VIDEOCONFERENCE MEETING

STATE OF CALIFORNIA

OFFICE OF ENVIRONMENTAL HEALTH HAZARD ASSESSMENT

PROPOSITION 65

DEVELOPMENTAL AND REPRODUCTIVE TOXICANT

IDENTIFICATION COMMITTEE

ZOOM PLATFORM

TUESDAY, OCTOBER 18, 2022

10:01 A.M.

JAMES F. PETERS, CSR
CERTIFIED SHORTHAND REPORTER
LICENSE NUMBER 10063

APPEARANCES

COMMITTEE MEMBERS:

Ulrike Luderer, MD, PhD, MPH, Chairperson

Patrick Allard, PhD

Diana Auyeung-Kim, PhD

Laurence Baskin, MD

Suzan Carmichael, PhD

Irva Hertz-Picciotto, PhD, MPH

Isaac Pessah, PhD

Charles Plopper, PhD

Tracey Woodruff, PhD, MPH

STAFF:

Lauren Zeise, PhD, Director

Carolyn Rowan, Chief Counsel

Marlissa Campbell, PhD, Staff Toxicologist, Reproductive Toxicology and Epidemiology Section, Reproductive and Cancer Hazard Assessment Branch

Vincent Cogliano, PhD, Deputy Director, Division of Scientific Programs

Julian Leichty, Special Assistant for Programs and Legislation, Proposition 65 Implementation Program

Francisco Moran, PhD, Chief, Reproductive Toxicology and Epidemiology Section, Reproductive and Cancer Hazard Assessment Branch

Martha Sandy, PhD, MPH, Chief, Reproductive and Cancer Hazard Assessment Branch

APPEARANCES CONTINUED

PANELISTS:

Bruce Draper, PhD, University of California Davis

Stephanie Padilla, PhD, United States Environmental Protection Agency

Jennifer Panlilio, PhD, National Institute of Child Health and Human Development, National Institute of Health

Dan Wagner, PhD, University of California, San Francisco

ALSO PRESENT:

Sean Burgess, PhD, University of California, Davis

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PROCEEDINGS

**WELCOME AND OPENING REMARKS**

DIRECTOR ZEISE: Good morning and welcome everyone to this year's meeting of the Developmental and Reproductive Toxicant Identification Committee. The meeting is being held virtually. My name is Lauren Zeise. I'm Director of the Office of Environmental Health Hazard Assessment. This is a department within the California Environmental Protection Agency.

So I'm really looking forward to today's meeting. Before we start with introducing the Committee and staff. I'll high level go over the agenda and also how the public may comment during the meeting. So our main agenda item today is the use of zebrafish in assessing developmental and reproductive health hazards. Zebrafish are increasingly being used as a model organism in toxicity testing, including for developmental and reproductive toxicity testing. OEHHA has included zebrafish study date and other types of new toxicological data and our hazard identification documents. We've prepared today's session to discuss the scientific underpinnings and further explore the use of zebrafish evidence in identifying chemicals posing reproductive hazards. A conversation we hope will help inform our future use of these data in our hazard identification documents and Committee

deliberations. We are looking forward to presentations from four invited speakers, as well as the Committee's questions and discussion of scientific issues and the public's comments.

After the zebrafish agenda item, the Committee will take up a consent item on the Section 27000 list of chemicals, which -- for which testing has been required but has not been adequate. This is different -- a different list than the Proposition 65 list. There aren't going to be any listing decisions before the Committee today.

For the third and final agenda item, staff will present updates on chemical listing via administrative listing mechanisms, safe harbor levels, and other regulations as well as litigation from the past year.

Then we'll -- we'll -- during the meeting we'll be taking a 45-minute break for lunch around noon and we'll also take a short 15-minute break around 2:15 in the afternoon.

So this meeting is being recorded and transcribed. The transcript will be posted on OEHHA's website.

Okay. So for public comment, during the meeting there will be an opportunity to provide public comment after the zebrafish agenda item. And you can see on the

screen here individuals who wish to make oral comment at the meeting are asked to do two things, first, join the zoom webinar. And so for those of you watching by CalEPA webcast, you'll be able to watch the meeting, but you'll need to join the meeting by Zoom in order to speak. Information on how to -- how to join via Zoom is shown on this slide. You go to the -- let's see -- yes, this web address, https://bit.ly/dartic_registration_2022. So you'll receive a link to join the webinar at the end of the registration process. And if you provided a working email address, you'll also receive an email with a link to join the webinar.

Then once we begin public comment, please raise your hand using the raise hand function on Zoom to indicate you would like to speak. So again on the Zoom bar on at least my screen, you have a menu bar on the bottom of the screen and you can see the raised hand there. It might not be the same on every screen.

When your name is called, you'll be prompted to unmute yourself. Please unmute and then state your name and affiliation, if you wish to state your name and affiliation, and provide your comment. And comment will be limited to five minutes per commenter. Okay. So that's the public comments.

Now, let's turn and introduce our committee.

So I'm very pleased to introduce members of the Developmental and Reproductive and Toxicant Identification Committee, or DART IC. If -- when you're introduced, if you could turn on your camera and state your name and affiliation. So we'll start with Dr. Patrick Allard, who will be actually chairing the first hour of this meeting today, absent our Chair during the first hour.

So Patrick.

COMMITTEE MEMBER ALLARD: All right. Good morning, everyone. My name is Patrick Allard. I'm an Associate Professor at the University of California, Los Angeles, UCLA, in the Department -- or the Institute of Society and Genetics.

Thank you.

DIRECTOR ZEISE: Thanks, Patrick.

Dr. Auyeung-Kim.

Oh, Diane, you're muted.

COMMITTEE MEMBER AUYEUNG-KIM: Okay. Hi. My name is Diana -- Dr. Diana Auyeung-Kim. And I am currently Executive Director at Genentech in the Department of Safety Assessment.

DIRECTOR ZEISE: Thanks, Diane.

Dr. Baskin.

COMMITTEE MEMBER BASKIN: Hi. Larry Baskin. I'm Chief of Pediatric Neurology at UCSF Children's Hospitals.

And thanks for including me on the Committee for all these years.

DIRECTOR ZEISE: Great.

Dr. Carmichael.

COMMITTEE MEMBER CARMICHAEL: Good morning, Suzan Carmichael. I'm a professor at Stanford University in pediatrics and OB/GYN.

DIRECTOR ZEISE: Dr. Hertz-Picciotto.

COMMITTEE MEMBER HERTZ-PICCIOTTO: Sorry. Good morning. I'm Irva Hertz-Picciotto. I'm Professor in epidemiology and in environmental and occupational health at the University of California, Davis, where I also direct the UC Davis Environmental Health Sciences Center.

DIRECTOR ZEISE: Thank you. Dr. Ulrike Luderer is next alphabetically in line, but she will be joining us in -- in about an hour.

Dr. Pessah.

COMMITTEE MEMBER PESSAH: Good morning, everyone. Isaac Pessah here, Professor of toxicology at UC Davis School of Veterinary Medicine in the Department of Molecular Biosciences.

DIRECTOR ZEISE: Okay. Dr. Plopper.

Dr. Plopper, you'll have to unmute.

COMMITTEE MEMBER PLOPPER: Yeah. I'm -- I'm unmuted. This is -- good morning, everyone. Charlie

Plopper, Professor Emeritus, UC Davis, School of Veterinary Medicine. Very -- like Dr. Baskin, appreciate being included on this very important Committee.

Thank you.

DIRECTOR ZEISE: Great. Thank you.

Dr. Woodruff.

COMMITTEE MEMBER WOODRUFF: Hi. My name is Tracey Woodruff. I'm a Professor in the Department of Obstetrics, Gynecology, and Reproductive Sciences at University of California, San Francisco.

DIRECTOR ZEISE: Thank you. Thank you all for joining us today. Really looking forward to the discussion. Now, I'm going to introduce the OEHHA staff and also invite them to turn on their cameras as they're introduced. So Carolyn Rowan, our chief Counsel. This is Carolyn's first DART IC Committee meeting.

CHIEF COUNSEL ROWAN: Hi. Thanks, Lauren. I'm Carolyn Rowan. I am the Chief Counsel at OEHHA and I just started in August. So thank you.

DIRECTOR ZEISE: Yeah. Welcome. Okay.

Dr. Vince Cogliano who's our -- go ahead Vince. Give your title. That would be great.

Oh, you're muted.

DR. COGLIANO: Good morning, everyone. Sorry about that. I'm Vince Cogliano Direct -- Deputy Director

for Scientific Programs here at OEHHA. Pleased to meet you all.

DIRECTOR ZEISE: Dr. Martha Sandy.

DR. SANDY: Good morning to everyone. I'm Martha Sandy. I'm Chief of the Reproductive and Cancer Hazard Assessment Branch. Thank you all for joining.

DIRECTOR ZEISE: Okay. Dr. Francisco Moran who is our new Section Chief of the Reproductive Toxicology and Epidemiology Section. This is his first meeting in his new position.

DR. MORAN: Good morning. Happy to be here. Thank you very much for the introduction.

DIRECTOR ZEISE: Dr. Melissa -- Marlissa Campbell who we'll be hearing from today.

DR. CAMPBELL: Hi. A staff toxicologist specializing in development -- mental and reproductive toxicity. Thank you, Lauren.

DIRECTOR ZEISE: Sure. And then from our Office of External and Legislative Affairs and Proposition 65, Program Doctor Amy Gilson.

DR. GILSON: Good morning, everyone. Amy Gilson here, Deputy Director for of External and Legislative Affairs.

DIRECTOR ZEISE: Julian Leichty.

MR. LEICHTY: Good morning. Julian Leichty,

Special Assistant for Programs and Legislation.

DIRECTOR ZEISE: And Esther Barajas-Ochoa.

MS. BARAJAS-OCHOA: Ho. Good morning. Analyst here.

DIRECTOR ZEISE: Great. Well, thank you, all. And now thousand I'm going to turn the meeting over to Carolyn Rowan for some introductory remarks about Bagley-Keene or any other legal issues related to participation in the virtual meeting of the Committee today.

Carolyn.

CHIEF COUNSEL ROWAN: Thanks Lauren. Good morning, everyone. I just have a few points to make before we get underway today. I just want to remind everyone that this is a public meeting, and under Bagley-Keene all discussions and deliberations for this group needs to be conducted during the meeting, not on breaks, or during lunch, or within individual members of the Committee. And that includes both on or offline, including, phone, emails, chats, or text messages.

So just generally, we're going to have the discussion regarding topics of the agenda during the meeting time. Please feel free to ask me any questions at any time during the meeting. I'll be here the whole time. If I do have to step away for some reason, Senior Staff

Counsel Kristi Morioka will cover for me.

Welcome, Kristi.

And so there will always be an attorney here if you have any questions.

And that's it from me. Thank you.

DIRECTOR ZEISE: Great. Thanks, Carolyn.

All right. Now, we're ready to start the main body of the meeting. I'll turn the meeting over to Dr. Allard. And again, Dr. Allard is serving as the Acting Chair for the first hour of the meeting until Dr. Luderer arrives.

**SESSION ON USE OF ZEBRAFISH DATA IN DART HEALTH HAZARD ASSESSMENT**

COMMITTEE MEMBER ALLARD: All right. Well, thank you, Lauren and thank you Carolyn.

So good morning. It is my pleasure to welcome the Committee members and all the members of the public. I see we have quite a few attendees, who are all joining us today for this DARTIC Committee meeting. So we are ready now to move to the main agenda item, which is the session on the use of zebrafish data in developmental and reproductive toxicity health hazard assessments.

So we will be therefore discussing the use of zebrafish data for DART, for development and reproductive -- reproductive toxicity, sorry. And as

Lauren already alluded to this is important, because the use of zebrafish in toxicology has really grown exponentially in the last 15 years. As the toxicity testing in the 21st Century paradigm and endeavor gain momentum. So it's really important to understand the strength and limitations of this model with regards to hazard identification.

So the way that we'll go ahead and start with is with an introductory presentation by OEHHA staff starting with Dr. Moran.

Francisco.

DR. MORAN: Good morning, thank you, Dr. Allard. As you were saying, every year we -- we find that the use of zebrafish as an animal model for developmental and reproductive toxicity is increasing. If one does a quick search on PubMed on DART effect for a particular chemical, it is not unusual to find that the search results include a number of studies published within the last ten years or so using zebrafish as an animal model. And interestingly, the number of zebrafish studied that came up in this search can be similar to the number of studies performed in classical mammalian tested species.

We, in OEHHA, have been included in this type of data and in our recent hazard identification documents. After today's meeting, we will have a better understanding

on the physiology, application, and value of the use of the zebrafish in DART hazard assessment.

I would like to thank all the participants and invited speakers and the -- of course, the DARTIC, and the public for joining us today.

Now, I would like to give the podium to my colleague, as Esther said in the slide here.

Okay.

(Thereupon a slide presentation.)

DR. MORAN: So Dr. Marlissa Campbell that will give a more extended introduction on this topic.

Thank you very much.

DR. CAMPBELL: Thank you, Pancho. Good morning. I'm going to be giving just a brief overview of how OEHHA has been using data from zebrafish in our hazard identification documents and how that's evolved through the years, given that increasing availability of data, and the understanding of the relevance of zebrafish to human health.

Pancho, can you put it in presentation mode?

DR. MORAN: Yeah, it was. Yes. I was trying to do --

DR. CAMPBELL: There. Okay.

DR. MORAN: Sorry about that.

DR. CAMPBELL: That's okay. Can we go to the

Next slide.

DR. MORAN: Yes.

NEXT SLIDE

DR. CAMPBELL: Our earlier hazard identification documents were -- that were prepared on chemicals under consideration by the DARTIC for listing as reproductive toxicants under California's Proposition 65 have included summaries and discussion of relevant studies conducted in zebrafish where those were available, as well as other non-mammalian models of different kinds, cell culture, whole embryo culture. But generally, they were presented as part of additional relevant information rather than give a more equal wait with mammalian and human data streams.

More recent hazard identification documents have taken advantage of advances in the application of the zebrafish model to incorporate the zebrafish data alongside the mammalian whole animal data as well as with mechanistic considerations. And just to illustrate some -- some of the comparisons and questions that have arisen from this more integrative approach, I just have a few slides to share based on the two most recent hazard identification documents, the documents on cannabis and on PFNA and PFDA.

Can -- can we go to the next slide.

NEXT SLIDE

DR. CAMPBELL: In OEHHA's 2019 hazard identification document evidence on the developmental toxicity of cannabis smoke and delta-9-THC, there were four neurobehavioral studies conducted in zebrafish that were included among the animal-derived data that were presented. Three of these studies used a visual motor response test, which is a behavioral test relying on the integrity of the central and peripheral nervous system, including the visual system as well as on normal locomotor and skeletal system development.

The fourth study involved exposure of zebrafish embryos to delta-9-THC during gastrulation period of development. And the effects observed included changes in locomotor responses to sound as opposed to vision, and also they observed effects on heart rate motor neuron morphology and synaptic activity at the neuromuscular junction, all findings which could be related to changes in calcium ion homeostasis during neurodevelopment.

In zebrafish embryos by 48 hours post-fertilization expression of the endocannabinoid receptor CB1R is widespread throughout the zebrafish central nervous system and it's found within the preoptic area, the telencephalon, the hypothalamus, tegmentum, and the anterior hindbrain. And overall, generally, the

findings in zebrafish supported effects that were also seen in mammalian models.

Can we go to the next slide.

NEXT SLIDE

DR. CAMPBELL: Turning to consideration of the effects of the compound PFNA on the male reproductive system, it becomes a little more complicated, since in these experiments, both male and female zebrafish were experimentally exposed. The findings of reduced egg production and hatching rate could potentially have resulted from effects on either or both sexes. It's unclear whether a male-mediated mechanism driving these outcomes in zebrafish would be analogous to something that would occur in mammals. Although for what it's worth, there was a mouse study that showed reductions in fertility index and litter size with PFNA exposure for 90 days prior to mating with untreated females.

Increased levels of serum testosterone were seen in the adult male zebrafish exposed to PFNA over 180 days. In contrast, PFNA exposure of male rodents was generally associated were decreased serum testosterone. Although, under some experimental conditions, testosterone levels were either unaffected or even elevated.

PFNA treated zebrafish showed alterations in gonadal expression of genes related to

hypothalamic-pituitary-gonadal, or HPG, access. While there's some overlap in the markers that were measured in the male gonads of rodents and also in zebrafish, expression was not always altered in the same direction.

PFNA can also bind to transthyretin, or TTR, a transport protein that impacts thyroid hormone levels and function. Disruption of the thyroid hormones may in turn contribute to male reproductive effects. A finding of increased TTR transcription in treated zebrafish could reflect induction due to competitive binding of PFNA. In these same treated zebrafish, plasma thyroid hormone levels were significantly higher than controls contrasting with rodent results, which tended to show reduced thyroid hormone levels with PFNA exposure.

The authors of the zebrafish study proposed that PFNA could act to induce TTR transcription across species while at the same time resulting in opposite effects on -- on the more downstream effects on thyroid hormone levels in zebrafish versus rats. So overall, these inconsistencies between the zebrafish and rodent data could be related to species differences or to other aspects of experimental procedures such as dose timing of exposure and so on. You know, just there's more work to be done to fully understand.

Now -- next slide, please.

NEXT SLIDE

DR. CAMPBELL: With PFDA exposure of male zebrafish, effects included an increased plasma estradiol to testosterone ratio as well as increased plasma estradiol to 11 ketotestosterone ratio. PFDA exposed male zebrafish also showed a dose-dependent increase in gonadal expression of the aromatase gene. Aromatase is a steroidogenic enzyme, which may affect the conversion rate of testosterone to estradiol.

Vitellogenin is an egg yolk precursor protein increase blood levels serve as a biomarker in both male and female vertebrates for exposure to environmental estrogens. In this case, the zebrafish data were consistent with other evidence suggesting involvement of affects on the HPG axis in PFDA mediated male reproductive toxicity.

Next slide.

NEXT SLIDE

DR. CAMPBELL: Just to go over the potential increase in the use of zebrafish for evaluating toxicity, we just wanted to note that in recent years both the U.S. and the European Union have been making commitments to reduce the use of mammalian test species for purposes of environmental health -- testing for environmental health.

U.S. EPA released a memorandum in 2019 stating

their intent to reduce requests for and funding of mammalian toxicology studies by 30 percent no later than the year 2025. Further reductions to effectively zero requests and funding is targeted for 2035.

The EU currently prohibits completely animal testing for cosmetic products or ingredients as of 2013. The EU is also currently developing plans to phase out the use of animals in research and testing for purposes of environmental health assessment. Zebrafish are increasingly becoming the go-to whole animal alternative to mammalian test species as the understanding of the comparative biology and -- and as well as validation of the use of zebrafish as a relevant model have been rapidly increasing in recent years. Fish, of course, are animals and there are guidelines for ensuring consideration of their welfare that have been published.

The Office of Laboratory Animal Welfare from the U.S. Public Health Service interprets aquatic species as live vertebrate animals at the time of hatching. For zebrafish, this is approximately 72 hours post-fertilization. The EU uses as their guidance commensurate of independent feeding by zebrafish larvae, which occurs at about 120 hours post fertilization point. And for that -- for their guidelines that's a point a which the welfare regulations start to apply.

Last slide, please.

NEXT SLIDE

DR. CAMPBELL: Pancho, next slide.

Oh, there they are.

Okay. Sorry.

As for today's presentation, we're going to be learning about aspects that are generally covered by these four topics, comparative reproductive and developmental biology of zebrafish, zebrafish as a model for large-scale screening for potential DART hazard and risk, zebrafish as an experimental model for investigating development at the cellular, and zebrafish as an experimental model for investigating development at the molecular level. And that concludes my presentation for this morning.

Thank you.

COMMITTEE MEMBER ALLARD: Thank you, Dr. Campbell.

Are there any questions for Dr. Campbell before we move on?

If I may actually, I do have a couple of questions. I was wondering when -- when you build the hazard identification document and you -- you review the literature available, what kind of criteria do you use for inclusion or exclusion of zebrafish data? Is it different from other mammalian data?

And it's a two-parter question. Related to that, basically do we need to build an expertise in non-mammalian model if -- if that does not exist already on the -- on the staff side to really accurately review that kind of literature.

DR. CAMPBELL: It depends, I think, on whether it -- you know, it fits the toxicity data, the same way we would use mammalian data, then we would fold that zebrafish data in there. In other cases where it's more mechanistic data, that's a little bit more -- it's harder to predict. We just have to kind of see where things go and what we find. I don't know if that fully answers your -- your question. And I don't know if anybody else from the staff would want to comment.

Pancho, you're muted.

We still can't hear you.

DR. MORAN: Okay. Thank you. Sorry. I have a second backup mute button. Sorry about that.

Yeah, you're right Marlissa, we don't -- Dr. Allard, we don't make any special adjustment for our literature search according to zebrafish or other mammalian models. We base our findings on what is relevant to reproductive and developmental. And it could be a final effect or it could be mechanistic effect on zebrafish as in any other animal species. So we don't

make a difference at this point.

So I hope that help.

COMMITTEE MEMBER ALLARD: Thank you.

I see Diana has a question. Diana.

Diana, you have your hand raised, but you're muted.

Okay. Maybe that was not a real hand raise.

Okay. Well, I am pleased -- thank you, Dr. Campbell. And I'm pleased to welcome the real Chair of this meeting, Dr. Luderer, who will be taking over the duties from now.

Thank you.

**PART 1. ZEBRAFISH BIOLOGY AND SUITABILITY FOR TOXICITY SCREENING**

CHAIRPERSON LUDERER: Thank you very much, Dr. Allard for stepping in. I really appreciate that.

So let me just get situated here. So our -- next, we're going to, I believe, switch to Part One, Zebrafish Biology and Suitability for Toxicity Screening. And this -- OUR first speak I'm delighted to introduce is Dr. Bruce Draper of the University of California, Davis. And our second speaker will be Dr. Stephanie Padilla of the U.S. EPA. After these presentations, we'll have 30 minutes for Committee discussion with Drs. Draper and Padilla.

So to introduce Dr. Bruce Draper, he's professor of molecular and cellular biology at the University of California, Davis. Dr. Draper's research uses a combination of gene knockout and single-cell transcriptomics to identify genes required for zebrafish gonad development and function and sex determination and differentiation.

So our first presentation by Dr. Draper will be comparison of zebrafish sex determination and reproductive developmental biology to humans as well as mammalian test species.

And welcome, Dr. Draper. It's a pleasure to have you here.

**PRESENTATION BY DR. BRUCE DRAPER**

(Thereupon a slide presentation.)

DR. DRAPER: Well, thank you very much and it's a -- it's an absolute pleasure to be here.

All right. So I've been tasked with giving a sort of general overview of zebrafish biology, early development, and what is my field of study reproductive biology.

NEXT SLIDE

DR. DRAPER: I'm going to divide this talk into sort of three general areas. The first is going to be the general overview of zebrafish development. And there are

-- and aspects of that development that make them advantageous for toxicant screening. I'll then give a -- a quick comparison of zebrafish to humans and other vertebrates. And then finally, I'll end with an overview of zebrafish reproductive biology including sex determination and how it compares to mammalian species.

Let me get my laser pointer going here.

Oh, that didn't work.

Okay.

NEXT SLIDE

DR. DRAPER: This is an overview of zebrafish early development. On the outside are some nice illustrations of the different embryonic stages. And on the inside of this diagram are the time scales in which these occur. One of the major advantages of zebrafish over, for example, mice for developmental studies as well as using them for toxicant screening is that all aspects of early development happen outside of the mother as opposed in utero in the mouse.

Zebrafish when we sent them up to mate, they're programmed to spawn in the -- when the sun rises, and in our fish facilities, that's when the lights come on generally around eight or nine o'clock in the morning. And a single female can spawn hundreds of eggs that will then relatively synchronously developed. So fertilization

happens outside the mom and then they go through these rapid cleavage stages. And at about six hours post-fertilization is when they initiate gastrulation, which is going to create the three germ layers, to ectoderm, mesoderm, and endoderm from which all of our organs are derived.

