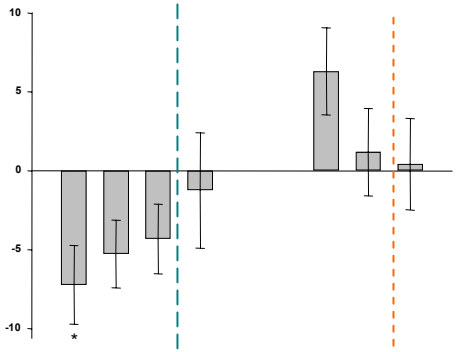
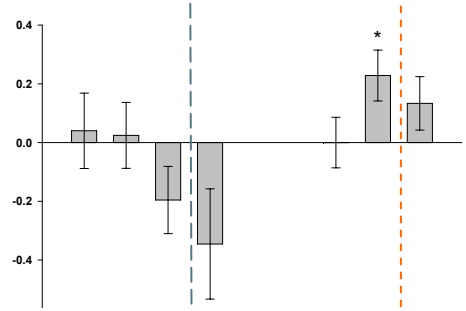
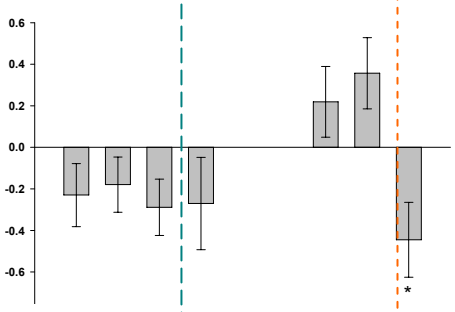
lactate