Gastrulation is complete by about 10 hours post-fertilization. And at that stage, we enter into somitogenesis. And already at this stage, you can start to make out the basic vertebrate body plan, where in the anterior you have the head and the developing brain, and then the posterior you have the developing somites, which give rise to the musculature and bone structure of the fish.

By 24 hours post-fertilization, this fish has the basic vertebrate body plan and many of the organs have already -- the primordium of these organs have already been set aside, and patterned, and in some case have already started to function. By three days post-fertilization is when the -- the larva hatch and become free swimming. And then by five days is when they can actually start feeding.

The entire life cycle from fertilization to becoming a reproductive adult can take anywhere from two months to three months depending upon how well they're fed

during the -- this time period and issues like crowding. So about the earliest you can get them to go through one cycle is two months. But, in general, I think in many of our facilities, it takes about three months. So in that aspect, the reproductive cycle is about similar to the mouse.

But importantly, because all of these stages happen externally, you can basically apply any toxicant to test their effects on various aspects of development whether it be early development effects on -- on the morphogenesis movements that are required for grastrulation, and also as larva juveniles and adults.

NEXT SLIDE

DR. DRAPER: A typical fish facility, or zebrafish facility looks something like this. These are manufactured facilities from any of number of companies. But the main point here is that we can raise very large numbers in a fairly small footprint. My facility at UC Davis is about 450-square foot facility and we have an average census between 15 and 25 thousand adult fish in this facility. And this is relatively cheap to maintain relative to mammalian species.

Another aspect, which is important is that if you keep the proper light cycle for zebrafish, they will breed year-round, so we can get embryos on any day that we want

to get them, and we can get them in the thousands, if necessary, for high throughput screening, which I think you'll hear more about from the other panelists.

NEXT SLIDE

DR. DRAPER: One reason why zebrafish was chosen for biomedical studies in the beginning was that their embryos are relatively transparent. So this is a picture of a 24-hour old embryo and just with a dissecting microscope you can actually make out very -- various developing tissues in the fish. For example, if you look here in the head, you can make out the forebrain, the hindbrain -- the midbrain and the hindbrain, you can make out a developing ear, eyes. By about a day and a half, the heart is functional and begins to beat, and you can follow, you know, blood flowing through the various vessels. Posteriorly, you can see a notochord, this transient struck -- which is a transient structure, which makes us chordates and the neural tube, and then the musculature.

So this is just in a light microscope, but we can combine this with transgenesis and create animals that actually express the green fluorescent protein and various specific tissues or cell types. This particular one is expressing the green fluorescent protein and all the blood vessels of the embryo. And so you can combine this with

toxicant screening to get a more refined view or to really hone in on a particular tissue type that you're interested in. So maybe this wouldn't be used for a primary screen, but for secondary screens to look at more mecha -- mechanistic studies. This is just one of many cell type specific transgenics that are available in zebrafish.

NEXT SLIDE

DR. DRAPER: So some other advantages of using zebrafish, I've already mentioned that they have the basic vertebrate body plan, which is similar to -- to mammal -- mammals, including humans. There is a molecular conservation of the genes that regulate development with other vertebrates. They're very easy to maintain in the lab. They were -- you know, one of the reasons they were also chosen is because they were a very robust fish species. And that also leads to them being relatively inexpensive relative to mammalian species.

We can get large numbers of embryos at any time that we want for doing large scale screens, which is also maybe easier by their external development. They're optically clear, amenable to high throughput screens, and the molecular, cellular conservation of the reproductive organs, which I'll get to, which is, you know, part of what this Committee is tasked to look at.

NEXT SLIDE

DR. DRAPER: So to do a more direct comparison between the genes that regulate development and reproduction in zebrafish relative to humans, the entire genome sequences are known for -- for both humans and zebrafish, so we can really do a direct comparison of gene orthologs between the two. And 70 percent of the genes that are required that are found in humans are orthologs are also found in zebrafish. And, in fact, 80 percent of the genes that have already been associated with human disease, also have orthologs in zebrafish.

Zebrafish have livers, pancreas, gallbladder, a circulatory system, an analogous digestive system. They obviously don't have lungs, because they are -- they are aquatic species, but they do have a structure called a swim bladder, which has a similar developmental origin, and as well as the central nervous system. And I'll just emphasize the fact that they have a liver is important also for toxicant screening, because the liver has many enzymes that can convert toxicants into other derivatives, and so you wouldn't have this -- this contribution if you were doing, for example, cell culture type screening for toxicants.

NEXT SLIDE

DR. DRAPER: I want to spend a brief moment on talking about the evolutionary history of zebrafish

relative to humans to point out mainly the numbers of genes that zebrafish have relative to humans. So there's one main branch invertebrate evolution. The branch that gave rise to us is what is called the lobe-finned fishes branch. And one of the species that is still alive today is the coelacanth, which is a precursor to -- which is a lobe-finned fish.

This other major branch is called the ray-finned fishes and that's where zebrafish is a part of. Two-thirds of all living vertebrates are in the ray-finned fish lineage, one-third in the lobe-finned fish. The other fishes that I put on here are here mainly because these are animals that we have whole genome sequence for, so they can be used to really allow us to do very careful analysis of gene orthology when we go from zebrafish to humans.

Now, these arrows back here are looking at genomic events that happen during the course of vertebrate evolution which were important for evolution and what are called whole genome duplication events. So predating the split between the lobe-finned and ray-finned fishes, there were two whole genome duplication events that took, for example, a gene that might present as a single copy in drosophila which is another -- you know fruit flies, which is another important species for -- for biomedical

research. Humans would have four copies of that gene, because the first genome duplication went from one to two copies, the second genome duplication went from two to four.

Now, after these split, and a little bit farther down, the teleost lineage underwent an additional whole genome duplication. Now, teleost is, particularly to zebrafish, are a diploid species, but in comparison to humans, in some instances where humans have a single copy gene, zebrafish would have two copies of that gene. And it's about 25 to 30 percent of the gene orthologs between humans and zebrafish actually have a -- a duplicated copy in zebrafish relative to humans. So this is important what comparison -- when comparing gene function and gene orthology between mammalians and fish species.

NEXT SLIDE

DR. DRAPER: Now, this is a -- I gave a talk to the staff of DARTIC a couple of months ago, and one of the questions that came up was how do you compare zebrafish lines relative to mouse lines, in particular how inbred are they? So mouse lines are typically very inbred and are very homozygous at most loci.

Zebrafish lines are not as inbred. These lines -- for example, this is not in an exhaustive list of the various lines that zebra -- that people use for

zebrafish, but three of the main ones that are used across the world are the AB line, which was derived at the University of Oregon, where zebrafish really got its start as a genetic system for studying vertebrate development. The other main line was developed at Max Planck Institute in Tübingen called the TU line. Both of these were pet store derived and they were put through a genetic bottleneck, because the point of developing these lines for these two institutions was to make lines that were essentially lethal-free, that did not have any heterozygous lethal mutations, because what they wanted to do was to use them for forward genetic screens.

So they were bottlenecked. Clonal lines have been produced, but they are typically much less robust and fecund than the non-clonal lines, so it's more practical to maintain the non-clonal lines for these studies. But at least for the -- the University of Oregon line, it's been estimated that they are about 70 percent homozygous, but there is, you know, that 30 percent diversity that we try to maintain when maintaining these stocks to keep these very robust Lines. Okay.

NEXT SLIDE

DR. DRAPER: Getting towards kind of more the reproductive biology. Zebrafish are sexually dimorphic. There are very subtle differences, but to the -- the

trained eye, you can start to pick these out pretty quickly. So what I'm showing here is various views of a female zebrafish on the top and a male zebrafish on the bottom. Female zebrafish are, in general, a little bit larger than males, a little bit wider.

And you'll see on the -- the next slide, that that's because they have a very large ovary relative to the testis size in males. So a lot of times, you can determine their sex just based on their overall body morphology. Zebrafish have three different pigment types that make up the stripes, one of them called the xantho -- xanthophores is this yellow pigment stripe here in between the -- the two dark pigment stripes. Females, this pigment is a little bit less saturated, so they don't look as yellows as the -- as the males do. So that's one way that's it's easy to tell males from females is -- is the males look yellow.

There are also other secondary sexual characteristics, for example, the genital papilla, which is where the eggs are released from relative to where the sperm is released. Back here, the genital papilla is kind of the swollen structure in females, whereas in males, you don't really see. It's just like a little flat structure over the pores. So -- so we can also use these to determine the sex of the fish.

NEXT SLIDE

DR. DRAPER: Now, going back to the internal organs. Here, we've just dissected off the -- the skin from the fish, so we can look inside and compare, in particular, the size of the ovary versus the size of the testis. So these are just light microscope pictures on the top, but these animals are also transgenic for a transgene that drives the green fluorescent protein expression in all germ cells. And so if we switch to the fluorescent channel, we can see that this is where the -- the germ cells are, you know, brightly fluorescent in the ovary. And you -- it's a little bit fainter fluorescence in the testis there.

NEXT SLIDE

DR. DRAPER: Now, one main difference in reproductive biology between female zebrafish and mammal -- and mammals, is that in mammals, all germ cell proliferation and production of oocytes happens in utero. Females are born with their germ cells arrested in prophase meiosis I and there are no new oocytes added for the remainder of the life of the individual.

By contrast, most fish species that have been looked at, and in particular zebrafish, have the capacity of producing new oocytes throughout their life, because like males of almost all species, they process a

specialized cell type called stem cells, in particular oogonial stem cells. And you can't see them in this picture. You would have to look at a higher magnification, but this is where they localize in the ovary. And these cells are capable of mitotic divisions to create new cells that can enter meiosis and become oocytes.

And so if you do a histological section through a zebrafish ovary, which is what this picture is, and stained with -- this is an H&E stained section, you can find oocytes at all stages of development from the early stages, like Stage 1B, all the way to later stage oocytes that are starting to take up vitellogenin form the blood, what are called the vitellogenic stage, stage 3 oocytes all the way through to the mature eggs, which is not shown here.

So if you are interested in studying toxicants that are affecting the early aspects of female reproductive biology, in mice, you would have to treat mothers that have female embryos to affect their germ cells. Whereas, in zebrafish, you can just treat adult females with these compounds because they are constantly producing new oocytes. So that's another advantage because of the reproductive biology of zebrafish.

NEXT SLIDE

DR. DRAPER: I want to give an overview of zebrafish reproductive organ development and then we'll turn to sex determinations to give you a reference for the timing at which these are happening during zebrafish development. So on top is a typical vertebrate timeline for -- and developmental stages for development of the ovary versus the testis.

I'll start back here at the -- during embryogenesis for both species, for -- for mammals and zebrafish. There are somatic gonad precursors that are set aside, as well as the precursors to the germ cells called primordial germ cells. And these are one of the first cell types to be set aside during embryogenesis. The sites of somatic gonad development and early germ cell development are at different locations in the embryo. So the first thing that has to happen is the germ cells need to migrate to where the somatic gonad is going to form.

Once they have reached that site, we call this initiation of gonad development. And then in both mammals and in zebrafish, there is a stage where the gonad is what we call bipotential. If you compared gene expression between the somatic cells of a -- what will be a male versus a female, there are no differences. So early gonad development is identical in males versus females. But once sex has been determined, then the bipotential gonad

in females switches to a trajectory that will lead it to developing female-specific cell types of the ovary, whereas, in the male, it will switch over to producing male-specific cell types to make up the testis. So this is what we call sex differentiation and then between here is sex determination.

So both mammals and zebrafish go through these same developmental stages. They just happen at different times. So in zebrafish, in particular, the specification stage happens during the first about 10 hours of development and then for the next five days of development not much happens, but around eight days of development, we start seeing a lot of proliferation of both the somatic gonad and the germ cells in the stage, which is called the bipotential stage. And I'll -- I'll say a little bit more about this on a -- on a subsequent slide.

The bipotential stage of gonad development, as far as we can tell, happens -- is basically between about eight to 20 days of development, by which time sex has been determined. And then starting around 20 days of development, we can actually, with appropriate markers, start to see differences between what is going to develop into an ovary versus what is going to develop into a testis, so that happens about 20 days post-fertilization.

NEXT SLIDE

DR. DRAPER: So again as -- just to reem -- to emphasize this -- this timeline and also when we can first tell the differences between ovaries versus testis. Back here in the bipotential stage is actually when we start to see the first signs that germ cells are starting to differentiate. And I'll say a little bit more about this in a -- in a second, so we can start to see evidence of the first meioses around 14 days post-fertilization, so the specialized cell cycle that's required for the production of gametes. And then between 20 and 30 days post-fertilization is when we see the somatic gonad and sexual differences.

So at 30 days, we can absolutely, with appropriate markers, either by dissecting the gonads out or what's shown down here is that same transgenic, which expresses the green fluorescent protein and germ cells, we can look in living fish and tell an animal that's going to develop as a male versus a female based on the sheer size of the -- of the gonad where the testis is very thin and faintly staining, while the ovary has already grown quite large relative to the size of the fish. And we can, at this stage, with almost 99 percent certainty, if we sort fish, that have this fluorescence versus this, these animals were developed as females versus males.

NEXT SLIDE

DR. DRAPER: So females versus males begs the question of what determines whether you have females versus males. And so one of the questions that came up in our previous meeting was -- are the sex ratios, outcomes that -- like is sex ratio an outcome that typically is or could be evaluated in a DART study using zebrafish in mammals, because there's chromosomal sex determination XX versus XY, you get a relative 50/50 ratio. So let's talk about how -- what we know about how sex is determined in zebrafish.

NEXT SLIDE

DR. DRAPER: So I'm not going to talk about initially how sex is determined in the domesticated fish, which we use a line in the lab, but I'm going to talk about how sex is -- what's known about sex determination if you go and collect zebrafish samples from the wilds of India where they are endemic. And so this study was done by John Postlethwait in Medford Shartle. John is at the University of Oregon.

And what they did was they did what's called a genome-wide association study. It's not important that you understand how that works, but what they're looking at is is there a particular chromosome or regional of a chromosome which is predictive of sex. And the important thing of this graph down here is on the X axis are the 25

chromosomes of zebrafish and on the Y axis is a score for predictiveness of whether a chromosomal locus is tightly associated with one sex versus the other as you would expect, for example, the Y chromosome to be in mammalian sex determination.

And what they found was that on -- on the distal end of chromosome 4, there is a highly predictive region which segregated specifically with animals that became females. And the females were heterozygous for this. And when the females are the -- what we call the heterogametic sex. So in mammals XY is the heterogametic sex, XX the homogametic sex. And if we have that situation that we -- that we use XX/XY nomenclature regular, that if the heterogametic sex is females, we use ZZ/ZW. So the ZW chromosomal situation is female, the ZZ is male. So this is the same in birds actually.

So at least in wild zebrafish, there is chromosomal sex determination, but somehow this has been lost in the domesticated zebrafish. We do not have any evidence for the lines that I showed you that there is a chromosomal basis of sex determination. And, in fact, sometimes we can get fairly skewed sex ratios, you know, 90 percent males versus 10 percent females or vice versa, that in general, and under standard laboratory conditions, we are somewhere in the 50/50 sex ratio region. So if

it's not chromosomal, what do we know about the mechanism of sex determination?

NEXT SLIDE

DR. DRAPER: So I'm going to apologize for this slide. It looks quite complicated, but I'm going to walk you through it. What I'm showing here are some simple diagrams of different cell types in the zebrafish gonad. On the right here are the germ cells going from the mitotic germ cells, which we -- in the larva, which we can call gonocytes and their early meiotic products. On the left is a representation of the somatic gonad cell types.

During the bipotential stage, it truly is bipotential if you look at genes that are -- eventually will be expressed in males versus females. We find a salt and pepper mixture of the expression of those genes in the bipotential gonad so this is prior to sex determination. So an example of that would be CYP19A1A, which encodes the aromatase the Marlissa referred to earlier. This is involved in estrogen synthesis. We can find cells that are expressing that -- and this is a female-specific gene eventually, whereas there are male specific genes, such as sox9a or the anti-mullerian hormone, which would be expressed also in cells that are adjacent to CYP19. So there's really this salt and pepper mixture.

What we know is that if you completely get rid of

germ cells, there's various techniques for doing that, for completely ablating the germ cell component of the gonad, 100 percent of those animals will grow up and be phenotypically male. They will look like males and they will behave like males, but they will be sterile. So that suggests that germ cells are playing an essential role in female development. And based on what we know, it is that -- one thing I haven't mentioned yet, which is a quirk of zebrafish, in that a hundred percent of animals initially start to produce early stage oocytes, even animals that will become male.

But what happens is is that what we believe is is that there is a threshold number of oocytes that need to be produced, because the oocytes are producing a cell signaling molecule which signals to the somatic gonad to stabilize the female gene expression. And so if you can reach this threshold number of oocytes and therefore the signal, you will stabilize female development and those animals would become female. If you do not reach that threshold, the oocytes will eventually die as the somatic portion of the gonad transitions to a testis and you start producing sperm.

Not only is this signal required for primary sex determination, but even -- we have evidence that even as an adult, you require constant signaling from germ cells,

in particular oocytes, to the somatic gonad. And if there's anything that prevents the production -- the continuous production of oocytes, we can actually have a female that initially develops as female producing oocytes will sex reverse and become a male. And in some situations, and some tricks we can do, we can get those males to actually be fully fertile and they behave like males.

So there's this constant signaling that has to occur. So any toxicant that prevents either this early signal or perhaps prevents this later signal will lead to either an overproduction of males versus females or cause animals that started off as females to sex reverse and become males. So while this is kind of a quirk of zebrafish, we can leverage it to, you know, fairly -- to do fairly high throughput screens looking for toxicants that skewed the sex ratio relative to the controls.

So this is really a cellular view of sex determination, what about the comparisons of genetic sex determination between fish and mammals.

NEXT SLIDE

DR. DRAPER: And this is just a slide showing you some key genes in mammalian sex determination and then I'll compare that to -- to zebrafish. So up here at the bipotential stage, all gonads sort of equally produce

these two cell signaling molecules. It's not really important what they are. One is red, one is blue. In mammalian males, because they have a Y chromosome, they have a transcription factor called SRY, which is kick-starts the entire sex determination process. If you had SRY, you are a male. If you lack it, you become female. SRY then leads to the upregulation of this FGF9 gene, the red gene up here, which inhibits the blue gene WNT4.

So in this situation, FGF9 wins out because of the help of SRY and then we turn on downstream genes which are important for sexual differentiation, one of which is this gene called DMRT1. If you lack SRY, if you're XY or XX, then went for is -- is set to win out over FGF9. And then you turn on the downstream female specific transcription factors, for example, FOXL2 which lead to sex differentiation.

So how does this compare to zebrafish?

Well, zebrafish do not have orthologs of SRY, this mammalian-specific gene, nor do they have orth -- orthologs of FGF9. By contrast, they do have orthologs of WNT4, FOXL2 and DMRT1.

On the next slide --

NEXT SLIDE

DR. DRAPER: -- this is essentially the same

information, but I've stripped out now in showing you a direct comparison of mammals versus zebrafish. And what I really want to emphasize is -- is that at this level down here of these transcription factors, which are really driving the genes that are required for sex differentiation, turning on the genes likely that are required for hormone production, secondary sexual characteristics, this is highly conserved in all vertebrates. And, in fact, DMRT1 is an ancient gene that it -- that even is regulating sexual differences. In most metazoans, for example, it was first discovered in drosophila the fruit fly and it also functions in nematodes. This is a very highly conserved level of sex differentiation.

How these get turned on is not known in zebrafish. But once they are turned on, they're doing very analogous functions in mammals and fish.

NEXT SLIDE

DR. DRAPER: And so to wrap-up the reproductive biology part, we -- there was already reference to this in Marlissa's introduction and I just want to kind of close this part with looking at the various hormones versus receptors that are required for -- for female versus male sex and what are similarities and differences between mammals and zebrafish.

So if we look at the females, the bioactive -- most bioactive form for females is the same in both mammals and zebrafish, essentially 17 beta-estradiol, also called E2. So exactly the same. And in fact, the receptors -- the orthologs for the receptors that -- that bind to the hormone and regulate gene expression, there are orthologs of estrogen receptor 1 and 2 in zebrafish. But zebrafish has a single copy of estrogen receptor 1 has got a duplicate due to that whole genome duplications of estrogen receptor 2 called 2A and 2B.

NEXT SLIDE

DR. DRAPER: Now, turning to males, here's where we see some differences. The most bioactive form of testosterone in mammals is 5-alpha-dihydroxy testosterone, whereas that in fish is 11-keto-testosterone. Now, these are derivatives of testosterone, but they're slightly different. Regardless, they both function through the androgen receptor, which are both single copies in mammals and zebrafish. So although there are slight differences in the testosterone, they're still functioning through the same -- same androgen receptor.

NEXT SLIDE

DR. DRAPER: Now finally, you'll hear more about this I think from Dan -- Dr. Dan Wagner's talk using single cell transcriptomics for analyzing at cellular

resolution gene expression in the gonads of zebrafish and being able to compare that to mammals. My lab has recently done a single sell RNA-seq study. The only thing important to understand here is that each of these dots represents a single cell from a zebrafish ovary. And the dots that are -- that we're comparing the genes that are expressed in these cells. And so the dots that are most close together have a more similar gene expression pattern.

But we've been able to identify all of the major cell types and cell subtypes in the ovary and compare them to -- to their counterparts in -- in mammals. And I just want to end with saying that there are more similarities than differences between the cell types in the zebrafish ovary and mammals, the follicle cells, which are the main producers of the -- the -- the estrogen for example and the gene, for example, and the theca cells which produce the precursors to that.

And so this type of study, you know, using this to also look at gene expression changes upon toxicant treatment I think is going to be incredibly powerful. I think we're going to hear more about that later today.

NEXT SLIDE

DR. DRAPER: So just to -- to wrap this up, when we're talking about the time points that we -- if we want

to look at developmental toxicity versus reproductive toxicity of particular compounds, you know, when are the optimum times for using zebrafish? So for developmental toxicity, that would be basically between the zero to five days post-fertilization, because that's when the major events of development are happening, organ production, et cetera. Whereas, reproductive toxicity treatments should really start on or after 10 days post-fertilization, because there's not much going on with the development of the gonads until after 10 days post-fertilization. But basically, any time, you know, even throughout adulthood, zebrafish can be used for screening for reproductive toxicants.

And so I would just like to end --

NEXT SLIDE

DR. DRAPER: -- that there are more similarities than differences I think in -- between zebrafish and humans, and therefore, they really do give us a good platform for doing screening for reproductive and developmental toxicants.

So with that, I will end and I don't know if we're going to take questions now or wait until the next talk.

CHAIRPERSON LUDERER: Okay. I think what we have time for now is some clarifying questions from Committee

members, so about five minutes. So please, I'm going to ask the Committee members. I already see some raised hands, so I'll start from the top left of my screen.

So Dr. Hertz-Picciotto.

COMMITTEE MEMBER HERTZ-PICCIOTTO: Yes. Thank you. Really intriguing, Bruce, to see this coming as an epidemiologist here. I just have a question about in the early embryonic stage, humans undergo an almost total or massive demethylation of the -- of the genome. And I just wondered if there's any data on -- on that in the zebrafish.

DR. DRAPER: Yeah. So -- so there's not as much -- so there's no like paternally versus maternally inherited epigenetic states in zebrafish. You know, basically, because there are no sex chromosomes, they really can -- we can push them to become male versus female and we don't see any difference in like imprinting like you would in -- in other species -- or mammalian species. So to my knowledge there's not a, you know, large scale eraser and then reestablishment of the epigenome, though that does happen during germ cell development just as it does in mammals.

COMMITTEE MEMBER HERTZ-PICCIOTTO: Okay. Thank you.

DR. DRAPER: Yep.

CHAIRPERSON LUDERER: Thank you.

Dr. Pessah, I see that you have your hand raised too.

COMMITTEE MEMBER PESSAH: Hi, Bruce. Thank you for --

DR. DRAPER: Hi. Isaac.

COMMITTEE MEMBER PESSAH: -- your helpful talk. I was wondering, do you want to comment on sort of the challenges of having external development, the chorion providing a barrier to actually getting chemicals where they would be mammalian systems and dechorionation and how that might influence everything? So --

DR. DRAPER: Yeah. So what Isaac is referring to is that the zebrafish have essentially what is, you know, an eggshell, which is very impermeable to a lot of chemicals. However, you can either manually remove that or there's actually and enzyme called pronase that you can treat, you know, en masse, the embryos to digest off that chorion.

One of the problems with not having a chorion during the first 10 hours of development is that the -- prior to the end gastrulation, is that the animals are very -- very fragile, but you can -- so if you just put them in like standard tissue culture dishes or 96 well, you know, plastic dishes, they can lyse when they hit the

plastic. But there are workarounds with that. You just have to put a thin layer of agarose coating the dishes, so that -- so that when they hit that plastic, they don't lyse. So there are workarounds with that.

But it does -- it is a little bit of a challenge up until -- the chorion does not -- normally, they don't hatch out until about three days of development. So doing the earlier study is, if you want to study things that aren't going to pass the chorion, you have to -- to remove the chorion, but it can be done.

CHAIRPERSON LUDERER: Okay. Thank you.

Dr. Baskin.

COMMITTEE MEMBER BASKIN: Yes. Thank you. Outstanding presentation. And this question may be for a future speaker and/or Dr. Campbell kind of for my own edification. How are you measuring -- I think it was actually Dr. Campbell's talk, but alluded to in this talk, plasma levels of, for example, the reproductive hormones in the zebrafish.

DR. DRAPER: Yeah. So that's something that we don't routinely, do but it's my understanding there are ELISA based kits, though it -- it's a sensitivity issue. There -- those kits are not all that sensitive. And maybe Marlissa has a better -- a better answer for that. I mean, there are very sophisticated kind of metabolomics

approaches that you can use that are much more expensive, but there are ELISA-based kits for measuring hormone levels, but I -- what I don't know is really how fine-tuned you can get those for looking at small differences.

DR. CAMPBELL: You know, I don't remember that detail off the top of my head. I'd have to go back and look at the original paper, but I can do that maybe at lunch and try get and answer for you.

COMMITTEE MEMBER BASKIN: Let me get my questions out. Are you essentially grinding up the -- the zebrafish?

DR. CAMPBELL: That would be my guess, but I don't -- I don't remember the details of the methodology.

DR. DRAPER: For the -- for the embryos, you absolutely would have to do that, because you can't -- there's not enough blood to do it. But for the adults, you can get enough blood to -- to look at plasma levels is my understanding.

COMMITTEE MEMBER BASKIN: Thank you.

DR. DRAPER: But, you know, a lot of times, they're -- they're looking at testosterone and not the 11-ketotestosterone and how testosterone and 11-ketotestosterone levels really correlate with each other. I think they're -- they're fairly closely

correlated, but generally most people are looking at testosterone because the're not kits for the 11-keto.

CHAIRPERSON LUDERER: Dr. Woodruff.

COMMITTEE MEMBER WOODRUFF: Yes. Thank you. That was really an excellent presentation. Yeah, I wanted to follow up on the question about the transfer across the chorion. Have people done measurements outside and inside for varying type -- different types of chemicals to confirm that it's completely as you're saying not penetrable?

DR. DRAPER: So I should have mentioned this before, I am not a toxicologist.

COMMITTEE MEMBER WOODRUFF: Oh.

DR. DRAPER: I'm a developmental biologist, so I think those --

COMMITTEE MEMBER WOODRUFF: I thought you were a toxicologist.

DR. DRAPER: -- so I think those -- those things have been done, but --

COMMITTEE MEMBER WOODRUFF: Uh-huh.

DR. DRAPER: -- I'm going to punt and -- and it looks like stephanie wants to address that. Let's get a card carrying toxicologist here.

COMMITTEE MEMBER WOODRUFF: That's fine. Thanks.

DR. PADILLA: Yeah. So I was -- I do have a lot

of strong opinions about the chorion. It is -- it is a membrane, but it actually has pores in it. And the pores --

COMMITTEE MEMBER WOODRUFF: Yeah.

DR. PADILLA: -- are large enough for most molecules -- most molecules, drug or toxicant molecules, even -- even some of the very large herbicide molecules can -- can go through the pores. And it is more experience that there is -- there are very few molecules that don't go through the chorion.

COMMITTEE MEMBER WOODRUFF: Right.

DR. PADILLA: There are some that do. And there is a price to pay besides time and energy for removing the chorion. There's some very good studies to show that development does change if the chorion is not there and also in -- for some of the experiments that we've done, that if they're dechorionated, the behave -- the behavior is different later on. So we -- you know, we can talk more about that later, but --

COMMITTEE MEMBER WOODRUFF: That -- that is really excellent. I really appreciate both of your answers. I mean, it just kind of reminded me about this discussion about, you know, all days about the placenta, and we found that really --

DR. PADILLA: That's exactly right.

COMMITTEE MEMBER WOODRUFF: -- wasn't that accurate.

DR. PADILLA: Yeah, it is -- it is --

COMMITTEE MEMBER WOODRUFF: And I'm not saying -- I'm not -- it's no judgment. Just it's like, oh, this is a very interesting component to the whole --

DR. PADILLA: Yeah.

COMMITTEE MEMBER WOODRUFF: -- exposure piece of this. So I really appreciate your answers.

DR. PADILLA: It's more of a sieve than it is a barrier.

COMMITTEE MEMBER WOODRUFF: Right. Thank you.

CHAIRPERSON LUDERER: All right. Thank you.

I do have one question also, which is you're talking about the shift from heterogametic sex determination that's been lost in the laboratory species that are commonly used. So has anyone really kind of tried to trace when that occurred? And that's one question. And the other one is would you see any potential benefits to using, you know, wild type zebrafish versus the -- these species -- or these strains that have lost that sex determination mechanism?

DR. DRAPER: Yeah, so -- so I should also point out that even in the wild strain where there is a high correlation with a particular locus for females, it --

they did not find that there was a hundred percent correlation. So they actually found some females that were ZZ and they found some males that were ZW. So even in the wild, it's not a hundred percent this, you know, what looks like genetic sex determination. So the way I think of that and I think others that is is that the sex determination mechanism, which, in general, is a very rapidly evolving system and many species, even closely related fish species have different genes that are -- that are the primary drivers of sex determination, and fish can be ZZ/ZW or XX/XY. So it looks like it's either an evolving system in zebrafish or a devolving system, so they're going, you know, towards this more rigid chromosomal or away from it.

So to your second question about using the wild strains. So the -- the advantages of the -- of the laboratory-bred strains is they really have been selected for being lethal-free. And so if you want to be able to compare, you know, effects in seeing -- seeing an effect and knowing that that's not some, you know, genetic predisposition, then the domesticated strains, I think, are better than the wild strains.

The wild strains, I mean, we have some in the lab there. They're also -- in general, they're more temperamental to use. I don't know why that is. They

don't like to breed as well as the ones that have been selected for, you know, good breeders. You know, there might be other wild strains that would be good. I don't have as much experience with those.

CHAIRPERSON LUDERER: Thank you very much.

DR. DRAPER: So hopefully that answered your question.

CHAIRPERSON LUDERER: Yeah. And thanks again for a really fascinating presentation.

DR. DRAPER: Yeah.

CHAIRPERSON LUDERER: All right. Now, I'm going to go ahead and introduce our second speaker, Dr. Stephanie Padilla from the U.S. EPA. So Dr. Padilla is a research toxicologist in the U.S. EPA's Center for Computational Toxicology and Exposure. She has extensive experience with the use of Zebrafish larvae in large-scale screening assays for development and neurodevelopmental toxicity. And the title of here presentation is, "Overview of Zebrafish as a Screen for Developmental Toxicity", with examples from our CASE chemicals as possible.

So Dr. Padilla, welcome. Looking forward to your presentation.

**PRESENTATION BY DR. STEPHANIE PADILLA**

(Thereupon a slide presentation).

DR. PADILLA: Welcome. So can you -- I'm just curious, can you see my screen? What screen are you seeing, you're not seeing?

CHAIRPERSON LUDERER: We're seeing -- we're not seeing presenter view, but we are seeing your screen.

DR. PADILLA: Okay. Just a minute. Let me stop the share. Sorry.

How about now?

CHAIRPERSON LUDERER: No, now we're not -- whoops. Yes, now we're seeing your presenter view. Perfect. Okay.

DR. PADILLA: So thank you all for inviting me. I really appreciate it and I'm very interested in the discussion as this -- as this meeting continues on today. And I also appreciate Dr. Draper's introduction. So some of what I've got on my slides is redundant with what he's presented, but I will skip over that part, so we don't have to -- have to go through it twice.

Let's see. Just a minute it's not progressing like it needs to.

NEXT SLIDE

DR. PADILLA: Okay. All right. There we go.

So I have a movie up here that I would really like to show, because we had a nice -- the movie is very impressive with how quickly zebrafish develop. So I

started working in developmental toxicity, neurodevelopmental toxicity with rats. And when I started working with zebrafish, it was a wonderful thing, because everything happens so quickly. So I'm going to show you this movie.

And over here are the hours of development, so this is the first hour of development. This is about the high blastula stage, which is when we usually begin our exposures. It is going through gastrulation now and going through epiboly. And in a few seconds, or hours as the case may be, you're going to see the embryo begin to form on top of what is going to become the yolk. And so the eye is going to appear over here on this left-hand side. And the right-hand side is the tail region, the somites are beginning to form, the eye is beginning, the brain is beginning to form. We're only 14 -- 14, 15 hours into it. The tail is going to separate here from the yolk. We're about 20 -- we're about one day into this development. You can begin to see the blood coursing through the -- the embryo. You can begin to see the heart starting to beat.

These things are melanophores, which are sort of like -- I guess the best way to say it is they're sort of like the spots on a fawn. They're designed for camouflage. You can see the eye has already developed. The heart is beating furiously over here at the front of

the animal. We're about two and a half days into the development. The jaw is beginning to move forward. You're -- almost all the -- it is going through organize -- organogenesis here. Pretty soon, you're going to see things flowing through the digestive system and he/she is going to swim away.

And so we're about 85 hours into this. This animal was probably reared at 28 degrees centigrade, so they develop a little bit faster. We rear most of our animals at 26 degrees centigrade. So they develop -- we actually do our experiments from day zero until day six.

And so some of the advantages -- and I'm going to reiterate some of these and sort of emphasize the ones that are important for how we do our research are there's a very rapid development. There's a transparent embryo. The developmental pathways are homologous with many other vertebrates. The genome is easy to manipulate.

And for me, in toxicology, I was thinking about working with zebrafish mostly as to extrapolate to human toxicity, but it is also a great model for extrapolation to other fish and ecotoxicology. So you're able to inform both types of toxicological assessments by doing research with zebrafish.

One of the things that we also do in our laboratory that I'm not going to talk about is we do

functional assessments. We do behavioral assessments to look for developmental neurotoxicity in the animal, so we're able to ask questions of, you know, are they -- are they -- are they behaving normally as -- as the controls are?

As mentioned before, the liver has a metabolic capability to both activate and deactivate chemicals. Some really beautiful work that's been done by Dr. Jed Goldstone has shown that they -- the zebrafish possess P450s from many of the same categories that humans do. They have a thyroid axis. They have a stress axis. They have an HPG axis, so they have all the communication pipelines that vertebrates do when they develop.

Now, some of the challenges of working with zebrafish models is when you're looking at either development or neurodevelopmental toxicity, if -- it is difficult to -- it is difficult to assign mechanism, unless you've -- unless you've got a very special test. We just usually know that something has happened, something abnormal has happened, but we're not too sure why or how, and so you've really got to delve into that. But for me, from a screening context, usually we're just looking for did something bad happen.

And then they also -- something that I'm going to talk about -- touch on towards the end of this -- at this

talk is talking about it's difficult to know the internal dosage of the chemical. It's not simple, but a lot of progress is being made.

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DR. PADILLA: So this is -- I wanted to go through this. This is sort of our baseline experimental design. So we get the embryos on the day of fertilization. We usually wash them with a very dilute bleach solution to get rid of any fungi and begin our exposures, usually about six hours post-fertilization.

And for the data that I'm going to present today, we change that solution every single day. So we renewed that chemical solution every single day until day five. And on that day, we actually wash the chemical out for our assessment on day six just so we don't have to handle as many toxic chemicals. And also, we do most of our chemical exposures blinded, so we don't know what chemical we're working with and we just consider everything really dangerous. And so when we do this assessment on day six, it requires quite a lot of interaction with the embryo, and so we'd rather not have the chemical around.

So on day six, we look at the embryos. This is a human assessment. And we look at the embryos and ask if it's dead or alive. And if it's alive, we ask if it's hatched, because there's -- we have -- you haven't seen

any pictures, but there's a -- there's a membrane around the zebrafish. And it's a -- I don't know how to describe it. It's not like, of course, a chicken -- chicken egg, but it's sort of like a hard jello. It's kind of like the consistency of a jello shot, if that's helpful to anybody. And it does have pores in it.

And so we're looking to see if the embryo has hatched, because there are classes of chemicals that will decrease hatching in the embryos. And if it's hatched, we record it as did not hatch. That is -- that is a toxic endpoint at six days. And then if it's -- if it is hatched, we perform a basic malformation assessment, just looking at various aspects, which I'm going to show you on the next slide, that can be abnormal about the developing embryo.

And I wanted to show this illustration down here, which shows the zebrafish -- this is a six-day old larval zebrafish this in a 96 well plate, because it -- it gives you an idea of proportion here with regard to how -- you couldn't do these experiments in a 384 well plate, but the fish is relatively comfortable in a 96 well plate.

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DR. PADILLA: And so these are the kinds of things that our human assessment is looking at. This is what a normal six-day old zebrafish looks like in a well.

These are -- they're very visually oriented. These are their big eyes. They've got ears, the otoliths on either side. You can't really see them very well, but they do have pectoral fins, and of course, there's a nice straight -- nice straight spine there. This is an inflated swimbladder. And this is what an abnormal fish would look like. There's a lot wrong with this fish. It has a curved axis. There's a lot of edema. It has a very small head. It has a very small eye. The swimbladder is not inflated. So there's a lot -- this is a severely abnormal fish. It is like -- unlikely to -- to reach adulthood.

And then this is -- we have some really nice pictures. We have a -- we have a system that is able to pull the fish up into a capillary tube and take -- take pictures of the fish. And it gives us a very, very detailed view of the fish and how it's developed. And this is again a normal six-day old zebrafish. You can from the side, the eye, the mouth is -- the jaw has developed normally, the mouth is towards the front. You can see the individual organs here. You can see the liver. You can see the digestive tract. You can see the heart. And this is the otolith, the ear, and a nice straight spine.

And here is one that is -- is abnormal, not severely abnormal, but abnormal. And you can see that

it's got quite a bit of unabsorbed yolk, which shows, and it's got some pericardial edema him. It's got a small head. The jaw is misshapen

And you go down to even more and more misshapen and dysmorphology in the developing animal. All these were alive, but they were very shaped of malformation. Taking these pictures -- this is something that we've just been able to start. Taking these pictures, we're able to enter them into software called, freely available, FishInspector software, that is able to then take measurements of different aspects of the fish. And so we -- we are able to not only do human assessments and say whether they look abnormal or normal and how they look normal/abnormal, but we're absolutely -- ab -- now, we can take measurements of the fish and we can ask how big is the pericardial space, how big is the -- is the -- is the area around the yolk, how big is the swimbladder, and how long is the fish, how big is the eye. All of these are going to be tied into the toxic assessment and whether some dysmorphology has occurred.

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DR. PADILLA: And so when I was thinking about this presentation, I was thinking about, well, if I was trying to make -- if I was trying to use zebrafish for risk assessment, what are the types of questions that I

would want to ask about the data? And so these are the ones that I was thinking about. I want -- first of all, I want to know how good are the data. And to ask that, I would want to know about consistency of the data within a laboratory, and consistency over time within a laboratory, and then a consistency among laboratories. And then, of course, you would want to ask how does it -- how does it compare with the mammalian data? And so I'm going to touch on -- on all of those.

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DR. PADILLA: So the first is how -- how consistent are the data within a laboratory? So the laboratory I'm talking about, of course, is my own, because that's the one where I've got the data. And this is a couple of chemicals that I know that you all -- this first chemical is BPA. And as I said, we do most of our assessments blinded to the chemical, so we only find out what the results are afterwards. And this was the same chemical, but different sources. So oftentimes when we get our chemicals, they'll -- they'll put the same chemical in the -- in the library of chemicals that we're testing from two different sources.

And the way that this is arranged is the dose of the chemical or the concentration of the chemicals. These animals were all exposed in the chorion by emersion, and

is the can -- concentration of the chemical that was in the water that they were exposed to. And then this is sort of the toxicity index, I guess. We sum up our assessments and come up with a number between zero and 100. And anything that scores 100 was -- the animal was dead -- was -- it had killed the animal.

Anything in between here, basically in the yellow region scored between 20 and let's say 99. These animals were more morphogenically not normal. So they either weren't hatched or there was something wrong with them. And the higher the score means the more things went wrong with them. And the animals that were here in this range, were within the control range. And each circle represents an animal. And sometimes there are lots of animals. It's difficult to tell, because they're circles on top of circles.

And so if you're looking here, as you can see the increase in the concentration of BPA, caused an increase in such that -- at the highest dosage, there was some death. And here there was one, I think, out of three animals that died but two were normal. And so this is often what the curve looks like. And then you can calculate an EC 50, an effective dose, basically. And for this -- for this run of this chemical, it was 55. And for this run of same chemical, but from a different supplier,

basically it was 63.1 micromolars. So these are very similar. And if you look at -- I think I have another one.

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DR. PADILLA: Yes. Chlorpyrifos on of my favorite chemicals. If you look at chlorpyrifos, it turns out we use chlorpyrifos as an internal control. And so we have lots and lots of our own assessments as well as the internal control that was within the library that we tested. And you can see here that same thing here, that at the lower doses, there tends to be -- most of the animals are within the control range. As you increase the dose, you see an increase in dysmorphology. Even going even higher, you see it -- basically, it moved from dysmorphology into lethality. And that's the kind of -- that's the kind of curves that usually see with this. It's a -- it's a gross curve. But from it, you can get an EC 50.

And again, these EC 50s, even though the chemical was from two different sources, were very close. Here, it's 8. Here, it's 10. So it's not bad about consistency -- above -- testing the same chemical from different sources.

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DR. PADILLA: And how consistent are the data

over time? So it turns out that we had tested -- we've tested multiple laboratories. We probably tested two or three thousand chemicals. And this was the same chemical that was three years apart. This is azoxystrobin. This is another chemical, and this is the triclosan. So we're looking at the EC 50s that were done with two different libraries that we tested. And here the EC 50 for azoxystrobin was 2.9, 3.6. So this is very consistent over time. Oryzalin we had 16 and basically 12. Again, very consistent over time. And for triclosan, it was 4.6 and 2.7.

So we're seeing -- we're seeing consistency not only between the chemicals, but also over time. And keep in mind, those of you that work with zebrafish will get this right away, but the population of fish that we were working with are very -- was very different three years apart. Now, we try to make everything -- and we need to talk about that, but we try to make everything as consistent among our populations as possible. But this also helps us realize that our populations are -- we're not -- we're not seeing a gradual change in the sensitivity of the population over time, because these, of course, were very -- were different fish than the ones that I tested three years before.

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DR. PADILLA: And how consistent are the data among laboratories? And so that's -- that's a little bit difficult to get at, but it turns out that to Ducharme et al. published a paper about ten years ago now comparing data from many, many different laboratories and many, many different studies to, it turns out, our data, which was really nice. And I think the reason they did that is because we -- we were one of the few laboratories at that time that had published a very large survey of a large library with regard to LD 50s and dysmorphologies.

And so basically what they did was they looked at all the different studies that they had reviewed and they had -- they had calculated a metric that they call loaded, that has to do with how toxic the chemical was to the -- to the developing zebrafish and realized that those studies had 16 chemicals in common with our study, and just tracked how -- what -- what was the correspondence between the toxicity that we had study -- that we had published and the toxicity that had been published in these other studies.

It was -- I felt they had a really nice correlation and I felt that it was very encouraging that the data are consistent among laboratories. And in fact, in just comparing our own data and also comparing our date -- looking at data in other papers for chemicals that

we've tested, I would say that there is quite a bit of consistency among laboratories with regard to developmental toxicity of chemicals in zebrafish, embryos, and larvae.

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DR. PADILLA: And then if you look at concordance between -- or concordance between mammalian toxicity and zebrafish. So there's some -- there's quite a few papers that we can look at for that, but -- so for this one, this was a paper that was published -- they wanted to look at the concordance with regard to four organotin chemicals, And so this chem -- this paper was -- they looked at the in vivo developmental toxicity in mammals and then they looked at the zebrafish developmental toxicity. And here, they're looking at the ranking.

And so what they see was the ranking of these chemicals was basically the same between mammals and zebrafish, that the dibutyltin dichloride was the most toxic followed by the dimethyltin dichloride. The monomethyltin dichloride -- trichloride was not toxic in either mammals or zebrafish. And then they had not tested the monobutyltin trichloride in mammals, but in zebrafish it was not toxic.

So they were -- they were very heart -- they were very heartened by the fact that the ranking of the

toxicity of these organotins was the same between the mammal -- the mammals and the zebrafish.

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DR. PADILLA: And then in another paper, Kari et al., looked at the toxicity of drugs basically in zebrafish versus mammals. This is developmental toxicity. And it was interesting here, they did see some concordance. But in general, the zebrafish, if there wasn't a concordance, the zebrafish tended to overestimate the toxicity for the mammals. So, I mean, if -- if you have to go one way or the other, you might want to -- you might want a sentinel species that is overestimating the toxicity.

And then Nisha Sipes and her -- her co-workers published a paper looking at the concordance between zebrafish studies and mammalian -- different mammalian studies. And they found that concordance ranked somewhere between 55 and 87, I guess. And so, you know, that doesn't sound too good.

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DR. PADILLA: But when you compare it in this way, so they -- they also compared it in this way, and this is a very interesting graphic. They basically looked at the concordance between zebrafish and rabbit, which is the blue, which was about 47 percent. And then they

looked at the concordance of the zebrafish and the rat -- and this is both negative and positive concordance with developmental toxicity studies, and they got about a 52 percent concordance.

But then, just something to take home, the rat to rabbit concordance was really only 58 percent. So it's not horribly wonderful, but it's also not terribly bad, in the sense that the zebrafish and the rabbit, and zebrafish and the rat is between 47 and 52 percent and the rat and the rabbit is -- is really only -- two mammalians is only 58 percent. So it's -- it is in the right ballpark, I guess, is the best way to say it.

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DR. PADILLA: Now, most of what I've talked about is hazard. And I know most of what you all are interested in is hazard ID. But I also would like to talk a little bit about exposure considerations in zebrafish, because most of the studies that we -- we conduct and most of the studies that are in the -- in the literature are emersion type of exposure. So you're taking the animal and you're putting the larvae or the embryo into the solution and you know what the concentration of the chemical is in the solution, but you do not know what the chemical concentration is in the zebrafish. And sometimes it's a lot less and sometimes it's a lot more, and rarely is it

the same concentration that is in the solution.

And so it's really important, especially if you want to do a risk characterization, that you understand what the dose is to the zebrafish. And so how can a zebrafish embryo larvae be exposed to the chemical? Well, obviously it's being exposed dermally to the chemical, so the chemical can partition into the -- into the embryo just like it crosses any type of membranes.

It can also partition into the yolk. And then as the zebrafish grows it absorbs -- the embryo grows, it absorbs whatever is in the yolk. And this is something that can happen. And the yolk is, in general, a more lipophilic type of environment that maybe the embryo is. After about three to four days, the zebrafish can be exposed orally. So the -- the chemical -- the zebrafish begins to take gulps of the surrounding solution by about four days post -- post-development.

You can expose them by injecting the chemical directly into the zebrafish. This is done for some chemicals that don't -- aren't absorbed well by the zebrafish, but rarely ever, and it's not really applicable in a screening context. And the zebrafish gills don't really develop until about 10 to 14 days. And so if you're exposing an embryo, and assets assessing the larvae, then you're not going to get much exposure at all

through the gills. Although it's a very efficient way in adult -- in adult zebrafish for the exposure to take place.