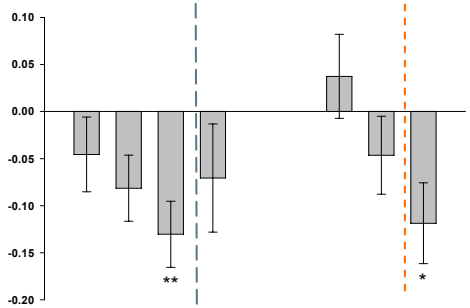
ATP/ADP



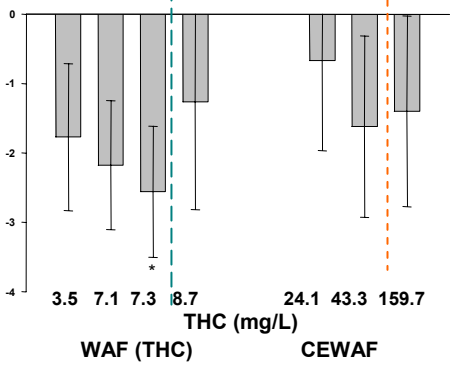
α-glucose



AMP



phosphocreatine



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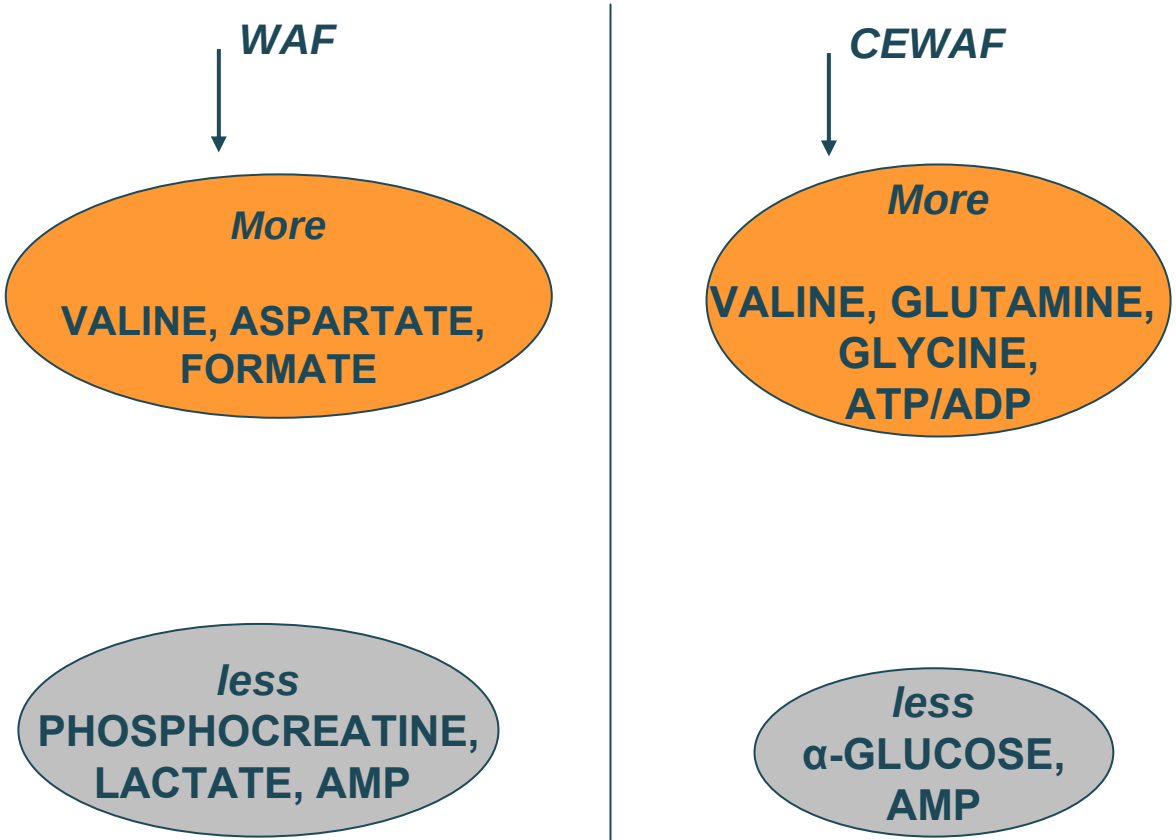
Summary of Metabolomic Results in Liver



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Summary of Metabolic Actions

- **Both WAF and CEWAF of PBCO result in an increase in amino acids – potentially to repair oil's damage to proteins and enzymes.**
- **Increased production of amino acids is at the expense of energetic molecules such as ATP and phosphocreatine.**
- **Loss of energy to damage repair leaves less available for growth, response to stress, and potentially future reproduction.**

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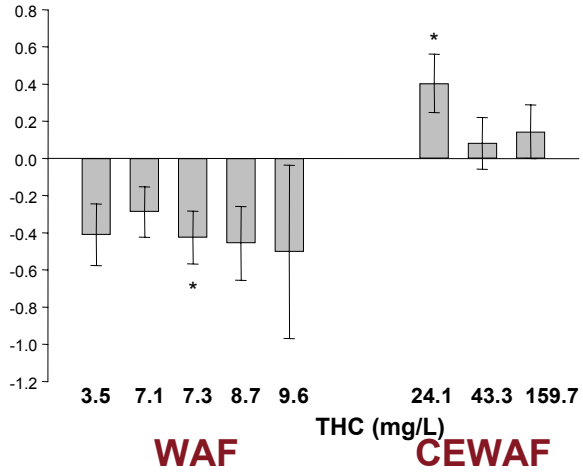
Summary of Metabolic Actions