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DR. PADILLA: So we do know that the physical chemical characteristics of the zebrafish are -- do determine how much of the chemical is absorbed by the zebrafish. So this is a study that was done where our laboratory and also Robyn Tanguay's laboratory tested basically exactly the same library. Now, their -- their protocol is a bit different from ours. They dechorionate, but they only dose once. We don't dechorionate, but we do dose every day. We renew the solution every day.

So we're looking at the distribution of the log P, which is the octanol water partition coefficient in the library that we tested. And you can see it was a pretty wide distribution. And now we're looking at the distribution of the -- of the chemicals that tested positive, that we saw changes in development. And the red, of course, is the chemicals that -- that we saw as positive and the blue is ones that -- that Oregon State saw as positive.

And, in general, the distribution is the same, that chemicals that have a log P below about minus one or above about eight probably are not useful. They're --

they're either too hydrophobic or too hydrophilic for testing in an emersion type of situation. So that can make a difference with regard to whether you can test the chemical or not.

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DR. PADILLA: We have also other aspects that can affect dose in zebrafish. You can have whether the chemical is present in the surrounding solution. So as long as a chemical is there, the zebrafish is probably going to absorb it. But as soon as the chemical is not there any more, basically it is going to be dep -- depurated. It is going to leave -- of course, the -- it's going to reach a new steady state. It's going to leave the fish and enter into the solution.

As I mentioned before, there can be hepatic activation of the -- of the chemical. It can be hepatic deactivation. The age of the time of the exposure determines how much is absorbed by the -- sometimes it's not only the presence of the chorion or not, but sometimes even if you expose them for the same number of hours, certain -- certain number -- certain developmental -- developmentals -- certain developmental stages can -- will tend to absorb or not absorb the chemical. The duration of the exposure is also very important.

Some chemicals like ethanol and nicotine will

reach steady state in minutes, whereas other chemicals will take days to reach steady state. Also, the chemicals can induce enzymes. And so the -- it could be that they induce enzymes that metabolize them or they could induce enzymes that actually pump them out of the cell.

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DR. PADILLA: But -- and I don't want to make this sound absolutely horrible. So even though this is very complicated, there's been some real progress in developing models that predict what the dose of the chemical is, what the internal dose of the chemical is in zebrafish. And these are some examples of that.

And so I'd like to thank you all for -- for your attention. And I would sort of like to summarize by saying that I think -- I think getting consistent results for developed -- screening for developmental toxicity in zebrafish is -- we're not only getting Consistent results within a laboratory, but also I think we're getting consistent results among laboratories, and the comparison with the mammalian data so far is -- is reassuring. And so I will be glad to consider any questions or comments and just remark that this -- this is a T-shirt that I found on Etsy that I thought was really interesting. I haven't ordered one for myself yet, but I might, so -- but

I'll be glad to answer any kind of questions or comments that you all have.

CHAIRPERSON LUDERER: Well, thank you very much, Dr. Padilla for that wonderful presentation. We have some time now for clarifying questions, about five minutes, and then we'll get into our discussion which -- for which we have -- with both Dr. Draper and Dr. Padilla for which we have 30 minutes allotted.

DR. PADILLA: Okay.

CHAIRPERSON LUDERER: So, please again raised your hands as before for -- with any clarifying questions. I will try to keep an eye out here for everyone.

Maybe -- Dr. Baskin, go ahead.

COMMITTEE MEMBER BASKIN: That was also a fantastic presentation. I feel like I'm at developmental biology meeting and just learning. The question I have is -- and I think I may have just missed this, so, you know, I'm a pediatric urologist and I'm kind of focused on genital development. And here, Dr. Draper gave some nice --

DR. PADILLA: Yeah.

COMMITTEE MEMBER BASKIN: -- as well yourself on kind of sexual differentiation. What are the major endpoints that the zebrafish community kind of considers, you know, super important? You know, like I saw the eyes.

I saw the cardiac development. I'm kind of seeing, you know, the development of like the tail and the whole fish. It looks like there's kind of a liver there. There's clearly an ovary which is very, very impressive, you know, compared to the testes, you know, but the genital development was -- was super subtle. It's kind of a global question.

DR. PADILLA: So we try to do our experiments and finish our experiments by day five -- day six. And by -- at that time, you cannot -- you can't really tell at all what this is -- is this going to be a male or a female. You cannot -- you cannot discern that. You can -- I mean, the types of endpoints that -- that most people look at, they -- sometimes they look at earlier endpoints and earlier in the development, but at day six, you're mostly looking to see, you know, is -- has the animal -- is it showing a curved spine, is it showing any kind edema, either pericardial or yolk edema, have they absorbed their yolk, is the eye, the head - things like that - is that -- is that normal?

I mean, having looked at thousands of animals that have been treated with chemicals, those -- it is a very generic type of report out at that point. There's only -- I mean, I often say this. There's only just so many ways that the development in the zebrafish can go

wrong, and it doesn't necessarily tell you about the mechanism. There are a couple of -- I think, there's a couple of chemicals that will affect the notochord development and sometimes you see a wavy notochord. There are some chemicals, as I mentioned before, that affect hatching. Hatching is actually kind of a complicated process, but most chemicals just show -- show just something went wrong, I guess, is the best way. And I -- at this point, it's six days, and I am not a reproductive biologist. I don't think there's anyway to tell if the animal has had some sort of misdevelopment with regard to -- with regard to reproductive organs.

DR. DRAPER: Yeah, the -- the -- the earliest what anyone has ever recorded gene expression differences is at around 14 days.

DR. PADILLA: Yeah.

DR. DRAPER: I think -- I think that's kind of early, but -- but that would be where you'd -- the earliest signs of skewing one direction to the other for male versus female would occur.

CHAIRPERSON LUDERER: Thank you.

Dr. Auyeung-Kim has her hand up. Go ahead.

Oh, You're muted.

DR. PADILLA: Yeah.

COMMITTEE MEMBER AUYEUNG-KIM: Can you hear me

now?

CHAIRPERSON LUDERER: Yes.

DR. PADILLA: Yes.

COMMITTEE MEMBER AUYEUNG-KIM: Okay. Thank you for the wonderful presentation and very detailed. My question is about the hepatic activation and deactivation that you mentioned. And I was just wondering how is -- has there been a comparison made between, you know, the -- whether the -- the metabolism would be -- how similar the metabolism is to that of the mammals?

DR. PADILLA: Is that I do know that the P450 complement is very similar to animals and they have all the same classes that the -- that the mammals have. I don't know -- I do know that there has been some plans to publish on that. I am not aware of whether it's been published or not, but there was a lot of effort to look at the metabolic profile, not necessarily the genetic profile, but the metabolic profile.

And so far, I mean, just from my own -- from my own experience in looking at the chemicals, I do know that they are able to activate many of the OP chemicals -- many of the OP chemicals -- organophosphate chemicals that require hepatic activation for real potency. But I -- I don't know -- I don't know about the other aspects of it, but it is quite similar.

COMMITTEE MEMBER AUYEUNG-KIM: Okay. Thank you.

CHAIRPERSON LUDERER: Dr. Plopper.

COMMITTEE MEMBER PLOPPER: Yeah. I just -- the thing that concerned me is that this bioactivation that was just brought up, do these animals have kidneys and they're -- and a number of other organs that also bioact in mammals, how is that addressed with this model?

DR. PADILLA: You know, I don't know that anybody has looked at -- they do have -- they do have -- they're not -- they're not called -- they do have kidney-like organs, I guess. Yeah. They even have gallbladders, which to me, for some reason, seems amazing, but -- so I don't know about the activation potent -- I don't know about the metabolic potential of the other organs, but they do have a liver that comes online about two days post-fertilization.

COMMITTEE MEMBER PLOPPER: Well, what do they have -- I don't know what you want to call them, some kind of -- let me think now, how do they absorb oxygen?

DR. PADILLA: They absorb oxygen by diffusion for about the first 10 to 14 days, and then their gills come online and they begin to absorb it through their gills.

CHAIRPERSON LUDERER: Okay. Thank you. I did have a question, which is when you were look -- sharing the data about the consistency among labs, whether that

analysis adjusted for -- it sounds like there's two main lab strains and did it make a difference which strain of zebrafish you were exposing to the chemicals. Like did that improve -- you know, if you only looked at the same strain, would it improve the consistency?

DR. PADILLA: Oh, gosh, so that was from many, many different laboratories --

CHAIRPERSON LUDERER: Um-hmm.

DR. PADILLA: -- and so they're all using various strains. And one of the things -- I don't want to make this any more complicated than it has to be. But one of the things that happens if you're rearing fish in the laboratories, you go through many -- you go through about four generations. You can go through quite a few generations each year. And if you're not careful to outbreed your animals, then your strain is going to become more and more sort of institution specific.

CHAIRPERSON LUDERER: Um-hmm.

And so even though you say you're working with the same -- like I'm working with AB or you're working with WIK, it doesn't mean that it's exactly the same strain. However, we have compared our strain to other strains and find that it is most like the AB strain with regard to behavioral characteristics, not necessarily sensitive to chem -- sensitivity to chemicals. But, you

know, were you're com -- in the -- in the graphic that I showed where we were comparing our data to OSU data, those are probably two vastly different strains, but yet the -- the amount -- I mean, the -- they -- we're picking up many of the same chemicals.

So I -- I don't know -- there has been some research on strain and effect on chemicals. And there is some difference, but usually you have to think about is it whether you're going to call it a hit, in other words, is the chemical toxic to zebrafish, or at what dose. And so in general, calling the chemical a hit is not going to be as strain specific as the actual sensitivity to the chemical.

So we handle strain a bit differently in our -- we -- we do our best to outbreed our animals as much as possible. When we breed the -- each time we have to raise up a new parental generation. We take it from all the different ages that we have. At least once a year, we order another completely different strain from some place and mix it in with ours. And so we try to keep our -- we've gone towards the randomization aspect of it rather than specificity aspect of it.

CHAIRPERSON LUDERER: Great. Thank you.

Let's see, I'm not seeing any more raised hands, unless, Dr. Plopper, did you have another question?

COMMITTEE MEMBER PLOPPER: No, I'm fine. I just didn't lower my hand.

CHAIRPERSON LUDERER: Okay. All right. Just wanted make sure.

Okay. So now we're going to start with the Committee discussion, the part one of that, with Drs. Draper and Padilla. And so we -- you know, just to kind of get the discussion going, we have some questions to think about. And so one of those is in setting up an experiment using zebrafish, how many adult fish of each sex would you typically start with as a source of ova and sperm. And either Dr. Draper or Dr. Padilla could -- could maybe -- maybe start responding to that.

Whoops, I think Dr. Padilla -- go ahead. Now we can hear you.

DR. PADILLA: In a screening context, we usually start with a lot of fish and a lot of embryos. We have group -- I don't know, we probably start with 30 or 40 of each sex. And we have multiple ages, parental ages, that we -- that we mate at the same time and we take samples from -- from the eggs that were produced by each group of parents. So again, we're -- we're trying to -- to basically randomize things as -- as much as possible.

If you -- if you do a one-on-one type of mating, you -- first of all, it's a lot of trouble, because you

would have to mate a lot in order to get the thousands of embryos you need to start with to set up the screening context, so -- and -- and it's much less successful when you use fewer fish. So anyway, that's -- that's what we would use, because we need -- we need quite a few embryos at each go.

CHAIRPERSON LUDERER: Um-hmm. Thank you.

Dr. Draper, did you have anything to add to that?

DR. DRAPER: I -- I don't have anything to add to that. As I -- as I said I -- my lab has not done a, you know, classic toxicology screen.

CHAIRPERSON LUDERER: Okay. I see that Dr. Allard had his hand raised.

COMMITTEE MEMBER ALLARD: Yeah. I wanted to circle back to that question of compare -- comparability between laboratories, both from a toxicity standpoint as well as from a endpoint measurement. So it's about standardization of practices across laboratories so that we can really understand differences that may emerge between studies. So the first part of the question is toxicology focused and less really relying on this paper from Windy Boyd is the first author in EHP that relied on your data, Dr. Padilla, and compared it to the Tanguay lab's data, and -- and saw a -- a decent but partial overlap between -- between labs. And then in the paper

they sort of talk about, you know, maybe it's the way that the -- the data is analyzed, I think. I mean -- I guess my question is where does the difference come from?

And then I'll have a second partner for both of you after that about standardization about endpoint Measurements, but maybe we can start with the toxicological angle.

You're muted.

DR. PADILLA: Got it. So the data that I showed that compared our data to Tanguay Laboratory on that graph with the physio -- physiochemical characteristics, we analyzed that data the same way. So it's different from the Windy Boyd paper. So we -- we took their data and our data and put it through the same analysis program. And on that one there, we did tend to get more hits. And from that, we interpreted it as dosing every day tended to give you more hits than the dechorionation aspect, because that was really the difference. I mean, there was some strain different, but I -- I don't think a strain difference would make a difference in whether you called it a hit or not. And that's basically what -- what that aspect was.

The Windy Boyd paper, there were differences in the analysis. There was quite a few differences in the analysis when she was comparing those data, and that's why we took it through the same type of analysis paradigm to

look at it.

I -- you know, we -- we are very concerned about differences in laboratory, and I think -- especially for behavioral assessments, but for doing developmental assessments, I have been -- I have been participating in OECD work group where we've been looking at developmental and behavioral assessments. In general, the developmental assessments of the chemicals among the laboratories is much more consistent than the results from the behavioral assessment, so --

COMMITTEE MEMBER ALLARD: Okay. That was -- that was my question about -- about standardization of measurements of behavior or other endpoints that often are done in alternate ways, but some people sort of design things in-house, some people use --

DR. PADILLA: That's right. That's right.

COMMITTEE MEMBER ALLARD: -- sort of commercial platforms. And I was wondering whether there's been some -- some common agreements --

DR. PADILLA: Well, I mean, that's --

COMMITTEE MEMBER ALLARD: -- about benchmarks that need to be met for those -- for those things to be used.

DR. PADILLA: Yeah. No. And actually, we just finished writing a paper and submitting a paper just -- we

reviewed the literatures just trying to figure out all the differences in -- in approaches, and it's -- and it's really scary. And this is for behavioral measurements. And it's not only approaches, it's reading a paper and trying to figure out what they did. The reporting is -- is something that we need to get a lot better in order to be able to determine if these two papers did run the assay in the same way or didn't run the assay in the same way. So I completely agree with you, yeah.

COMMITTEE MEMBER WOODRUFF: Yeah. Can I just follow up on that -- on that question.

CHAIRPERSON LUDERER: Yep.

COMMITTEE MEMBER WOODRUFF: -- which is I think one of the things that we found when we're doing this is that people compare active/inactive, which is not -- which can skew your comparisons and don't compare like a benchmark dose across assays. Have you done this and compared the difference, because the benchmark dose tends to be a better reflection of the experimental dose response --

DR. PADILLA: Right. Right.

COMMITTEE MEMBER WOODRUFF: -- and you get better comparability when you use --

DR. PADILLA: Yeah, and so the data that --

COMMITTEE MEMBER WOODRUFF: -- do have -- I think

that alludes a little bit to the statistical analysis component of it.

DR. PADILLA: Yeah. And so the data that I showed with the physiochemical characteristics and how well it corresponded was a re -- basically a -- I guess, a benchmark dose type of calculation. So it wasn't limited by the doses that were chosen. It wasn't like a LOEL or something like that, yeah.

COMMITTEE MEMBER WOODRUFF: Okay. Great. Thank you.

CHAIRPERSON LUDERER: Dr. Pessah, you have a question.

COMMITTEE MEMBER PESSAH: Yeah. I still want to get back to this issue of testing compounds at the extremes, where, you know, many of the persistent organic pollutants that are thought to be developmentally toxic, and, in particular, neurotoxic, they have to be in solution to get past the chorion. I mean -- and I -- I found many studies that I've reviewed where the dose response and the EC 50, whatever the measurement was at endpoint, were well above the solubility limit of the compounds in aqueous solution. Now, granted maybe the solutions that you're using have components in it that are analogous to serum proteins, which can help the compound get in if the pores are large enough. But could you

address that, because that really speaks to how seriously you should take some of these results.

DR. PADILLA: So most people that do the testing do look at the solubility characteristics and shouldn't be testing above the solubility of the chemical. Is that -- is that what you're saying.

COMMITTEE MEMBER PESSAH: I just read on -- I just read a paper on benzophenones, which are virtually insoluble in water and they got all sorts of results. And so I'm -- it left me wondering how do you interpret those results?

DR. PADILLA: And they're not measuring --

COMMITTEE MEMBER PESSAH: Again --

DR. PADILLA: -- they're not measuring the level of the chemical in the animal.

COMMITTEE MEMBER PESSAH: They are not.

DR. PADILLA: It sounds like -- it sounds like that would be the next question, right --

COMMITTEE MEMBER PESSAH: Yeah.

DR. PADILLA: -- how much of it got into the animal?

COMMITTEE MEMBER PESSAH: But do many studies actually take the expense of sending off extracts to -- I mean, is that routinely done so that one could --

DR. PADILLA: Well, no, but that's why they need

to work within the solubility characteristics of the chemical I guess.

COMMITTEE MEMBER PESSAH: Okay.

DR. PADILLA: No. There are some -- there are some -- there are some companies that do that, that will test your chemical and also determine, because they want to find out if the chemical is negative -- this is mostly European, but they want to find out if the chemical causes adverse effects in the developing vertebrate, so they send it. And if it doesn't, then they also need to ascertain the chemical got into the animal. And they -- they do that type of analysis, but -- and in eco -- in ecotoxicology, they do spend a bit more time looking at whether the chemical is in solution and how much of it is in solution and working below the solubility characteristics of the -- it's more so than in mammalian hazards ID, but, you know, think is a -- I this is a very -- a very valid concern.

COMMITTEE MEMBER PESSAH: But is there -- are there steps being taken to try to get that more standardized in terms of either normalization to internal dose or, you know, having a factor that you use in if, you know, you try --

DR. PADILLA: Well, I mean, as -- as -- sort of what I was talking about towards the end is there -- there

are people that are developing models that should be able to at least do a pretty good job of predicting how much of the chemical -- how -- what is the bioconcentration factor, how much of the chemical is in the -- in the embryo after a certain time -- after a certain type of exposure?

COMMITTEE MEMBER PESSAH: That's important.

DR. PADILLA: Yeah.

CHAIRPERSON LUDERER: Okay. Thank you.

We have a -- kind of another aspect we can turn to to discuss and to -- in our discussion, which is, you know, whether -- and I think you -- you mentioned this a little bit, Dr. Padilla, but I think we could have more discussion about it, whether the potential parental contribution is considered in study design or data analysis. And you mentioned that you really try to randomize that when you're doing these high throughput screens. But, you, know how -- you know, or do other -- you know, and perhaps do other groups, kind of getting into what is sort of the common practice potential -- you know, this type of parental contribution considered in the test group assignments or are all embryos just considered the same equivalent?

DR. PADILLA: Yeah. From what I know with screening large libraries, the -- the approach is to view

all embryos as the same.

CHAIRPERSON LUDERER: So there's no consideration to parental -- whether -- which parents they came from?

DR. PADILLA: Well, you won't know that unless you do a one-on-one type of mating.

CHAIRPERSON LUDERER: Um-hmm.

DR. PADILLA: And that is extremely inefficient for obtaining the number of embryos that you need.

CHAIRPERSON LUDERER: Right. So the key thing is really that you're mating many fish and then you're random -- and you're basically randomizing the embryos from those --

DR. PADILLA: Yeah.

CHAIRPERSON LUDERER: -- parents.

DR. PADILLA: Yeah. And I frankly don't know how much the results would differ, if you did one-on-one type of mating. I don't -- we don't see a lot of variability. I mean, I don't know if you noticed it, but we're -- we're dealing -- when we do these types of developmental studies, we can run an N of three to six and have a very good repeatable idea of what it's going to be in two years with a completely different group of fish.

CHAIRPERSON LUDERER: Um-hmm. Um-hmm.

DR. DRAPER: Where it -- where it may make a difference is if sex is being used as an endpoint --

DR. PADILLA: Yeah.

DR. DRAPER: -- because it has been shown that although, you know, you generally get 50/50, 40/60 sex ratios, if you made a single pair repeatedly, they will give very similar sex ratios from mating to mating that may be different from another pair. So there is, at least in the domesticated line, although there isn't a strong, you know, sex determinant, there are definitely loci that -- multiple loci that can affect sex.

So -- but -- but if I were using sex as an endpoint, I would do what Dr. Padilla does, which is basically, you know, use a very large randomized mating and just combine all those together, so that you basically have the average sex ratio of the -- of those fish.

CHAIRPERSON LUDERER: Yeah. I mean, I -- yeah, I think this comes up when, you know, we're thinking about mammalian studies, we usually -- if we're doing any kind of departmental exposure, you know, correct for litter effects, right? So we do some sort of statistical adjustment for that. And so, I mean, it sounds like that's --

DR. PADILLA: Oh.

CHAIRPERSON LUDERER: -- or it should do that.

DR. PADILLA: Well, actually, I mean -- well, this gets us off on a whole nother tangent. If you raise

those embryos in a -- in -- together in a solution -- this is why we put one per well. If you raise those embryos together in a solution, they -- they have an effect on each other and you really need to do statistics. I mean, if you're raising 50 embryos in a petri dish, let's say, then that needs to be your litter, that needs to be your statistical litter --

CHAIRPERSON LUDERER: Um-hmm.

DR. PADILLA: -- because there's some really good data to show that the condition of one embryo may affect the condition of the other embryos.

CHAIRPERSON LUDERER: Um-hmm.

DR. PADILLA: And so we don't have that maternal, but we do have the environmental contribution, so that is something you do need to worry about and to consider when you're looking at the experimental design. Yeah.

CHAIRPERSON LUDERER: Dr. Allard, I see your hand is raised.

COMMITTEE MEMBER ALLARD: Yeah, so I -- we had just talked here about potential -- in some cases, some studies that need higher numbers. So I guess a very basic question then, when I review literature involving zebrafish of those early stages for reproduction, what is a well-powered study? What is a good number of animal that would make the data appear more sound? Is that -- is

it really too study dependent?

COMMITTEE MEMBER WOODRUFF: Well, I -- can I just like -- isn't there partly though, like when you were showing that data between EPA and Oregon State, I mean part of it is length of exposure, right, like more chronic exposure resulted in more robust findings from the results, right? And it -- it's not -- it's partly about the number of embryos, though --

DR. PADILLA: Well -- okay. So in that study, they had 32 embryos per concentration. We had --

COMMITTEE MEMBER WOODRUFF: Right.

DR. PADILLA: We had five.

COMMITTEE MEMBER WOODRUFF: But you saw --

DR. PADILLA: But we were dosing every single day.

COMMITTEE MEMBER WOODRUFF: Right.

DR. PADILLA: They did not dose every single day.

COMMITTEE MEMBER WOODRUFF: Right.

DR. PADILLA: And so I think that -- so it wasn't -- they exposed -- we actually exposed for the same amount of time, except they just dosed once. And then we just -- we renewed the solution. So, I guess, that's -- I mean, it -- and also there's was at a different temperature than ours was, so -- so there are -- there were some differences between them. And we don't really

know which one contributed to, that all -- although I do know that there are people that are trying to decipher this out. You know, is it dosing every day? Is it removing the chorion? Is rearing them at 28 instead of 26? So there's -- there's various aspects that you could look at, but -- okay.

COMMITTEE MEMBER WOODRUFF: Yeah. Well, I guess what I would say is like that gives you a fine point on the level of the dose response, but in general what you were seeing is there was a response, though at different gradations, depending on some of these -- obviously experimental factors are important --

DR. PADILLA: Yeah.

COMMITTEE MEMBER WOODRUFF: -- for that, but you -- it looked like from that -- those study results, except for the issue about the (inaudible) which seemed to influence --

DR. PADILLA: Yeah, both ends.

COMMITTEE MEMBER WOODRUFF: -- the findings, that there was -- I mean, if you looked at the correlation between the responses, it looked like it would be very high. I'm not sure if you did that, but...

DR. PADILLA: No, we didn't do that.

COMMITTEE MEMBER WOODRUFF: Yeah.

DR. PADILLA: This was for a methods paper. So

Patrick was asking what was the number. I don't -- I don't know what -- I feel -- so when we first started -- maybe this is too much, but when we first started testing the ToxCast chemicals, we had three -- 400, 500 tests. I was very worried, because we were using two to three animals at each concentration. And the statistician explain to me that to calculate the EC 50, you're just looking at where it changes, right? So you've got nothing, nothing, nothing, happens, and all of a sudden everything happens, and then -- then at the higher doses, there -- it's all lethal.

And so to calculate that EC 50, you're looking for that change. And that's a bit different than if you're looking for -- if you need to have data to calculate a BMD. So for that, that data calculation requires more -- more doses, more animals in the area where the change is occurring, so you can accurately cal -- calculate that BMD, so --

COMMITTEE MEMBER WOODRUFF: Well -- yeah. No, I agree. I just would say though -- I mean, your EC 50 is like a BMD, it's just a BMD 50 not at BMD 10, or 5, or something like that.

So I mean, I think your --

DR. PADILLA: Okay.

COMMITTEE MEMBER WOODRUFF: -- your earlier point

about it didn't -- we didn't actually have to have too many of these, I guess, embryos to see some -- to at least identify the 50 percent response, right --

DR. PADILLA: Yeah, because --

COMMITTEE MEMBER WOODRUFF: -- is that what you're saying?

DR. PADILLA: Yeah, because it -- because usually nothing happens at the lower doses, then everything happens. You see malformations and then quickly you've moved on to death usually.

COMMITTEE MEMBER WOODRUFF: Right.

DR. PADILLA: There are some chemicals like the pyrethroids, which gets back to the solubility question, where you never quite reach lethality. It's just sort of probably because you can only get so much of the chemical in solution, and after that, nothing -- nothing -- you don't get any increase in solution.

And it was really interesting. We looked at some mixtures, some chemicals that tended to be mixtures, and you saw, in general, a very protracted dose response curve, that there were many -- there was much longer -- you know, many of those dose responses that I showed you were very quick. Within two or three concentrations, you've gone from normal to absolutely lethality, so you have to be able to catch it basically, if you want to do a

good calculation. I mean, it's a very -- in some ways, it's a very gross assay. You're going from control to death in most cases.

CHAIRPERSON LUDERER: Okay. Thank you.

COMMITTEE MEMBER WOODRUFF: I guess I -- can I just ask a question, but you did -- you did say that you can measure different developmental aspects, right, of the --

DR. PADILLA: Of the malform, yeah. And so what happens usually is you get a -- you get a dose or doses, we're nothing much is happening, and then the in-between doses you begin to see the malformations.

COMMITTEE MEMBER WOODRUFF: Right. Okay. Thank you.

DR. PADILLA: And then -- then the -- maybe a dose or two higher than that. I mean, these are half log. So a dose or two higher than that, you're beginning to see mostly lethality, yeah. And sometimes you see -- I mean, I -- sometimes you see, at the higher doses, the lethality occurring earlier, and earlier, and earlier. I mean, there could be also a time component of it too.

CHAIRPERSON LUDERER: Okay. It looks like we have a couple more hands raised. Thank you.

Dr. Auyeung-Kim.

I think you're muted still.

I think you're still -- we can't hear you. Yeah.

COMMITTEE MEMBER AUYEUNG-KIM: Sorry. I'm doing the -- the phone mute and computer mute -- unmute.

So I was just wondering with the -- you know, you were talking about like with the doses that you go -- you know, you see low levels. You don't see anything go higher and then you see lethality. How much of that has to do with like the different conditions that might be at each lab? You know, like you mentioned during your talk that there are some labs that, you know, did use different temperatures, some, you know, manipu -- or some, you know, keep the chorion and others don't, does -- how much of that can influence the toxicity -- the developmental toxicity that is observed?

DR. PADILLA: I don't know, but I would guess, because we have -- we have fiddled around with this a little bit by changing temperatures and also changing whether we dose once or whether we dose multiple times. In general, it does not affect whether you would call the chemical a hit or not. In general, it -- it doesn't -- that doesn't change too much, but the dose -- the effective dose, or the BMD, or the LOEL is what is usually affected when you change those. But it -- it may. I mean, I haven't tested all the chemicals in all the different protocols, and so it's hard to tell, yeah.

COMMITTEE MEMBER AUYEUNG-KIM: Understood.

DR. PADILLA: But I think -- I think if you came up -- if a chemical came up completely negative, it is likely it would be com -- there's -- there's not very much you could do to the protocol to make it a positive, but I don't know that. I mean, what situation are you worried about? Are you worried about the dose or are you worried about whether the chemical causes overt changes in development?

COMMITTEE MEMBER AUYEUNG-KIM: Well, I guess, it's over the dose and whether or not you'll see -- you know, because we make the decisions on, you know, whether the dose is going to -- or whether the chemical is going to be a reproductive toxicant.

DR. PADILLA: Yeah.

COMMITTEE MEMBER AUYEUNG-KIM: And so the dose kind of -- because if you dose too low, then you're not going to see it, then you may have the -- you know, make a different decision versus if you do see something. But then if we do see something, is it because it was, you know, a super high dose?

DR. PADILLA: Yeah. Yeah. I mean, we only go as high as 100 micromolar. That is -- that is our highest dose, and -- but you see papers that are using millimolar levels. And I mean, you have to figure out -- you have to

think about how real -- what -- how realistic that is basically.

COMMITTEE MEMBER AUYEUNG-KIM: Yep. Thank you.

CHAIRPERSON LUDERER: Thank you. Dr. Draper, did you have any other comments?

DR. DRAPER: (Shakes head).

CHAIRPERSON LUDERER: No. Okay. Dr. Pessah, you have your hand raised?

COMMITTEE MEMBER PESSAH: Yeah. So I was wondering if we could get some guidance when you are working at high EC 50s, where you're clearly seeing either behavioral or a morphometric change. But usually, those are at much higher levels than you would ever see in, let's say, serum or urine samples from humans that have been exposed.

Do we --

DR. PADILLA: So are we --

COMMITTEE MEMBER PESSAH: Do we --

DR. PADILLA: Are we talking about internal dose or are we talking about --

COMMITTEE MEMBER PESSAH: Do we use an uncertainty factor in interpreting the zebrafish data, much like we would do in a mouse study?

DR. PADILLA: I'm not a risk assessor, so I can't answer that, but -- I mean, are you talking about internal

dose or are you talking about nominal dose, what's in the water?

COMMITTEE MEMBER PESSAH: Well, I guess, we don't have that data for most of the studies, the internal dose. We have the external dose.

DR. PADILLA: Right, but pretty soon, there would be models that you ought to be able to at least guesstimate within an order of magnitude, I would guess, what the internal dose will be in the zebrafish. So that will help considerably, right?

COMMITTEE MEMBER PESSAH: Yeah, it would actually. Thank you.

CHAIRPERSON LUDERER: All right. Let's see.

Kind of turning to another question that we can continue our discussion thinking about. So studies including in OEHHA's recent hazard identification documents provide examples of similar biological systems or pathways being -- being affected in both zebrafish and mammals by a given chemical, but with different directionality of response or with a different downstream outcome. And so how do we consider differences as well as similarities between species and these kinds of evaluations? And either -- either one of you would like to start with that or anyone else have any kind of additional questions related to that?

DR. PADILLA: Well, that -- that appears to be more of a risk assessment question, but do they want it to be a biological question? I mean, I know from a risk assessment standpoint, even if you're working with mammals, it doesn't necessarily have to be the same -- exactly the same thing that's happening in rats or mice to -- to inform the risk assessment in humans, right?

CHAIRPERSON LUDERER: Yes. Although, I mean, I think -- I think, you know, there's more consistency among studies, that definitely tends to strengthen, you know, the evaluation of the association. If anyone else would like to jump in on that. But it would be -- I mean, if you do see -- I mean, I would think that for certain endpoints, you don't have an exact, you know -- analogous endpoint between a mammalian system and a zebrafish system. So that's not always going to be possible to look at exactly the same outcome either. I mean, that's one consideration that -- you know, and the question is really is there an effect? And it may not be the same exact out -- downstream outcome, but there -- but there are effects.

Dr. Woodruff.

COMMITTEE MEMBER WOODRUFF: Yeah. I'm wondering if -- I mean, just to say that from those DART reviews, it was a little hard to totally interpret, because I think,

as Stephanie was showing, sometimes the experimental conditions can influence the exact findings, so -- and I don't remember the papers from when we did those reviews, but I -- I do think -- I think what you're saying, Stephanie, is in -- there's a general concord -- or actually maybe you -- you and Bruce can comment on --

DR. PADILLA: Yeah.

COMMITTEE MEMBER WOODRUFF: -- general concordance of developmental effects compared to specific concordance. So, for example, in cancer --

DR. PADILLA: That's right.

COMMITTEE MEMBER WOODRUFF: -- you -- I don't know if actually zebrafish do get cancer and we aren't doing that in this committee --

DR. PADILLA: They do.

COMMITTEE MEMBER WOODRUFF: -- but it -- for example, when we look at animal and human concordance for cancer there's -- in general, you can see concordance, but the sites might be different.

DR. PADILLA: Um-hmm.

COMMITTEE MEMBER WOODRUFF: So maybe you could speak to that for developmental and reproductive endpoints.

DR. DRAPER: I can just comment on the reproductive endpoints. You know, one of the main

differences between mammals and fish is that fish are very easy to sex reverse. And so, you know, there's a lot of ways that we can affect, you know, the production of these signals that are required to maintain, for example, femaleness, and so -- at multiple levels. You know, a toxicant that affects the somatic gonad could cause sex reversal. A toxicant that affects germ cell development could cost sex reversal. So even though those toxicants wouldn't cause sex reversal in mammals because we don't sex reverse that easily, you know, that doesn't mean that it's not hitting the same pathway. It's just that fish are more labile and easier to get to flip. It --

COMMITTEE MEMBER WOODRUFF: Yeah, can I -- can I just -- so are you saying that sex reversal could be an indicator for a different type of sex-related effect in humans? Is that --

DR. DRAPER: Absolutely.

COMMITTEE MEMBER WOODRUFF: Got it.

DR. DRAPER: And once we --

COMMITTEE MEMBER WOODRUFF: So sometimes the mapping needs to be -- the outcomes may look different, but the mapping or the general issue is the same.

DR. DRAPER: That's correct. So once, you know, you see something that causes skewed sex ratios, then you can, you know, from that determine what the cell type is

or system that's being affected, and probably more often that not, it would be the same system in mammals. It's just the -- the endpoint is going to be different.

COMMITTEE MEMBER WOODRUFF: Right. Thank you.

CHAIRPERSON LUDERER: Patrick, I see your hand it raised.

COMMITTEE MEMBER ALLARD: Yeah. So I guess does that go back to the point that was made earlier that basically, zebrafish is a great sentinel species. If you see ovotestis going on, in a -- in the fish, then it might be even sign, indeed, that you would have probably a very strong hormonal imbalance going on in other species?

DR. DRAPER: Yes, I agree with that.

COMMITTEE MEMBER ALLARD: Thank you.

CHAIRPERSON LUDERER: All right. And I'm looking -- I don't see any other -- oh, Dr. Pessah, your hand is raised or -- or did you forget to put it down?

Okay. All right. Then not seeing any other raised hands, and I think we are just about at the time point that was allotted for our lunch break, if I'm not mistaken.

It's actually well past it apparently, so -- that's because we've been having such a wonderful discussion and such great presentations. So I'd like to thank both Dr. Padilla and Dr. Draper again.

And we will now -- do we want to take 45 minutes for lunch or are we -- let me see. I'm going to look in the chat and see if there's something about that.

Okay. It looks like -- no, we'll take our 45-minute lunch break as planned. And so -- let's see, I have that it's 12:30 right now. So we would come back at 1:15, unless some -- one of the staff members wants to change that.

Lauren, I see you cam on.

DIRECTOR ZEISE: I think that is fine, but I did just want to ask that we have Carol provide us our Bagley-Keene reminder.

CHAIRPERSON LUDERER: Yes. All right. Thank you.

DIRECTOR ZEISE: Great.

CHIEF COUNSEL ROWAN: Hi. Thank you. I'd just like to remind all of the members that during the breaks you aren't allowed to talk amongst yourselves about the subject matter of the meeting, so that includes once again phone calls, texts, and chat. And my recommendation would be that you also not talk to third parties regarding that same information. So if you do, then you'll need to disclose the fact that you had a discussion with someone and give the general content of the discussion, so that it's part of the public record.

So just generally, it's better not to chat -- it's just better to chat about something else over lunch. And that's it for now.

CHAIRPERSON LUDERER: All right. Thank you. So we'll see everyone at 1:15. Everybody have a good lunch.

Bye-bye.

(Off record: 12:30 p.m.)

(Thereupon a lunch break was taken.)

AFTERNOON SESSION

(On record: 1:15 p.m.)

**PART II: BEYOND SCREENING: ZEBRAFISH AS A MODEL FOR DEVELOPMENTAL MECHANISMS AT THE CELLULAR AND MOLECULAR LEVEL**

CHAIRPERSON LUDERER: All right. Welcome back, everyone. It's 1:15. So, let's go ahead and begin our afternoon session. So the part two session is entitled, "Beyond Screening: Zebrafish as a Model for Developmental Mechanisms at the Cellular and Molecular Levels".

And our afternoon speakers are Dr. Jennifer Panlilio, Woods Hole Center for Oceans and Human Health, and Dr. Don[SIC] Wagner from the University of California -- Dan Wagner, excuse me, from the University of California, San Francisco.

After their presentations, we'll have 30 minutes for Committee discussions, like we did this morning, and then we'll take a short break, and then move on to the last part of the session, a discussion with the Committee and all four of our invited speakers.

So I'd like to now introduce Dr. Jennifer Panlilio from Woods Hole. She is -- her research interests include circuit neuroscience and neurotoxicology. Recent publications based on her graduate work feature the use of zebrafish larvae to study

the effects the of an algae produced neurotoxin domoic acid on outcomes of neurobehavioral and neuronal development.

And her -- the title of her talk today will be use of a Zebrafish model to investigate how low doses of domoic acid affect the developing nervous system, including windows of susceptibility, structural and molecular changes in nervous system tissues, and links to behavioral alterations.

Dr. Panlilio, welcome and thank you for speaking today.

**PRESENTATION BY DR. JENNIFER PANLILIO**

(Thereupon a slide presentation).

DR. PANLILIO: Wonderful. Thank you so much for that introduction. So let me share my screen. Please let me know if you don't see the slides shortly.

CHAIRPERSON LUDERER: Yes, we see-- now, we see presenter view. Perfect.

DR. PANLILIO: Wonderful. Okay. So it's a pleasure to speak to you all about my research on essentially how we can use zebrafish to identify the developmental mechanisms of neurotoxicity. So for this talk I'll focus primarily uncovering -- uncovering the mechanisms of neurotoxicity that occur from exposure particularly to a harmful algal bloom toxin known as

domoic acid.

NEXT SLIDE

DR. PANLILIO: But firstly, what is harmful algal blooms and what's a harmful algal bloom toxin?

So it's probably familiar in the west coast, but harmful algal blooms are these mass accumulations of algae that are defined really by their adverse societal impacts rather by -- than by any strict scientific definition.

So there are plenty of ways that harmful algal blooms, or HABs, can cause harm. And so some of these HABs produce toxins that directly affect human health.

NEXT SLIDE

DR. PANLILIO: And so one particular harmful algal bloom toxin that I study is called domoic acid. So this toxin is produced by a diatom. So this is a type of phytoplankton that you see over here. And then shellfish and other seafood we eat can then accumulate domoic acid as they're filter feeding for these phytoplankton, which in turn we then can consume.

And so when domoic acid is consumed in high enough doses, particularly in adults, it can cause what's known as amnesic shellfish poisoning. So Symptoms from amnesic shellfish poisoning include gastro intestinal issues, memory loss, coma, and even death in the most severe cases.

NEXT SLIDE

DR. PANLILIO: And so really to prevent acute toxic in adults, we do have a regulatory limit set at 20 milligrams of domoic acid per kilogram shellfish tissue. But I do want to emphasize here that this limit is based on protecting adult humans from acute toxicity and those severe overt symptoms that I just previously mentioned.

However, we do know that humans are regular exposed to domoic acid below these limits, and that furthermore, humans can be exposed to these toxins during potentially more susceptible periods of early development.

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DR. PANLILIO: And so what do we know about domoic acid? So we know there's now ample evidence that it is a developmental neurotoxin. So we know, for develop, from rodents that both prenatal and postnatal exposures can lead to long-term behavioral defects, aberrant neural activity, and pronounced changes in brain tissue architecture. It's also been notably shown to heighten sensitivity to other toxicants that the rodents are exposed to later in life.

We also have used zebrafish in the past. And we show that we have -- previous work has shown that zebrafish exposed to high doses of domoic acid very early in development - here, it's the 1K cell stage - causes

behavioral phenotypes that are consistent with other animal models that are acutely exposed to high doses of domoic acid.

However, no zebrafish studies have been done up until, you know, the -- we published ours. More lower level doses, that may be potentially more relevant for human exposures. And further, there was no previous studies that looked essentially into those more potential windows of susceptibility for exposures.

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DR. PANLILIO: So knowing this, our studies really wanted to address these knowledge gaps. And so there were these three primary objectives. And so the first is to identify specific windows or developmental windows of susceptibility to domoic acid exposure, to identify the functional consequences for behavior, and then to determine whether there are specific cells types that are targeted by exposures to domoic acid.

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DR. PANLILIO: And so to accomplish this, and this is what this meeting is about, right? We're -- we utilize the strengths of the zebrafish model. So one thing that's really nice is zebrafish have these really simple and quantifiable behaviors. And so we've chosen one behavior in particular where the neural circuits that

underlie these specific behaviors are very well characterized. And so what that allows us to do is it allows us to link that be -- potential behavioral deficit to the underlying neural circuits that drive it.

And finally, we've also heard this, but they have transparent larvae that develop externally. So what this really allows us you to do is it allows us to directly image cellular processes that may be perturbed over the course of early development, and we can do this in real-time as the animals are living.

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DR. PANLILIO: So to identify the critical windows of susceptibility, we exposed fish in discrete periods in early development through a different method of exposure than what you've heard about, but we've used intravenous microinjection. And then we identified -- to identify the mechanisms of toxicity, we then took this multi-level approach where we measured the behavior as the readout of toxicity and then assessed the potential structural changes in the neural circuit that underlies this behavior. And then finally, we linked these to specific cellular targets and molecular effects from exposures.

NEXT SLIDE

DR. PANLILIO: So in particular, I exposed

zebrafish to domoic acid, again during these discrete periods in early development. And the way we could do this is through intravenous microinjections. So the microinjections were done into their caudal vein. So essentially, we took the little needle and we could find the caudal vein and inject them over time. And we did this at one, two, and four days post-fertilization, or dpf. And so we decided to do this over the more common route of exposing them, which is through chemicals in the water, you know, that emersion type exposure that Dr. Padilla spoke about, because we wanted to ensure domoic acid was entering in the fish, and we also -- and it also really allowed us to mimic that single acute exposure during these very discrete periods of early development.

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DR. PANLILIO: And so just to back up a little bit, why did we choose these three periods? I targeted these three key early stages, because they mark three neurodevelopmental stages that are quite important for the fish. So, for example, exposures at one day post-fertilization is when neurons and glia cell -- precursor cells are spec -- are first specified. It's when a majority of those early sensory and motor neurons differentiate.

And then at 2 dpf, this is the time where that

would target a specific glial cell called the oligodendrocyte, which I'll take about quite a bit. This is when it starts to migrate and differentiate and starts to wrap axons across the central nervous system.

And then finally, exposures at four days post-fertilization correspond to those later developmental periods where most of the cell types in that early nervous system are already specified.

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DR. PANLILIO: So following these exposures, I then did a detailed assessment of the startle response behavior in the larval stages between 5 to 7 dpf. And during the same larval period, I also imaged the different cells and structure that make up the circuit that drive this response. And so I mentioned startle, so that's the behavior we're looking at. So what is it and why -- why are we using it?

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DR. PANLILIO: And so assess the functional effects of domoic acid, so we're using the startle response behavior. And so the larval startle response behavior occurs in response to a sudden and intense stimulus. It's quantifiable and it really requires proper sensory processing and motor control, so it serves as a tool to determine whether these processes are disrupted by

exposure to domoic acid.

Finally, it's driven by underlying neural circuits that are pretty well known, so it makes it a really powerful behavior to study, because again it can allow us to link these behavioral results to the anatomy and the cell types that are involved in the circuit.

NEXT SLIDE.

DR. PANLILIO: And so to assess startle behavior, essentially these fish are placed in the 16 well plates that sit above a speaker and their startle responses are recorded using a high speed camera above.

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DR. PANLILIO: Okay. Okay. So here's just a zoom-in on a single well that you see over here. And the larvae is essentially divided into three segments, as encoded by these three different colors. So the changes in the curvature, as the fish undergo startle, are estimated by the changes in angle between these three segments. So what I'll do is I'll play a video, so you'll see -- you'll see the video over here. And what you'll see to the right of the video are the estimated changes in curvature as the fish undergoes a startle response.

So note that there's this initial really large bend angle. This is that C bend over here followed by a corrective angle, then the fish start swimming away, and

then eventually stop. So I'll play that just one more time, so you can see that. So that first really large bend angle and the corrective bend.

And so what's nice about this is you can -- from these videos, we can get some really interesting kinematic attributes. So, for example, we can get latency right, which is the reaction time when the -- from when the stimulus is produced to when the fish reacts. We can get, for example, that maximal bend angle from the first bend. We could get like the angular velocity, et cetera, et cetera.

NEXT SLIDE

DR. PANLILIO: So we used this behavior, right? And so we expose zebrafish at different developmental time points, and then we assess their startle behavior kinematics. And so what we found is that fish exposed to domoic acid, particularly at 2 dpf from the lowest dose all the way to the highest dose had reduce bend angles relative to controls.

In comparison, the fish exposed to domoic acid earlier at 1 dpf or later at four only had reduced bend angles at the higher doses, and the bend angle reduction was a lot less severe compared to those exposed at 2 dpf.

NEXT SLIDE

DR. PANLILIO: This is also true for other

kinematic attributes you would look at such as the maximum angular velocity that led to that bend as well.

NEXT SLIDE

DR. PANLILIO: So we know that there's this behavioral deficit that results from domoic acid exposure and that it is most true from when we're exposing fish, particularly at this 2 dpf time point. Again, just to emphasize, I love this behavior, because we know a lot about the underlying cells and their connections that drive it. And so knowing that there's deficits in startle, we then sought to look at the anatomy of the circuit.

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DR. PANLILIO: And one thing we started looking at first for myelin sheaths. And startle responses are extremely fast. This is why we really need those high speed video cameras to actually record them. So they happen been between 50 and 50 milliseconds after a stimulus is given, which ex -- which also requires extremely fast conduction velocities through the axons as they -- essentially the signal propagates down the axons and enervates the muscles across the trunk.

And so to allow for this rapid propagation of a signal axons within the startle circuit have heavily myelinated. And in fact, this -- this neuron that I'll

talk about a little later is the most heavily myelinated neuron in the nervous system of the fish.

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DR. PANLILIO: And so we assess myelination in this -- the spinal cord, again Because proper myelination is required for -- for proper startle. And so to accomplish this, we again employ the power of the zebrafish model, right? So we have this transgenic fish that have myelin sheaths that are labeled by GFP. And because zebrafish are transparent when they're younger, we can again image these structures in living animals, right?

And so here's just a fish on its side. Here's just a cartoon of a cross-section of its spinal cord. And in control fish what you see is that there's two regions here. This is the ventral region of the spinal cord and this is the dorsal. And what you'll see is it contains myelin that is both abundant and elongated.

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DR. PANLILIO: Now again, so that's our control fish, right? But -- and using these transgenic fish, we can score myelin phenotypes visually from those again that look like this -- this beautiful control you see the SEC up here to those that essentially have these disorganized sheets, where essentially the myelin and the axon doesn't follow the track perfectly, all the way to the most severe

form, where its -- there are very, very sparse sheaths and they contain all these like very unusual circular features, which I'll get back to you later about what we think those are.