- *toxicant dependent*

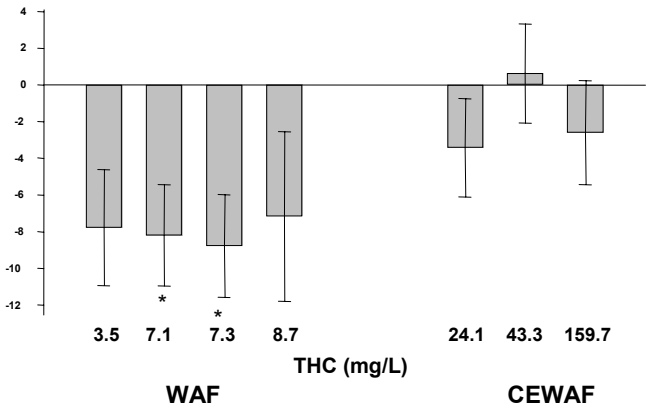
eg. succinate



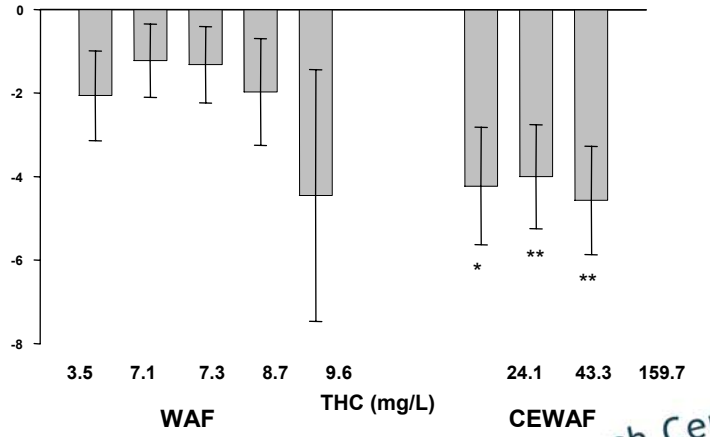
- *organ specific*

eg. glycerophosphorylcholine

livers



muscles



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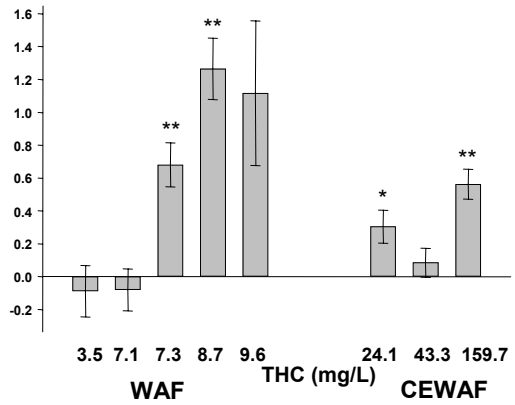
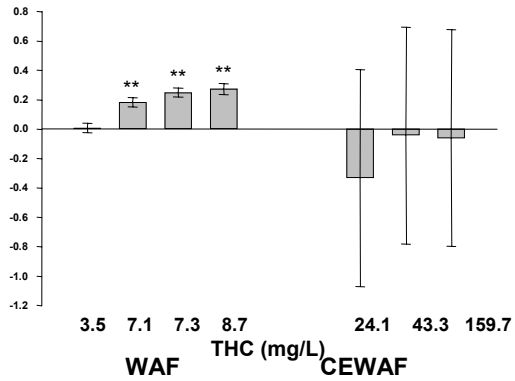
Summary of Metabolic Actions

- *dose dependent*

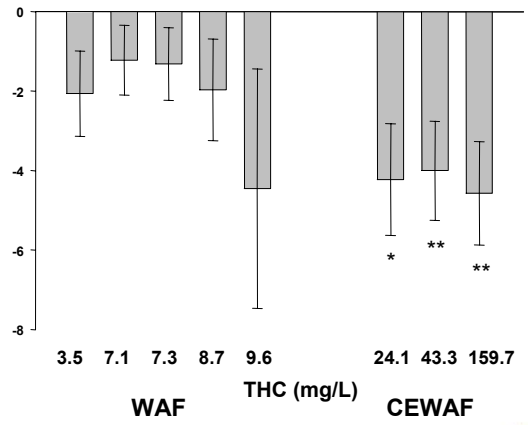
eg. valine



formate



glycerophosphorylcholine



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Conclusions

- **This investigation suggests that treatment of oil with a dispersant decreases its toxicity to Chinook salmon smolts approximately 20-fold – something to consider in response.**
- **^{1}H-NMR provides a more sensitive, sublethal assessment of toxicity, and may be used to help ascertain mechanisms of toxicity.**
- **Long-term impacts of acute oil exposure on salmon growth and metabolic function are still under investigation.**

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NOAA through the UNH Coastal Response Research Center

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and the UCD Oiled Wildlife Care Network

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NOAA National Oceanic and Atmospheric Administration U.S. Department of Commerce