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DR. PANLILIO: Okay. So what we did is we again exposed fish to domoic acid. And what I'm showing you right here is an exposure to an intermediate dose. And then we did this over different developmental time periods. And what we see is that the highest proportion of fish with myelin defects occur when domoic acid is exposed at 2 dpf and 2.5 dpf. And those exposed at 2 pdf have a larger proportion of the more severe myelin defects, so these -- these levels right here.

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DR. PANLILIO: So we also tested a range of doses and during a range of different developmental times, and we found that even down to 0. -- 0.09 nanograms, which is the lowest dose we tested, we still see an effect at 2 dpf, while not seeing this for fish exposed at the two other time periods you see up here.

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DR. PANLILIO: So note though that there are gaps in the developmental times we were -- and for example, there are places we were unable to test like this one. So

for example, within our highest dose of 0.18 nanograms, we did not test fish exposed at 4 dpf, because they did have other phenotypes that made them untestable. And so I do want to bring up that point, and I think it's also important, because one such phenotype -- severe phenotype these animals have would -- is essentially widespread brain necrosis, where you see essentially their head because they have -- they're transparent is -- it looks like a cloudy brain, if you will.

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DR. PANLILIO: And so in this case, right, over 40 percent of the embryos injected specifically at these later periods, so at 4 dpf, show this very severe phenotype. So it seems that at higher doses, those overt severe phenotypes can occur at the later stages of exposure.

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DR. PANLILIO: So both the behavioral data and myelin sheath data both now point to 2 dpf being an important period for domoic acid exposure, especially exposures at the lower ends of the dose -- the dose range. And so we wanted to continue to investigate whether exposure at 2 dpf disrupts other important players in the neural circuit. So to do that, I'll step you through the different players that make up the startle circuit that we

know pretty well.

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DR. PANLILIO: So in addition to myelin sheaths that are required for that proper, that rapid execution of that startle response, there are other things that are involved, right? So auditory and vibrational stimuli are first detected by the hair cells that occur both within the inner ear and within the neuromasts that make up the lateral line.

And so the mechanical deflection of these hair cells then lead to the activation of sensory neurons and then these sensory neurons can then send information to the hindbrain. And so in the hindbrain, cells like the Mauthner cell along with other homologs integrate all the sensory information. And once this -- the Mauthner cell reaches threshold, it then fires a single action potential, which propagates down its -- its axon that extends into the spinal cord.

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DR. PANLILIO: And then what it -- what this results in is that as the action potential rapidly travels down the spinal cord, it activates primary motor neurons along the way. And because the signal propagates so quickly, this leads to these really fast unilateral muscle contractions and these deep bend angles that you saw on

the video previously.

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DR. PANLILIO: So -- Okay. So let's step through the circuit. Let's look first at the sensory side. So let's look at the neuromasts and the inner ear hair cells as -- in addition to the other sensory structures.

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DR. PANLILIO: Okay. So to assess neuromasts, we used a live cell staining dye called GASPI. And so essentially, this is one of those cases where you can put a zebrafish in a dish and expose them in the water to this live staining dye. They will take it up. And what we found is that domoic acid exposed fish have the same number of neuromasts, both in their heads, so we looked at the number of neuromasts in the head, as well as their trunk region over here. So it doesn't seem at least in terms of absolute neuromast count that domoic acid alters that.

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DR. PANLILIO: We also looked at various sensory neuron -- neurons, their axonal tracts, and found no differences. For example, here's a -- an image of a zebrafish. This -- these are fixed fish, so these are now antibody staining at least on the top. And what you'll see here is that we see no differences, the inner ear is

outlined in this like teal dashed outline. We saw no differences in the inner ear hair cells. We also, using a live -- live imaging of another transgenic also found no noticeable differences in both the lateral line -- the neuromasts -- again over here. These are the neuromasts -- as well as that lateral line, which is the ganglia, or that nerve, the -- the nerve connecting, all these neuromasts over here.

So -- so I like to point this out that at least at this stage and at this resolution, it doesn't seem like domoic acid is targeting the sensory system directly.

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DR. PANLILIO: So again, so -- okay. So that's the sensory system. And so after sensory information is collected, right, by the inner ear hair cells and the lateral line, it then sends it to the Mauthner cells and its homologs. So we then assessed for the presence of this specific Mauthner cell, which is teal one in this cartoon over here.

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DR. PANLILIO: And so here show -- we use antibody shading -- staining to show that while all the controls we imaged had these two Mauthner cell pairs -- so here is one pair going this way, the axon crosses the midline, and goes down contralaterally. So it has these

two beautiful Mauthner cells here. Most domoic acid exposed fish have no Mauthner cells. So here -- here is an image where you just don't see the Mauthner cells here, but there were a few with two, but majority of them did not have Mauthner cells.

The other thing we can look at in the same tissue, right, is we can count the hindbrain tracts, so that would be these lines that you see over here. And when looking at these tracts, we found there's no significant difference between the domoic acid exposed fish and the control. So it -- again, emphasizing that it isn't all neurons that are altered by domoic acid, but potentially very specific targets.

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DR. PANLILIO: Okay. So these findings indicate that exposure to domoic acid led to these measurable startle deficits, particularly exposures at 2 dpf. And In conjunction with these, we also see that there's a loss of this Mauthner cell, that's really important for startle, as well as myelin defects during the larval period at 5 dpf.

So note that all of these endpoints were taken during the larval stages, which is after nascent myelination has occurred, which occurs between 3 -- it starts at like 2.5 and ends mostly at 5 dpf. So it's

after this -- this nascent myelination has occurred and also it's well after exposures at 2 dpf, right?

And so we then wanted to look more at the initial cell targets and the initiating events that perhaps may contribute to all those later larval phenotypes we were seeing.

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DR. PANLILIO: So recall, right? So in the larval stages, we both saw the loss of that Mauthner cell along with its axons in the spinal cord as well as myelin defects. So we no, of course, that myelin cannot. So here is like my little cartoon, right, of an oligodendrocyte, which wraps myelin around axons. So we know, of course, the myelin cannot wrap around axons that are just not present. So if an axon, for example, is lost first, we expect there to just be not as much myelin around, right, and there might be myelin defects.

But we also know that myelin provides really important metabolic support or axons. And so the loss of the myelin could then also lead to axonal defects. So the question then becomes what happens first, right? Do we lose myelin first and then the axons follow or vice versa?

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DR. PANLILIO: And so to -- to address this question, we first wanted to see how domoic acid exposure

affects those initial stages of myelination. And again, I'll emphasize here like that I think the power of this model is we cannot only easily image fish because they're transparent and they're small, but we can also visualize cellular processes that are occurring in real-time. So that's what we did here.

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DR. PANLILIO: We're essentially perform time-lapse imaging with transgenic fish, so these are live fish, where we looked for and we observed myelination actually happening within their spinal cords. And so what you're looking at here is the cartoon on the left is essentially this is a transgenic fish, where are cell bodies of the oligodendrocytes, those are the cells that make myelin, are labeled in red. And their -- and their membranes that become myelin are labeled in green.

And so what you'll see here, this is a time-lapse video that occurs over the course of around 12 hours. And so on the top, you'll see the controls are forming these beautiful myelin sheaths, so there's those little thick processes -- elongated process here and here for example.

In comparison, domoic acid exposed fish primarily do not. So what you'll see here is -- so here they are, right? These oligodendrocytes have these red cell bodies. And instead of traveling around and starting to myelinate,

they tend to form these shorter sheaths, right, so you don't really see those elongated -- elongated sheaths that you see here. And they also form these really strange -- I'll play that one more time.

They -- they tend to form these really strange circular features, which again I am pointing out, because this becomes relevant later to what we think these phenotypes are.

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DR. PANLILIO: All right. So these -- now we know this -- the initial stages of myelination is perturbed. And so now we turn more specifically to looking at again those oligodendrocytes, which is that lineage of cells that make myelin. So we looked at how domoic acid perturbed oligodendrocyte development, a little bit after the time myelination commences.

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DR. PANLILIO: So we used another fish line, so we have a lot of fish lines through this study. And this fish line essentially labels oligodendrocytes that you see here. And so what we did is we quantified the number of myelinating oligodendrocytes in the spinal cord during the period of myelination.

So as I said, so each of these individual points is an individual oligodendrocyte that we can quantify.

And then on the right, you'll see each point in this graph represents the number of oligodendrocyte counted from an individual fish that was exposed either to no on domoic acid or that was the control all the way to the two doses of domoic acid you see here.

And what you'll see is that there is this significant reduction in the number of oligodendrocytes per -- which is true for, you know, our -- our medium range dose, but it's particularly true for our highest dose of domoic acid.

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DR. PANLILIO: Okay. So now, I'm building this model, right? So we know that domoic acid disrupts that initial myelination and leads to the loss of oligodendrocytes like 4 dpf. And the loss of oligodendrocytes could conceivably contribute to the myelin defects we're seeing, because if there's a reduced supply, if there's less oligodendrocytes that can myelinate, this may just lead to the less myelin overall being formed, right?

The loss of the oligodendrocytes could be because domoic acid is directly binding to and affecting these oligodendrocytes, because they do have the receptors to which domoic acid binds to.

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DR. PANLILIO: Okay. So now we've looked at the oligodendrocytes, right? But we also know that they've lost -- they have this Mauthner cell loss. So let's revisit that. So we know that this occurs at the larval stages, but we wanted to determine whether these Mauthner cells were absent shortly after exposure, but before myelination even commences, right? So all we had to do really was image domoic acid exposed fish and look for Mauthner cells much earlier.

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DR. PANLILIO: So using the same antibody, it's a neurofilament antibody, we're aim -- we're able to image the Mauthner cell prior to myelination. So here's just a control brain to orient you and here are -- in these teal arrows are those two beautiful large Mauthner cells.

And so while control fish always have two Mauthner cells, domoic acid exposed fish have a range of phenotypes. But again, majority of domoic acid exposed fish don't have those two Mauthner cells even prior to when myelination commences.

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DR. PANLILIO: So now know again, that the Mauthner cell loss occurs even earlier than myelination. Thus, the domoic acid exposed fish may be targeting -- domoic acid, excuse me -- may be targeting these neurons

first, and then altering the cellular environment in the spinal cord and in the brain prior to myelination, which may, you know, have downstream effects for the oligodendrocytes and myelin later.

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DR. PANLILIO: So speaking of the cell environment, let's look at myelination in the spinal cord. So in a control animal, there's really this correct balance between the number of oligodendrocytes, as you see here. So this again is the cell that is responsible for myelinating in the brain and spinal cord. And -- and so there is a balance between oligodendrocytes and the amount of axons they have to myelinate.

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DR. PANLILIO: But what happens when these axon targets are reduced? What if there's just less axons around? So the Lyons lab addressed this questions by essentially using a genetic model that reduced axons within the spinal cord. And they found that the reduction of axonal surface area also led to the reduction in total number of oligodendrocytes. And they propose it's potentially because there's a feedback process, which attempts to now correct the mismatch between the oligodendrocytes to the axons.

What they also found is they found the appearance

of these circular profiles remember that I -- I've been point that out and saying what are those. It's very strange. And what they -- they found that to be this -- was this abnormal phenotype where they thought that it's due, and they actually show data, to show that essentially instead of wrapping just axons, because from there were not enough axons, these oligodendrocytes were starting to wrap neuronal cell bodies instead. So not just he axons, but the cell -- cell interface itself.

And so they attributed again these effects to the mismatch within the axonal surface area to the number of oligodendrocytes present, because as there is less axons in the environment to myelinate, oligodendrocytes start to myelinate other things, right, including again those neuronal cell bodies.

So it is possible, right, that such a thing is occurring the domoic acid exposed fish to and the loss of this Mauthner cell, as well as other like large axons within the spinal cord, could contribute to the effects we're seeing with the oligodendrocytes and myelin.

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DR. PANLILIO: So we wanted to see whether our circular features were very similar to theirs. Are these also incorrectly rats' neuronal cell bodies.

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DR. PANLILIO: And so to identify what these circular features are, we used another transgenic fish. So here in magenta, this labels myelin, which is the oligodendrocyte membranes and the green labels the membranes of neurons. And so in -- and what you'll see here, right, this is a domoic acid exposed fish. There are these again these really unusual circular profiles that are produced by oligodendrocytes primarily only in domoic acid exposed fish. And in green, in this picture over here, you're seeing these honeycomb-like structures that represent those densely packed neuronal cell bodies within the spinal cord.

And in the merged image on the right most, you'll see that these circular oligodendrocyte membranes appear to be in the same location as the neuronal cell bodies. So suggesting that the oligodendrocyte membrane is wrapping neuronal cell bodies. And so this is just a schematic of just that, right? So there are oligodendrocytes that wrap, you know, axons as they should, but in -- in addition to that, they're now wrapping these cell bodies, because there's just not enough axons around presumably.

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DR. PANLILIO: So we then did electron microscopy, which will give us a little bit better

resolution. And so to orient you, here's just a cross-section of a fish over here. And here -- so here's it's whole trunk and here -- over here is it's spinal cord, and if we zoom in on that, these circular -- you know, all these circles over these densely packed circles are under -- are -- are neuronal cell bodies. And in control fish, right, most of these are just wrapped by a double membrane layer, which is what you would expect.

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DR. PANLILIO: And so that's true for a lot of domoic acid exposed fish. But in addition to that, we also have cells -- neuronal cells, where there's more than just that -- that double membrane, but there's a second outline around them like here and here. So we think that these are also myelin inappropriately wrapping these cell bodies.

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DR. PANLILIO: And so with all this, we have a working model for how developmental exposures to domoic acid mediates toxicity. So the loss of the Mauthner cell precedes disruptions in myelin. And then the loss of the Mauthner cell contributes to this reduced axonal surface area in the spinal cord. And the loss of oligodendrocytes may result from that reduced axonal surface area as that feedback process attempts to correct the discrepancies

between the axon surface area and the oligodendrocyte present.

Myelin defects may also result in a reduced axonal surface area as oligodendrocytes start to myelinate neuronal cell bodies in the absence of just enough axons. So both these phenotypes have been found in the genetic model that lacks axons. So it's possible that all of these oligodendrocyte effects are secondary effects to the loss -- the initial loss of those -- the axons

I previously proposed that domoic acid may bind directly to the oligodendrocytes as well. And it is still possible that the -- you know, these cells do have receptors to respond domoic acid, so it might be that these processes occur in concert.

So with all of these elements can contribute to that observed aberrant startle behavior we see. And as you see this model has a lot of question marks, so the receptors to which domoic acid binds to are expressed by multiple cell types in the nervous system. So again conceivably, there could be multiple targets happening at the same time.

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DR. PANLILIO: So stepping back from all these very specific details, I want to discuss a little bit about what this means -- that this could mean for human

health. So this -- this current research identifies a potential mechanism of domoic acid toxicity that occurs during a susceptible window of exposure and early development. So I found pronounced changes in spinal cord structure following domoic acid treatment. And so this highlights how domoic acid targets not just the brain, which is where a lot of people look, but also potentially the spinal cord.

I also found that there's specific cells that were lost during domoic acid exposures. So humans don't have Mauthner cells, but they do have a group of functionally equivalent cells. And so even if this research does not identify specific neurons that may be targeted in humans, I think it can provide us with clues as to which -- what characteristics some cell types may have that may make them more susceptible to domoic acid toxicity. So perhaps neurons with really large axons that are excitable, that sort of span the range and have -- into the spinal cord may be more suscept -- sensitive to domoic acid exposure.

So I also found that the loss of axons right before the big wave of myelination leads to really aberrant myelin phenotypes. And we could, of course, speculate whether domoic acid exposures in humans prior to myelination could also have important consequences. But

what's interesting, right, is myelination is -- if myelination is truly a target of human exposure, it could have really important health consequences and exposure considerations, because unlike fish, myelination in humans spans from late prenatal development all the way to early childhood or even later, and thus leading to potentially a prolonged window of susceptibility to domoic acid exposure.

So, of course, this would all have to be tested, but it's just a -- it's just a framework for us to understand about exposures and how we think about it in fish and we relate it to humans.

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DR. PANLILIO: And so just my last slide to step back from the domoic acid story to just remind us that this could, in many ways, be a model for how we assess really any neurotoxicant, right? We can leverage the zebrafish model to really do these more multi-level approaches, where we start with a behavior, we step through the circuit, and we look at the cellular and molecular endpoints that underlie it. And so with that, thank you for letting me share this story, and I'm more than happy to take questions from everyone.

CHAIRPERSON LUDERER: Well, thank you, Dr. Panlilio for that wonderful presentation. We have just a

few minutes now for clarifying questions from the Committee. And then remember, we'll have more time for discussion later. So I see some raised hands already.

Dr. Pessah.

COMMITTEE MEMBER PESSAH: That's a wonderful presentation. I really like the sort of methodological approach. You mentioned excitability. And did you actually measure the balance of excitation inhibition was that different at two -- two days post-fertilization? Is it driving the axonal loss, I guess, is -- a hyperexcitation?

DR. PANLILIO: Right. And you know, I -- I sort of glossed over the -- what we know about domoic acid. So domoic acid binds to ionotropic glutamate receptors, so it is an excitotoxin. I did not measure that directly, but I would be very surprised if there were not changes in that for sure. And in terms of like why I speculate the Mauthner cell particularly at the two day post-fertilization time point is susceptible is there are papers that show that it actually will switch subunits right before 2 dpf for two subunits that are more excitable and that respond more, and it's a very specific subunit of a ionotropic glutamate receptor. So short answer is no, I didn't measure it directly, but I would be very surprised if it were not changed.

COMMITTEE MEMBER PESSAH: Thank you.

DR. PANLILIO: Yep.

CHAIRPERSON LUDERER: All right. Dr. Hertz-Picciotto, I see you have your hand raised too.

You need to unmute.

COMMITTEE MEMBER HERTZ-PICCIOTTO: So sorry.

CHAIRPERSON LUDERER: Now, we can hear you.

COMMITTEE MEMBER HERTZ-PICCIOTTO: Yeah, my video is also a little lag there.

Yeah, I love this talk. Your -- you really explain very well your step-by-step approach. And I'm just -- you know, I was real -- I'm really struck about the myelination, because, you know, at least two years postnatally it's -- it's really immature, the -- not complete. And what I don't know, and I don't know if you do, one -- so one question I have is the brain versus the spinal cord in humans, is the myelination happening more or less in both at the same. Is that extended period also for the spinal cord? I think the two-year that I had heard as -- or that you constantly see in the literature is based on brain specifically.

And then the second question I had also was I'm interested in -- you know, you are talking about -- you are interested in those low -- lower doses that human beings get. And I'm wondering about the comparability of

your exposure to this -- the zebrafish and how that might translate in terms of doses that humans are getting generally through seafood consumption, I presume.

DR. PANLILIO: Right. Wonderful question. So I will say I -- you were right for the human stuff all the -- my understanding of the ontogeny from myelination occurs from the brain. I do believe there is a paper that goes through different brain regions. I'm wondering if spinal cord is in there as well. So the short answer is I'm not sure for humans.

However, for fish, nascent myelination occurs across both the brain and the spinal cord roughly during that embryonic period between 2 to 5 dpf. So it's a pretty like relatively short window, but I guess like if you like stretch zebrafish out, it wouldn't be that short. But yeah, so great question about whether that's true for humans and I'm not 100 percent sure.

For the dose comparability, so I've thought about this a lot, and so the short answer is there's no direct comparison, partially because there's currently no information that I know of of domoic acid concentrations in both human fetal tissues or even maternal. Like we just don't know what domoic acid is in maternal, like human blood, you know, the concentrations in serum.

And so what we do know, for example -- so all we

really know is unfortunately, the reason it was brought up for the fourth round is that we know about an acute -- doses that can cause acute exposure in humans, because of an incidental exposure that occurred in 1987, but that was acute and that was for adults.

Our doses are comparable to if we assume that the embryos are 1.5 milligrams, which they can't change, right? Our doses are comparable to doses used when they injected rodents subcutaneously, postnatally to look at domoic acid.

But they are -- but they are higher than I would assume. You know, we talk about route of exposure, there very -- they're much likely higher than what potentially a human would see just because we're injecting it directly into the system, right? Like depending on how -- you know, if you were to, for example, eat domoic acid and it's an oral exposure, we find that oral bioability -- availability is pretty low. So if you were a young child, for example, you know, you can think, for example -- but they've also found, for example, that the concentration in fetal tissue is less than concentration in maternal plasma in rats. So like all of this is to say, we're using zebrafish as like a mechanistic model realizing that there are -- you know, there are some limitations in not being able to translate that dose directly.

COMMITTEE MEMBER HERTZ-PICCIOTTO: Thank you.

DR. PANLILIO: You're welcome.

CHAIRPERSON LUDERER: Dr. Woodruff, I see you have a question. Just time for a quick question. We'll have more discussion later.

COMMITTEE MEMBER WOODRUFF: Okay. I can wait.

CHAIRPERSON LUDERER: Okay. Great. Thank you.

So maybe keep -- keep that question in mind till the discussion. So now, I'd like to turn to our second speaker, Dan -- Dr. Dan Wagner, who is Assistant Professor of Obstetrics and Gynecology and Reproductive Sciences in the School of Medicine at the University of California, San Francisco. His research applies the techniques of cell lineage tracing and single cell transcriptomics to zebrafish embryos as a model for understanding the cellular changes that occur as development progresses.

And the title of his talk today is high throughput single cell genomics used -- as used to identify genes involved in regulating cellular differentiation during zebrafish development.

And now, I'll turn the floor over to Dr. Wagner.

Thank you.

**PRESENTATION BY DR. DAN WAGNER**

(Thereupon a slide presentation.)

DR. WAGNER: Okay. Thank you so much for the

introduction. Can everybody hear me and see my slides as well?

CHAIRPERSON LUDERER: Yes. Yes, to both.

DR. WAGNER: Okay. So very happy to be here today. Learning a lot. So I'm a developmental biologist by training. So I'm from UCSF. I started my lab about three years ago. And we study the zebrafish as a means to understand basic molecular mechanisms of sulfate specification in vertebrate embryos.

And here on this title slide, I'm showing you two images of a zebrafish. So left is obviously a micrograph showing individual cells labeled by an H2B localized fluorescent protein transgene. The right side shows a data modality that we use extensively in my lab and that's the single cell profiling. So I think Bruce Draper introduced single cell profiling very nicely in his talk.

Well, generally what we're doing in these kinds of measurements is we're collecting individual cells, measuring all the genes that are on or off in those cells, and then representing a very high dimensional data set in -- in -- in a two-dimensional plane. So in these -- in these graphs, each dot is a cell and the proximity of the cells on the graph denotes gene expression similarity.

And so I'll tell you a little bit about how we're trying to use this mode of data analysis to understand

embryonic developmental in a more quantitative manner than had been possible previously.

NEXT SLIDE

DR. WAGNER: The main motivations for the research in our group are to understand developmental defects, fate specification, but also how embryos respond to challenges and what sort of processes we might be able to dial up or down to assist embryos in -- in overcoming such challenges. So zebrafish, just like humans, face numerous challenges that can stand in the way of normal development. These challenges span from both genetic causes to environmental causes. And we've -- we and others have observed that embryos can have remarkable plasticity, so they can often recover from some -- some pretty catastrophic insults at the genetic or environmental levels. And so we'd like to understand the mechanisms that help modulate the responses to those challenges.

NEXT SLIDE

DR. WAGNER: As I alluded to on the first slide, so we use a lot of single cell genomics in our lab to interrogate developmental processes. And the reason we use single cell result assays are because really the enormous complexity of developmental systems. So every individual cell in a developing embryo has differences.

Those differences can be genetic. They can be epigenetic. They can be gene expression, stress, position in the embryo. So there's just a high dimensional vector of differences at the individual cell level.

And previously for gene expression profiling, we've been limited to -- to sort of bulk assays, where we would take many, many cells, analyze the DNA and RNA in those cells, collectively to arrive at a population average. Now, the downside of such assays like this are that we often arrive at non-physiological understanding of the processes because we're averaging between cells and losing important differences.

So single cell profiling by contrast preserves the individual states of each cell when we make the measurements. And this allows us to gain different levels of insights into the processes happening inside the embryo.

NEXT SLIDE

DR. WAGNER: So the motivation, as I said before, we want to understand and manipulate mechanisms of cell fate feedback control in embryonic development. Our vision is to combine in vivo studies with tools from single cell genomics. So we use zebrafish in on our lab, of course.

NEXT SLIDE

DR. WAGNER: And I think Bruce and the other speakers today gave fantastic introductions to the system, so I won't belabor this too much. I think Stephanie actually showed this exact same video, which was collected by a former lab mate of mine in our post-doc. But as we've -- as we've seen, zebrafish are transparent. They develop outside the mother. This makes them extremely accessible experimentally for perturbation as well as for analysis. So I'll just leave it at that, because we've -- we've -- we've well established why zebrafish can be powerful system.

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DR. WAGNER: So the methods that we use in my lab to generate hypotheses regarding developmental plasticity and fate specification rely on these technologies for single cell profiling. And these technologies are still fairly recent, probably in the last five years is when they took off. They generally use microfluidics -- microfluidic droplets or channels to encapsulate individual cells and make measurements at the single cell level. And then we have an accompanying set of computational tools that we use to analyze these very large data sets and produce graphs or landscapes that give us the ability to understand patterns in these data sets.

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DR. WAGNER: So I'll tell you a little bit about the technology we use. It's a called inDrops. This is a -- one of the classic original methods used to encapsulate individual cells into microfluidic droplets. And this allows us to perform barcoded CDNA reverse transcription reactions. Basically, we can create a library of DNA molecules that represent the expression states of each individual cell. And each cell gets a unique bar code that allows us to decipher those patterns when we sequence our data. And so we use this method in my lab extensively through a microfluidic setup and we can use this to capture thousands of cells from any stage of development that we choose under conditions of perturbation or from wild-type embryos. We really have a lot of ability to measure cells in different states and we use the zebrafish system.

NEXT SLIDE

DR. WAGNER: So the general sort of computational pipeline that we use for most of our data analysis is schematized here. So as I said, we start with staged embryos. We can grow the embryos to any stage of choice. If we were specifically focused on spinal cord or brain, we can grow them to a particular stage, collect either the whole embryo or specific cells from the organ of interest. We capture and using inDrops we record these high

dimensional measurements for every single cell in that tissue or in the whole organism.

Once we've done our sequencing of these data sets, we've now returned a data set that's very large. It's usually in the form of a large matrix of cells by measurements. And then this matrix can be subject to any number of machine learning data science algorithms. So the way we typically sort of portray these data sets is using a graph, like the one I showed on my first slide or the one Bruce showed, the beautiful depiction of the germ line in zebrafish. Where we have little dots, those dots are individual cells. And those cells can be thought of as points in a high dimensional gene expression space. So this -- this graph here I'm showing has three dimensions, but in reality we have tens of thousands of dimensions. And we generally use the machine learning algorithms to simplify this high-dimensional data set into something that we can -- we can see with our -- with our eyes.

NEXT SLIDE

DR. WAGNER: So a few years ago, we collected a large data set that described the -- the normal development of the zebrafish embryo. So this was published in 2018. And in this data set, we analyzed a few different strains of embryos for the first 24 hours. So this -- this takes us from cleavage, to gastrulation,

to epiboly, and the beginning of organogenesis in zebrafish. And so as you can see, we -- we assembled the data into one of those graphs. In this particular graph I'm showing, each cell is labeled by it's time point of origin, and I hope you can appreciate that at the early time points, the picture is relatively simple, all the cells of before hpf, that's four hours after fertilization. The cells are generally not that different from each other. They perform this sort of -- this ball at the -- at the middle of this graph in dark blue.

But as we move forward in time, the cells become more different from each other. They become more molecularly diverse. And the algorithms that we use to assemble this graph actually were able to recapture these trajectories, so these branches that extend forward in time. And these branches actually correspond to different lineages of the embryo, as they're developing from -- from this -- this sort of early pluripotent state.

NEXT SLIDE

DR. WAGNER: And so this -- this type of depiction, this depiction of developmental biology, this sort of landscape view is really the basis of -- of all the work in my laboratory. And so I'll give you some very, very high level quick vignettes about how we use this landscape representation for different kinds of

questions in zebrafish development. So I don't -- I don't have time to go through full stories for each of these, but I'm just giving a flavor of the kind of quantitation that we can now bring to zebrafish and to our understanding of developmental biology using data sets like this.

So I'll tell you just a little bit more first off about how we map all the cell states of this -- of this landscape and what we learned just from the shape of -- of that landscape, the shape of the manifold that came out.

And then in number two, I'll tell you a little bit about how we incorporate lineage measurements, a separate method we can use to incorporate genuine lineage measurements into these cell state measurements. And this allows us to gain some insights about the paths that cells take as they move forward in time across this landscape.

Probably of most relevance to -- to this meeting is something I'm very excited about and that's how we are using this landscape as power phenotyping tool. So most of the data I'll show you today is from wild-type embryos, but we can also collect single-cell profiling data from perturbed embryos and ask any number of different levels of questions about how a perturbation - and that can be a drug, it can be a mutant - has affected the developmental ontogeny of -- of -- of the embryo.

And then finally, I'll tell you a little bit about some -- some unpublished work that we're excited about where we're interested in getting -- gaining some molecular insights into how time is controlled in embryos, so what dictates how fast embryo -- cells move across this landscape during the process of embryogenesis.

NEXT SLIDE

DR. WAGNER: Okay. So just starting again just big picture, what -- what can we learn from these -- these landscape representations of an embryo? So we have detailed molecular data now for each cell from many time points all across the embryo. Because we have this transcriptome information, we can annotate our landscape with any kind of marker gene, so a gene that we might know something about.

So, here's any easy one, nanog marks the pluripotent cells. These are the cells of that four -- four-hour embryo that I -- I talked about before. So pluripotent cells are right at the center of the graph. So we use markers of different lineages to annotate all the different branches on this landscape on this graph that we found.

NEXT SLIDE

DR. WAGNER: So we had markers of neural, the neural plate.

NEXT SLIDE

DR. WAGNER: The eye.

NEXT SLIDE

DR. WAGNER: Epidermal lineages.

NEXT SLIDE

DR. WAGNER: Mesodermal --

NEXT SLIDE

DR. WAGNER: -- and endodermal lineages.

NEXT SLIDE

DR. WAGNER: Blood. Blood vessels.

NEXT SLIDE

DR. WAGNER: Neural Crest.

NEXT SLIDE.

DR. WAGNER: And of interest to this group, we did find germ cells. Germ cells were probably one of the least interesting lineages we saw in this time window of development. So we found germ cells. They didn't change too much as far as we could measure in the first 24 hours, but they were there and they were easy to collect.

NEXT SLIDE

DR. WAGNER: Interestingly, we're not only -- and this goes to this sort of high dimensional nature of these data sets. We're not just collecting sort of identity information about which cells belong to which tissue. This landscape view, this high-dimensional view of

development captures other information as well. So one of the things that we get for free is positional information. So in the 24-hour embryo, which is the final time point in the data set here, we still have expression of genes that are positional markers in the embryos. So CDX4 marks the posterior of the body. And this -- this is true for many different lineages, so mesoderm, ectoderm. And we can see that our machine learning graph that we've generated actually incodes this information as well as the cell type information. So the posterior cells that express high level CD -- CDX4 are organized on this landscape in a particular manner --

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DR. WAGNER: -- and reciprocally, we have markers for anterior tissues that are expressed in a -- in a complementary fashion.

NEXT SLIDE

DR. WAGNER: So we've done a lot of annotating of this data set. We've made public portals. In the last few years, many, many groups have actually published similar data sets, dates sets at different times, a few perturbation data sets, and we really have just not a wealth of data in the public domain that gives us detailed molecular information about how all of these different cell types, what defines them, and also what are the

changes -- what are the transcriptional changes that coincide with their formation during development.

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DR. WAGNER: And so one of the -- yeah, one of the cool observations we made from just looking at this data, this is wild-type data, was related to the topology of cell fate specifications. So typically in our -- in our textbooks, you know, ontogeny or lineage is represented as a tree. So as a bifurcating tree of progressive cell fate choices that lead to very diverse cell fates as you go forward in time.

And what we found is that that's actually not the case, in terms of the transcriptional identity of the cells. There are many branching regions of the tree, but there are also looping regions So there are cases where cells that differentiate, but then might have different origins, but then start to look more similarly. So this was particularly the case for the cranial neural crests, which we -- we know differentiates into mesenchyme in the head region and the pharyngeal arches.

But I think what our data showed that was a little surprising is to me personally at least was just how similar those transcriptional states were to the point where they become indistinguishable. So some interesting insights about topology kind of came from just anal --

analysis of the shape of that landscape.

NEXT SLIDE

DR. WAGNER: So what is it good for and what we are using it for? The zebrafish embryonic landscape we found does not resemble a tree, So we don't use the term tree to describe this -- this data representation. We either use manifold or landscape, so we can collect data from many different embryos and it's -- we generally get reproducible information, despite the fact that it's an indeterminate lineage. I can go more into that, if anyone is interested.

And we have sort of -- in our analysis pipeline, we have no presumption of topology. And this really opens us up to ask questions about converging, differentiation, dedifferentiation, transdifferentiation, things like this. So those are -- those are things that we're very interested in in my lab.

Okay. So one of the questions -- one of the initial reasons we did this is that we wanted to build trajectories that describe the detailed molecular instructions making any tissue in the vertebrate body planned. And we thought in the early days, oh, we'll just sort of look -- we'll look for these branches. We'll build, what they call, trajectories, and we'll read out gene expression information or every single tissue and

organ and reconstruct the molecular history.

NEXT SLIDE

DR. WAGNER: And it turned out this was not so easy to do just from the single cell RNA sequencing data alone. And in some cases, it was -- it was okay. So the two examples I show here are the epidermal lineage as well as the retina, the eye. And so there are a few cases on landscape where we could -- we could try to draw a path and it made sense that it represented sort of -- of a stepwise process by which cells are differentiating along a particular path.

But there were many places in the landscape where the data were messy or just confusing. And it was very hard to establish directionality, so which way would cells be moving across this landscape. So there are emerging toolkits for building this type of information back into our RNA-seq data sets. One of them is a computational method call RNA velocity, which can be very useful.

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DR. WAGNER: What we used to tackle this was genuine lineage tracing measurements. And I'll tell you a little bit about how we do that and the kinds of things that we're hoping to do next with lineage tracing information.

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DR. WAGNER: I'm actually going to skip this slide just due to time.

NEXT SLIDE

DR. WAGNER: The method -- the lineage tracing method that we've incorporated into our single cell profiling is called TracerSeq. It's based on a transposase system, the Tol2 system, which we routinely use in zebrafish to build transgenics. So what Tol2 the transposase enzyme allows us to do is insert a cassette of DNA into the zebrafish genome and we can control exactly what goes in the cassette. So in the case of TracerSeq, the cassette is a -- is a transgene. So it's a -- it's a -- it contains a promoter and a reporter for a fluorescent protein. In this case, it was gfp.

And then in the three prime UTR of that transgene, we placed a barcode -- a unique barcode. So we're actually not injecting one species of DNA. We're injecting a library of -- of DNAs into the -- into the embryo.

So we co-inject this library into this single cell stage with our transpose mRNA. And what we've shown is that TracerSeq works very well by inducing unique insertion events into the genome in this asynchronous fashion. So in this diagram here, I've shown just a little -- a small portion of the lineage tree of the

embryo. And what we've depicted are integration events. So each of these color boxes is a particular TracerSeq barcode that's been inserted into the genome. And now any that cell divides, all of the descendents of that cell will inherit that same barcode. And we have many barcodes that get integrated into many different cells and sometimes into descendents of a cell that was previously barcoded with a different insertion. And so this produces very rich data sets that we can -- whoops.

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DR. WAGNER: -- that we can actually combine with our single cellar RNA-seq landscape. And this allows us to now have ground-truth information about which cells are most related to which other cells and where those cells ended up in their differentiation trajectories.

So these data sets are very big. We use them in lots of different ways. One of the things we can do with these data sets is -- is analyze individual clones and how cells that are different -- how cells differentiating within a clone, within a clade diverge on the landscape and become different.

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DR. WAGNER: We can also aggregate data from many different embryos and many different TracerSeq integrations to come up with aggregated pictures of which

lineages are often more related to each other. And this -- this has produced very satisfying trees. Now, I know I told you that -- it's development is not always a tree, so we have a few violations in this tree. But in general, we've been able to use this data to corroborate places where the single sell RNA-seq data both conforms to a tree as well as when it violates that -- that sort of tree assumption. So now we have these two interdependent pieces of information that allow us to establish ontogeny.

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DR. WAGNER: So the TracerSeq lineage data has also allowed us to resolve some complex scenarios. So that looping event that I told you about before, where trans -- neural crests appear to be transdifferentiating into a mesodermal like mesenchymal state, our TracerSeq data confirms what we know from the developmental biology textbooks about which direction cells might be moving through this complex topology. This particular loop our TracerSeq data tells us that there's particular mesenchymal cells that have a neural plate origin that have transdifferentiated from crests into something that looks very much like mesoderm.

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DR. WAGNER: So these are just a few examples. I don't -- I don't really have time to tell you about some

of the things we're doing with TracerSeq now, but just very briefly, we're combining lineage barcodes with perturbation barcoded and we're also using TracerSeq to examine how lineage relationships change under conditions of perturbation. But even in the first pass, which I just went over now, the TracerSeq lineage data has been very helpful in helping us resolve ontogeny on the landscape. It's confirmed tree violations. And it's also given us quantitative ways to sort of look at lineage similarity and lineage trajectory

NEXT SLIDE

DR. WAGNER: Okay. I'm going to talk a little bit about the next way that we've used these landscapes, and that's to assess perturbations.

NEXT SLIDE

DR. WAGNER: And really our -- our long-term goal is to understand not only how embryos become defective in response to genetic or environmental queues, but how they could recover from such -- from such insults and what molecular mechanisms they might use to recover.

NEXT SLIDE

DR. WAGNER: So just to show, you know, what we can do with this kind of data, I'll describe a really simple experiment we did, which analyzed a classic patterning gene that's chordin. Chordin is a BMP

inhibitor gene that affects the dorsal -- early dorsal ventral patterning of the early vertebrate embryos, all vertebrates, zebrafish included. And so what we did is -- and chordin, because it's a BNP inhibitor, when you inhibit chordin, it leads to overactivity of BMPs and a ventralization phenotype.

And so what we did with chordin is we -- we did our inDrops single cell RNA sequencing. We collected individual cells from control embryos as well as embryos where we'd knocked out the chordin gene using you CRISPR. And we compared the transcriptomes of the cells that we got from each of those two samples and we had replicates back to our reference landscape of wild-type embryos.

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DR. WAGNER: And what we found was encouragingly what we expected. So we found that in the chordin mutants ventral tissues were expanded at the expense of dorsal -- dorsal tissues. In this plot what I'm showing you is I've broken down the embryo into distinct clusters of cells. And these clusters correspond to particular tissues. And I've colored the tissues based on where they sit in the embryo. So whether they're generally in a ventral location or a dorsal location.

And what you can see is that on the Y axis and the X axis of this particular graph, I've show -- I'm

showing you how a particular tissue changed from -- going from a wild-type normal control scenario to the chordin defective scenario.

And I hope you can appreciate that the ventral tissues have all moved to the right on the X axis. And what this means is that those tissues were expanded. There were -- there were more cells in those tissues than there were in the control and the opposite was true for the dorsal tissues.

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DR. WAGNER: We can also show this -- this same data by projecting these cells back onto that landscape, so this landscape representation that I have been showing throughout the talk. Now, instead of coloring cells by time point or by gene expression, I'm coloring cells based on how abundant a local neighborhood was in the chordin mutant embryo relative to the control embryo. And so here, purple regions of the graph correspond to regions that were overrepresented in the chordin mutant and green regions correspond to regions that were underrepresented.

And so what we see is that this dorsal ventral transformation is actually again it's organized on the landscape. It's not just sort of random, but it corresponds to particular branch points, which we think are chordin sensitive fate decisions. And so this was a

really cool result that sort of confirmed what we already knew about this pathway, but it also suggested a relationship to fate choice that wasn't so obvious. And we also found examples of chordin-dependent cell fate switches that -- that occur later than the -- the classic early embryo DD access specification.

The other result that we got from this was that although the -- the predominant change that we saw with the chordin embryos was this -- this changing of cell type ratios, so how abundant or -- or not abundant a particular tissue was. The individual cells themselves actually look almost -- essentially identical from a -- from a normal embryo.

So this graphy on the bottom left is measurement of how similar those gene expression profiles were at the single cell level between either our control or chordin and our reference landscape. And you can see similar distributions for these.

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DR. WAGNER: So this led us to a conclusion that, at least in this particular perturbation, we've affected a patterning change in the embryo. We've skewed ratios along particular fate choices. But because each individual state looks essentially normal, we haven't actually created new states or new pathological states by

modifying chordin. We've simply changed how cells flow through these -- these cell fate decisions and they're flowing differently in a predictable fashion when we knock out chordin or overexpress BMP. So this has led to a number of questions. We have a couple projects in the lab that are sort of examining in further detail what's going on with chordin.

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DR. WAGNER: But in the final couple minutes, I want to talk about a -- a new project that we've started more recently, and that examines the landscape from one sort of different respective and that's -- and that's through time. And so I've -- I've -- I've sort of shown you the landscape with time points and how, as cells advance over time, they're becoming more differentiated, more diverse at the molecular level, but we're interested in understanding what really controls the speed at which cells are making these decisions.

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DR. WAGNER: And the speed or tempo of zebrafish development is something that we've long known can be altered by the environment. So Chuck Kimmel back in the nineties and some of the early staging observations that he made noted something that we all use, probably every lab, is that the speed of development is very sensitive to

temperature. So zebrafish are not warm-blooded. They depend on in the environment for -- for their temperature.

And we know, we've seen it many times, that at higher temperatures development precedes more quickly than at lower temperatures. So there's -- there's sort of environmental influence that changes the rate at which cells move through the developmental process.

And it's actually quite fascinating that this is -- that this can work. I mean, we're -- we're not talking about one cell type. We're talking about dozens or hundreds of cells types that coordinately know how to remain synchronized at faster or slower speeds at different temperatures. And so it's not only temperature, so we've -- we've been investigating this. We've -- we've seen a few other environmental conditions that can modulate tempo. One of them is oxygen. Another is simply crowding the embryos, which we think could be oxygen, but there's some evidence that it might be something else as well.

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DR. WAGNER: And so we embarked recently in the lab on a -- on a chemical screen to see if we could Get some molecular insights into processes that might be affecting tempo, sort of tempo regulation. So we performed a chemical screen using this Cayman chemicals

that had a -- this particular library we purchased off the shelf. So it had 160 drugs, so a fairly modest screen, that was targeted at metabolic enzymes, and so glycolysis, citric acid cycle. And I can talk about why we -- we targeted this, but this was just sort of a first -- first attempt to see if we could modulate this process using drugs.

So we used a fairly simple metric to -- to -- to determine whether tempo had been affected. And this goes back to Chuck Kimmel again, but the measurement of the head-trunk angle. So as an embryo -- as a zebrafish embryo in between 18 hours and about 30 hours is finishing segmentation and organogenesis. The head of -- head straightens basically. So as this -- as this angle between the head and the trunk increases, it's a fairly linear readout of where that embryo is in time. And so we use both head-trunk angle as well as embryo length as a quick readout for developmental progression.

And we exposed zebrafish to -- to this chemical library at two concentrations between the hours of 19 and 25 plus fertilization. We did this because this is the window when head-trunk angle and length have very linear relationships to time. Let's see. And we -- just because this came up earlier, we did this on dechorionated embryos.

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DR. WAGNER: So here's just an example of sort of the head-trunk angle measurement and how this fairly simple thing that we can measure using a micrograph corresponds linearly to -- to time and as well as developmental progression.

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DR. WAGNER: And so we performed the screen in a -- in a few different ways at a few different doses. And what we were looking for in the end were drugs that caused a tempo deceleration both at -- both according to the head-trunk angle as well as length and ideally at multiple concentrations of the drug.

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DR. WAGNER: And so we found a few hits. We're just beginning to investigate the sort of molecular context of these different hits. But we've implicated a few processes that I think are pretty interesting, so there's electron transport chain, there's catecholamine methylation, NAD, NADH metabolism, and one hit that was really related to autophagy.

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DR. WAGNER: And so we're -- we're excited about where this is taking us. I'll say that when it comes to tempo, we're also doing single-cell RNA sequencing right

now to -- to try to understand, you know, what happens in embryos that are going at different speeds. So we're approaching this -- this question from a few different angles.

So I think I'm -- I'm like a couple minutes over, so I'm going to wrap up now. Hopefully, I've been able to give you an impression of how we can use our quantitative single-cell approach to understand the process of development in a sort of new way, in a quantitative way that let's us make specific statistical measurements and to do so across the embryo both in normal conditions and ways that allow us to infer ontogeny using lineage barcodes. We can use this approach to understand the details of perturbations. And hopefully, we'll -- we'll get some insights into timing mechanisms as well.

And So I just want to quickly thank the group, my lab. The tempo project that I talked about at the end, was -- is carried out by a grad student Chris Chen here and his summer student Sarah Foust. And so just want to thank the lab and our collaborators at UCSF who we work with on a number of projects. And thanks to all of you. So I -- I guess I'll answer any clarifying questions that may have come up. Thank you.

CHAIRPERSON LUDERER: Thank you, Dr. Wagner for that fascinating talk. Just looking to see whether we

have any raised hands for clarifying questions for Dr. Wagner.

DR. WAGNER: Okay.

CHAIRPERSON LUDERER: Patrick.

COMMITTEE MEMBER ALLARD: Yeah. Thank you so much for this -- this wonderful talk, fantastic talk. I had a question regarding the -- the loss of function work that you did so -- with no gain, right? So you can -- you can capture by single cell these pretty dramatic changes from a -- from a functional perspective, but I was wondering what that looks like when you do perhaps -- when you look at the hypomorphic situations where things may not be as dramatic or when you do pharmacological interventions, if you've tried that, and -- and whether the single cell data still gives you clear answers or whether it gets a little bit messy because cellularization is tough and doing single cell in general is very computationally challenging.

DR. WAGNER: So I -- I'll say that the results we've gotten from single cell analysis of perturbation data have -- have been a bit surprising. I would say that we've -- I'm not aware of a case where we have seen nothing yet, but I think chordin demonstrated a principle that I think has been borne out in -- in many other studies by other groups too. And that -- that's a first

observation. I think it's just interesting, and that's that perturbations that exert massive patterning defects on the embryo's body plan. So dorsalization, ventralization, loss of entire organs, or, you know, states that -- that are completely lethal that look terrible when you actually look at the embryo under a microscope, that the individual cells in -- in those embryos often still look quite normal. And so that's not to say that the proportions are normal, but the actual granular cell states themselves look generally normal.

So this is something we're really interested in -- in exploring more systematically, so some of the projects in the lab were comparing sort of different loss of function phenotypes to gain of function phenotypes. So I think gain of function phenotypes or overexpression phenotypes are probably more likely to produce novel states or pathogenic states, and we're very interested in how an embryo would resolve states like that.

So I think the -- yeah, in some ways, we didn't really see what we -- what we kind of expected to see more aberrant states and we just haven't seen that and that's been true for other groups as well, generally for loss of function mutations. So we're gearing up right now to do single-cell analysis on some drug perturbations starting with retinoic acid. And I guess it -- we have yet to see

if -- if that's going to be a similar case here.

But, yeah, I think the one thing that's been very, very robustly seen in single-cell analysis is -- is sort of whether specification fails or not. So that's -- that's something that's easy to see.

COMMITTEE MEMBER ALLARD: Thank you.

DR. WAGNER: I will say one more thing related to that too, and that's, you know, these single cell measurements are just a particular set of measurements. We could -- this is the transcriptome. So there's a lot of other variables related to cell identity. So there's epigenetic signatures, there's -- there's, you know, post-translational modifications. We're not seeing any of those things. So to the extent that changes in a particular pathological context eventually feedback and change the transcriptomes, we'll see that. But there are things that we wouldn't expect to be able to see with this.