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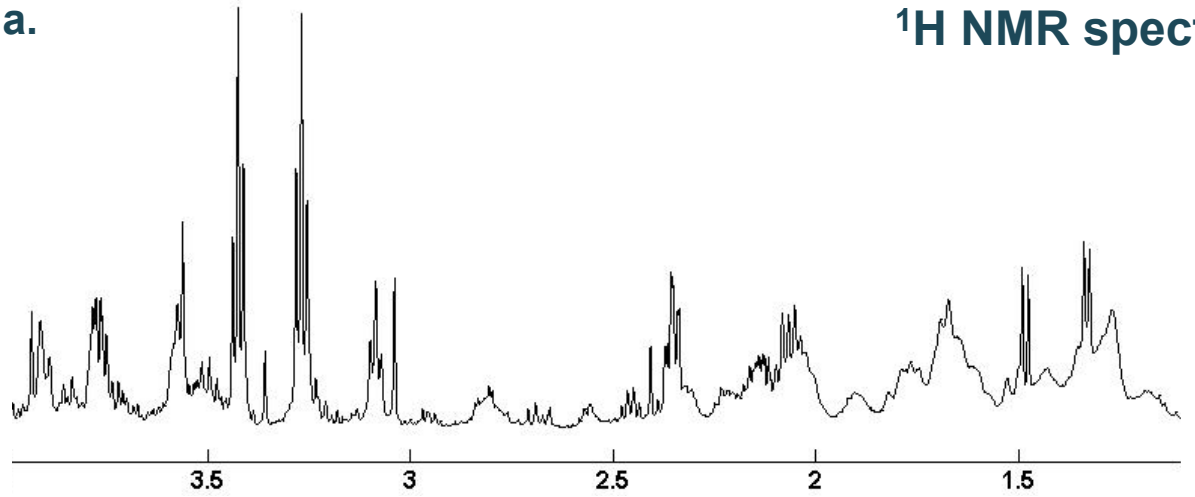
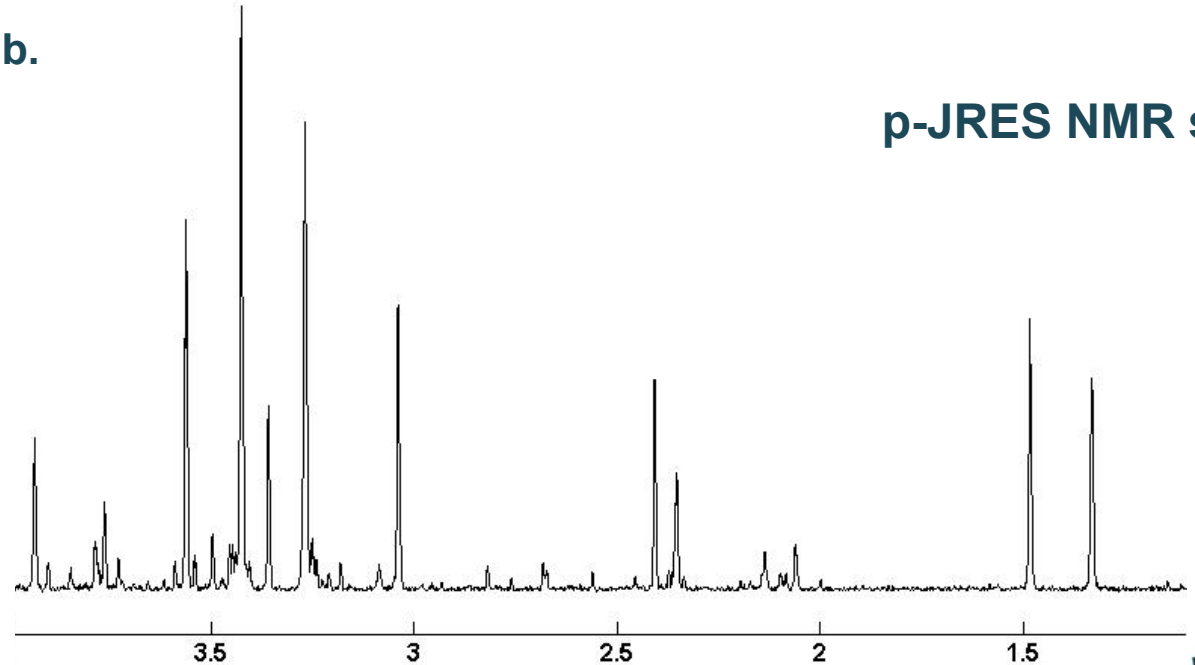
a.

^{1}H NMR spectrum



b.

p-JRES NMR spectrum



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