CHAIRPERSON LUDERER: Thank you. I see we have a question from Dr. Baskin.

COMMITTEE MEMBER BASKIN: Hi. Larry Baskin. Fantastic talk. More of a technical question. You showed some beautiful data that early on in embryogenesis the cells were basically the same and then you move out, you know, four or five days, case and very, very -- very

different. How do you know where -- where you're getting the cells from and are you using markers, for example, for mesoderm, ectoderm, endoderm or is it just where you place the needle so to speak.

DR. WAGNER: So, in general, we can do surgical enrichments, and we've done that for a few experiments. For the data I showed today, we're actually disassociating the entire embryo, so we're taking all the cells from everywhere and we're allowing our computational algorithms to -- to put it all back together based on transcriptional signatures. And so then we use markers in silico to -- to sort of determine which -- which clusters of cells go where.

And so I just showed a few little examples of some marker genes that we use to say, okay, this branch is expressing all the mesoderm markers we expected and this other branch is expressing all the epidermis markers. And generally it's -- when you get into those data, it's very convincing, because the cells are the -- you know, the cluster doesn't just express one marker, it expresses dozens of markers that are all specific to a -- to a particular branch.

So, yeah, we've -- we've generally done pretty well allowing the data to tell us which cells are -- are which. I don't know if that answers the question, but...

COMMITTEE MEMBER BASKIN: It kind of does. It seems likes it's a little more conducive to the earlier embryos as opposed to later on when you're -- you know, have a lot of, you know, blood vessels and all types of, you know, different -- the complexity, I think, I guess increases but in the system of the zebrafish earlier on, it seems like this is -- pretty kind of spot on.

DR. WAGNER: Yeah, and I think the -- you know, the loop that I talked about where, you know, cells that have distinct ontogenies as they differentiate, they start to look more like each other, even though they're not related in their lineage. This is abound to increase dramatically after 24 hours. So hox genes are only expressed for so long. So some of the markers that -- that allow us to distinguish these spatial domains, we don't expect those to remain on. And so we -- we may see a collapsing of these things.

But, you know, within -- within this -- this particular time window, it's -- you're right, it is -- it is pretty good. I think if we wanted to, you know, preserve other -- sort of other identities or spatial origins, you know, we can turn to other methods or include other modalities in this. So optogenetics to mark cells with -- of a particular region, such that they would have a label that we could read out with our transcriptional

profiling and sort of see that in the data as well. Surgical Techniques, there's -- there's sort of ways to go about it, if needed.

COMMITTEE MEMBER BASKIN: Thank you.

CHAIRPERSON LUDERER: Okay. Thanks.

Dr. Pessah.

COMMITTEE MEMBER PESSAH: Hi. Very nice presentation. I was wondering, have you segregated on sex, but you don't know the sex at that point or -- and different lines of zebrafish, do they converge? Does the machine learning algorithms converge on a common pattern?

DR. WAGNER: So, let's see, the answer to the first question is we don't know the gender. We haven't looked for that at all yet. It's too early. The second question is, yes, we actually had two -- two strains in our initial data set and I didn't show that they -- they lay on top of each other almost perfectly. And another data set that hasn't been published yet by a colleague, they've seen the same thing.

So, I think -- I mean, that's something interesting I didn't really go into, but, you know, we -- we often think about or wonder about strain differences, but at least at the -- at this level of transcriptional similarity across tissues, the signatures that distinguish tissues from each other at that level, we haven't really

seen any trends strain by strain.

COMMITTEE MEMBER PESSAH: Thank you.

CHAIRPERSON LUDERER: Great. Thank you.

So it looks like we don't have any additional clarifying questions, so then we can move into our discussion part two with both Drs. Panlilio and Wagner. And we have about -- maybe a little -- about 30 minutes for this.

So to get us started, maybe something to think about some suggested questions for discussion. So in the absence of mammalian and mechanistic data on effects of chemicals like domoic acid or bisphenol A, could zebrafish screening studies for general and/or neurological effects alone have indicated the need for additional studies to fully characterize toxicity in mechanisms of action? So that's one thing to think about.

Another question is to design appropriate zebrafish studies, what other types of evidence could help define appropriate lines of experimentation? So, for example, with the domoic acid and bisphenol A, we have documented adverse effects on wildlife that correspond to those observed in humans and test animals, and which can then suggest detailed laboratory examination of adverse effects and mechanisms.

And finally, another thing to think about is

should zebrafish data generated for the purpose of acute fish toxicity testing conducted for environmental hazard assessment, be more generally incorporated into consideration of likely adverse harm to humans.

So those are some sort of food for thought. And any discussion from members of the Committee or from any of our speakers, including from this morning, want to kind of go -- take -- riff on any of those or -- or have some other points of discussion they'd like to the bring up?

I know Dr. Woodruff had some -- had a question earlier today that -- which she deferred. Would you like to start with that?

COMMITTEE MEMBER WOODRUFF: Oh, you're calling on me, because everyone is like, whoa, that was a lot of really amazing content.

(Laughter).

COMMITTEE MEMBER WOODRUFF: Okay. I was listening to your questions and I'm thinking through -- yeah, I guess I had a couple of thoughts, right? So, I mean, you guys presented a lot of really detailed and, you know, biology and mechanistic information. And, you know, kind of trying to think how that -- I'm thinking through the -- that kind of piece of it and some of the -- I mean, I -- it's interesting to look at the different ways to measure influences on development. And you both talk a

little bit about this, including, you know, the response, or how fast the head unfurls, and -- but I think we're -- you know, when we're trying to think about how to use this information, it all points towards, and what I'm hearing from you, is that these are different ways to interrogate development using the zebrafish model, that -- and maybe in the domoic acid, I think it's -- and I wanted to follow up on this question, is that what -- you know, that's definitely a toxic chemical and that you're -- you're -- a lot of the work that you were doing was looking as these windows of susceptibility.

And I thought what was pretty interesting about that, which was a follow up on the question, Ulrike, that I had, which was you were looking at acute exposures. But if you had done a chronic exposure across all the windows, you still would have identified that effect, right, because what you saw is that -- I think it was post-fertilization one and post-fertilization four, you got kind of a mild effect that was higher at the high doses, but at the two, everything was depressed.

So I think, you know, when we're thinking about trying to interpret data for the kinds of chemicals that we're looking at, it's useful to see, you can identify windows of susceptibility, but a chronic exposure could also, right, is what I would take if you had exposed your

embryos chronically. You probably still would have seen it, but you wouldn't know exactly when that -- what was the timing that was influential, right -- is that right?

DR. PANLILIO: Right. And I think -- so in addition to that -- so that's right. So like let's say that would have required me essentially to be microinjecting every day from one to four. But then one thing that that -- that may do is that may miss sort of lower dose exposures that can in -- and happen in a single hit. That's my first thought on that.

But most importantly what I like about using windows of susceptibility is it also gives you a clue for the mechanisms of action, right --

COMMITTEE MEMBER WOODRUFF: Right.

DR. PANLILIO: -- because you're -- if you're exposing them through the entire period of time, you don't really know which neurodevelopmental processes are potentially targeted by that. So part of the reason why I did that was like, okay, so we know that the developing nervous system is a target for domoic acid, but what within the developing nervous system and what processes are perturbed. And so that sort of gives you a little bit better of a clue as to like what we need to be watching out for. Like kind of going to that guide question about how we can use zebrafish to understand like, you know, the

mechanisms by which we like a toxin or toxicant works, right? But then --

COMMITTEE MEMBER WOODRUFF: Yeah, so I'm -- oh sorry. Go ahead.

DR. PANLILIO: Go ahead.

No and --

COMMITTEE MEMBER WOODRUFF: You want to just -- you go first.

DR. PANLILIO: Go ahead.

(Laughter).

COMMITTEE MEMBER WOODRUFF: Well, I guess what I'm thinking is -- and it's with both those examples is that from the purposes of where we're looking at the chemical -- environmental chemical exposures that you -- you could -- if you don't hit a particular mechanism, that you have been -- as you are working through these experiment -- experiments, if you don't hit that particular mechanism, and you don't evaluate that, you could miss a chemical as opposed to, you know, if you're doing this chronic exposure domoic acid and you -- you have an experimental condition that, I guess, I think is a little bit broader, so you make sure you don't miss something, it's kind of -- I think our -- you know when people have been asking questions about exposure, and that was interesting about how you did your dosing, I -- that's

very important and the chart -- what we're -- I think we -- you know, as scientists, we're super interested in all the shape of the dose response curve and the mechanisms, but our charge on the Committee is that it can be a response at any exposure.

So even though you have these higher exposures -- I guess, you didn't -- weren't really sure -- or there hasn't been some experiments done to look at the difference between the injection versus if you stuck them in a dish of the --

DR. PANLILIO: Right. Actually, no there has been. So I will say, so two things. So it also matters what type of injections. So the paper that I referenced above where they do -- it's very -- it's much more common to do yolk injections, for example, at earlier stages. To see any sort of effects, they had three to 260-fold higher doses they had to use. So I think method is important.

And also I -- I did try water waterborne exposures and we did up to 40 micromolar of domoic acid and we didn't see any effect whatsoever. So I do -- I do think that, you know, the problem is, of course, this is not high throughput at all, and that's hard. So it would be, for example, I suspect missed in a screening process to do this work, because I just didn't -- I was not able to see it in waterborne. And this was through a chronic

exposure from I believe I started at 1 dpf all the way to five with my highest dose of 40 micromolar. And so -- yeah, so that is -- you know, we talk about exposure and exposure route and how that's important, so that's part of it for sure.

COMMITTEE MEMBER WOODRUFF: But did you say -- you said also they sell this in the animal studies, right, response?

DR. PANLILIO: Which part?

COMMITTEE MEMBER WOODRUFF: The domoic acid. Did you mention that all --

DR. PANLILIO: Yeah, absolutely. So they did. So like -- so, for example, in rodents either they injected the mother, right, and they looked at the --

COMMITTEE MEMBER WOODRUFF: Okay.

DR. PANLILIO: -- you know, or they injected, you know, neonates as well. And so I will say like what was kind of assuring, right, is that we saw very -- at least on the very acute neurotoxicity side, we saw very similar phenotypes. So that sort of allowed us to think more about using fish as a model to look at this.

COMMITTEE MEMBER WOODRUFF: Thank you.

CHAIRPERSON LUDERER: All right. Thank you.

DR. WAGNER: I wanted to -- I wanted to comment on this thread a little bit too, just about the -- the

sort of potential benefits or use of the methods I talked about today and sort of where they fit into -- into these kinds of goals. So I think -- I mean, Stephanie Padilla talked about this a little bit. And, you know, there's -- it sort of depends on the goal of the experiment how many animals you use, what level of resolution we choose to ask a mechanistic question.

So, you know, because our single cell methods that are -- you know, they're getting cheaper every day, but they're still fairly expensive. It's never your first experiment, so we do the same dose response curves. Even for CRISPR or mutants, we establish using morphology the general phenomenology defect first. And then when it's very reproducible, we use our method to go in and get deep mechanistic insights into what's specifically going wrong under a very small number of conditions. We can't -- we can't effectively apply -- apply this technology to a thousand conditions. We can apply it to a few.

I would say one area in which the single cell approach could be very powerful and maybe different is that it can be -- it can be good in assessing defects that might be mysterious, or pleitropic, or where it's just difficult to nail down with markers like one at a time, like which -- which -- which sort of tissues might be affected or if multiple tissues are simultaneously

affected by perturbation.

Those are things that we can actually evaluate pretty easily and without the need -- without even the need for a hypothesis. So you know, we just look at every single gene and every single cell and then ask the fish, you know, what changed. And so we can -- I guess, we could be more open-ended in terms of mechanism, as long as we know we're looking at the right time and place. So I don't know if that helps, but that's -- that's sort of how I see a method like the single cell fitting in.

COMMITTEE MEMBER WOODRUFF: Right. And I was kind of thinking also, but your -- your approach could identify kind of unique pattern signals that could -- could interrogate later, right? Rather than doing more broader evaluation, you could perhaps target some of your exposures also on particular unique patterns.

DR. WAGNER: Absolutely, yeah.

COMMITTEE MEMBER WOODRUFF: Thank you.

CHAIRPERSON LUDERER: Thanks.

Irva, you've had your hand up for a while.

And I think you might still be muted.

COMMITTEE MEMBER HERTZ-PICCIOTTO: Yeah. I wasn't muted any more, but I was trying to get the video on. It seems to have a lag.

Okay. So I guess this has been really

interesting, because I have to say that I tend to be skeptical as we get more and more away from humans, and then away from mammals, and then away from, you know, whole organisms, where when we talk about exposures, which is, you know, what DARTIC is about, where, you know, the models seem, you know, to be getting further and further away from what we're trying to do.

But I will say that I -- I have been really impressed, and I -- I think the bottom line that I've kind of come out with from today and all of the presentations really, you know, beginning with -- with Bruce's, you know, comparisons of the, you know, the organs, and all the way through Stephanie's presentation and, Jennifer, yours, and Dan's well, that this -- there are a lot of similarities that make, you know, the comparisons I think useful and certainly from the perspective of just hazard identification, which, you know, is sort of that -- that first step that -- that -- that we are, as a panel, are -- are, you know, supposed to be trying to -- to take based on the best available evidence.

And, you know, I -- I understand that there's been sort of that -- the level for, you know, in vitro testing has been kind of -- is always seen as being, oh, it can be confirmatory, but -- you know, and so forth. And -- and I -- I still think that the -- the -- the

translation from species to species needs -- needs -- needs work to establish when things translate and when they don't. And when people bring out that -- I don't know, one of the examples early on, and I can't remember what the chemical was, but you got negative effects in one set of experiments or one species, and then positive in the other. And, yeah, that tends to erode the confidence that we can use that data. You know, that that's going to actually be useful for -- for our purposes.

But I -- I think the kind of careful mechanistic step-by-step sort of laying out of -- of biological responses and the consequences of those responses, and the pathways that -- that follow one after the other of these consequences is the kind of data that to me does speak to the relevance for -- for -- you know, for regulatory, you know, purposes such as -- such as the Prop 65 that we are -- we are working under.

So, yeah, I think that -- that's really -- it's not really a question, but just a comment on, that I -- I feel I really gained a much big -- better understanding that the zebrafish is more than just, oh, we -- there's this transparency and you can see things happening internally, you know, from this.

And, yeah, that's -- I mean, I think that's kind of the question before us is what -- what -- what is the

utility of zebrafish modeling? I see that it's really got a lot of features that make it very relevant and that the right kinds of studies can really help us in the regulatory realm.

CHAIRPERSON LUDERER: Yeah. Thanks. And I think that gets to kind of one of the questions that we were -- that I, you know, read through at the beginning, which is this idea that using some model like the zebrafish model could potentially use -- be used as screening -- you know, in screening for various different kinds of effects, and that might indicate the need for additional studies to characterize -- you know, more mechanistic studies to further characterize the mechanism of action of a toxicant.

Dr. Pessah

COMMITTEE MEMBER PESSAH: My question is for Dr. Panlilio. I was wondering, so you found a critical window at two days post-fertilization for domoic acid. Would there be any advantage to letting those fish go past day 10, when they've got a more complex array of functions that you can test? Is that something in the work, because I think that would be very informative.

DR. PANLILIO: Absolutely. It was actually a slide I took out, but thinking about sort of the long-term consequences of setting up a nervous advertise and

perturbing it during these critical windows and looking at what that looks like in adulthood is absolutely critical.

The one thing that is a little bit harder is that when we start going to adulthood and just with complex behaviors, we don't know as much about the sort of underlying neural circuits for a lot of these behaviors. And I will argue that while there are some really wonderful labs and wonderfully established behavioral protocols to test for, for example, adult behavior or adult endpoints, it's a little less established than like, for example, mouse behavior in some ways.

And so while we can do that and we can test, for example, the adult behavior, what does this mean in terms of, okay, yes, we know that there was a perturbation prior in development and now let's say there's this deficit in social behavior that we see in adulthood, how does -- how do we connect that prior insult to that later adult behavioral endpoint is still tricky, just because we know less about the circuits that drive adulthood.

I mean, just because that's -- you know, there are still blank boxes there, that doesn't mean it's not important. And I do think growing the fish up and doing that sort of study is important. And I do believe there are people working on that.

CHAIRPERSON LUDERER: Okay. Thank you.

I know we are supposed to take a break of about 15 minutes and I'm wondering whether this would be a good time to do that.

So if I'm not hearing anything from the staff that we should not take a break now, why don't we go ahead and take our 15-minute break. And then when we come back, we'll be talking further, discussing more about the use of zebrafish data in developmental and reproductive toxicant -- toxicity health hazard assessment.

So it is 3:02, so why don't we come back around 3:15 and then reconvene then. All right everyone have a good break

(Off record 3:02 p.m.)

(Thereupon a recess was taken)

(On record: 3:15 p.m.)

**PART III. USE OF ZEBRAFISH DATA IN DART HEALTH HAZARD ASSESSMENT**

CHAIRPERSON LUDERER: Okay. Welcome back, everyone. We're now moving on to part three of our discussion and session today. And that's on the use of zebrafish data in DART health hazard assessment.

We have about an hour -- up to an hour to discuss this among ourselves and with all four of our invited speakers. So we have several questions that we could discuss and think about. So starting with the first one,

given that biological differences between zebrafish and mammals, for example, lack of internal fertilization and pregnancy, what are the issues that be should be discussed in considering zebrafish data in human health hazard assessment?

Another is how might considerations of life stage and windows of susceptibility be used in evaluating data from zebrafish?

What are some ways routes of exposure in toxicokinetics could be considered in interpreting the results from zebrafish assays?

So those are some things -- some points that we might want to discuss.

Also, applications of the zebrafish model focus on upstream essential processes in reproduction and development, rather than final apical outcomes and how should this be considered when we consider different data streams?

And finally, another question to think about is given the diversity of zebrafish study types and outcomes measured, how might these studies be best evaluated for quality of the studies for purposes of hazard and risk assessment.

So if any one of our speakers or panel members have thoughts upon -- about any of those, please go ahead

and raise your hands and I will call on people.

Let's see, I think -- I believe that's, yes, Patrick. Dr. Allard.

COMMITTEE MEMBER ALLARD: Yeah. Thank you. I -- I guess what I'm struggling with, when I'm thinking about mammalian development and zebrafish development is to think about the timing of things and thinking, for example, of -- that's just one example, the blood-brain barrier formation and whether it forms in a way that's analogous to the generation, the birth of the same neuronal types that we're going to then try to examine and compare with in -- in mammals.

So -- so I guess this is -- this question is for the panel members and maybe we can just think about the blood-brain barrier formation. What do we know about it and how is the timing comparable or dissimilar between the two, and what are some of the lim -- related to that, what are some of the limits to the extrapolation that we can make between zebrafish and -- and mammalian species? Should I direct the question to one of the speakers?

CHAIRPERSON LUDERER: To one of the speakers?

Yes.

COMMITTEE MEMBER ALLARD: I think this would be for Dr. Panlilio perhaps.

DR. PANLILIO: Hi. It's a really interesting

question. I've thought about this. So, I mean, it occurs slowly over time where the largest chemicals get -- start getting excluded at 2 dpf, so I was thinking about that in terms of my -- my windows of susceptibility. And so -- but it is a really interesting question to think about, okay, so how does that relate -- so I know how that looks like in terms of how does the blood-brain barrier formation occur in fish relative to the developmental processes in fish.

One thing that I think this question is really interesting, because I'm trying to think about now how that may be uncoupled for example with myelination that happens, you know, in humans, that occurs like well into, you know, early adulthood, right? And so you can imagine that there are potentially processes where it will be -- depending on the structure of the chemical, that, you know, you'll see it in fish and you won't necessarily have to worry about it as much in humans.

It's not necessarily true for domoic acid, just it's -- it's a very small, you know, neurotoxin, but it is -- it is definitely something to consider is like again not only expose -- exposure route, but like also like how does a chemical toxin and toxicant, like how is it able to get to target tissue? And, you know, and one thing about domoic acid too in particular is there are specific brain

regions that we know of in adults that are more -- that are more targeted, so for example the hippocampus, because of how domoic acid can sort of shunt that blood-brain barrier.

So, I mean, again, I -- so even if it does form over time, and that's an important consideration, there are also still target tissues that sort of allow for more accessibility even without that. So I do think it's an interesting question. I haven't -- I don't have like an exact graphed out answer for how, you know, different processes, neurodevelopmentally parallelize with that, but...

COMMITTEE MEMBER ALLARD: Thank you.

CHAIRPERSON LUDERER: Dr. Pessah.

COMMITTEE MEMBER PESSAH: I guess I need to question -- I think I heard that in -- in the injection model with domoic acid, the dose may be high, but it's a single exposure, and we don't really know the pharmacokinetics, but you do get a very, very robust response. And I believe the response, based on the data I've seen and read about, but then you also just told us that if you put domoic acid on postnatal day two up to 40 micromolar in the water, that you don't see any effect. And this goes to my comments last year when we were reviewing a particular set of compounds where I said,

well, these studies seem to lack face validity. And then the question came up, well, what is face validity?

It means that what you think you're doing at the very fundamental stage, which is the exposure stage, really is occurring. And we -- we heard about, well, small molecule domoic acid has a favorable log P. It should be getting in past the chorion, if we understand that the chorion has pores, and yet it doesn't do anything, at least in this context.

When we then review papers, how are we to know which ones have face validity and which ones don't? Because unless you have internal concentrations and target engagement as part of that data set, it may not be relevant. I guess it -- and that -- and that goes for a lot of animal studies, I mean, not just the zebrafish. I like zebrafish by the way. They're great.

(Laughter)

CHAIRPERSON LUDERER: Any -- oh, Dr. Padilla.

DR. PADILLA: So, Isaac, I have -- I've thought a lot about this, because I started working with zebrafish at about the same time I started screening chemicals. And screening -- and I thought, oh, zebrafish is going to be such a big change, and it is. But screening chemicals is a really big change too. And you have to -- you have to get comfortable with not knowing everything. That's the

first thing.

And what I've always said with chemicals and screening is there's lots of reasons to get a negative. There are just lots of reasons to get a negative and you really have no idea why you've got the negative. The positive on the other hand, you do have something happening. And so usually I -- I view the positives with a lot more certainty than I view the negatives, because there's just -- I mean, the chemical could have fallen, the chemical could be sticking to the -- to the plastic, the chemical could be sticking to the chorion, the chemical could be sticking to the -- to the outside of the embryo and never get inside the embryo, or it could be breaking down, it could be pumped out by reverse pumps in the zebra -- I mean, there's all kinds of things that could be happening to give you a negative.

Put the positive means that something has happened and let's go after it or let's let somebody else go after it. But I guess that's one of the things. That's -- and if you're only looking at negatives, then you either need to know the chemical is there in the fish or else you need to have -- the other thing you need to have is some positive controls. So that's another thing that you need to look in papers is are they running positive controls. Do they -- can they pick up a

chemical, if it's a positive? And some papers don't do that.

COMMITTEE MEMBER PESSAH: Agree.

DR. PADILLA: I mean, this is all pretty obvious, but for me, it was -- it was a different mind set when we started doing screening.

CHAIRPERSON LUDERER: Thank you.

Dr. Wagner, did you have a comment on that?

DR. WAGNER: Hi. Sorry. I had a comment on the previous topic.

CHAIRPERSON LUDERER: Um-hmm.

DR. WAGNER: I just wanted to mention the blood-brain barrier --

CHAIRPERSON LUDERER: Um-hmm.

DR. WAGNER: -- before we got too far away from it.

CHAIRPERSON LUDERER: Sure.

DR. WAGNER: So, yeah, there is a -- there have -- has been some really cool recent work done on the zebrafish blood-brain barrier by Dr. Natasha O'Brown and Sean Megason. So basically, the barrier forms at 3 dpf. And there have been some -- some elegant studies that show what you can do tracer dextran injections into the bloodstream to investigate when -- when the barrier forms and which regions of the brain and spinal cord become

protected and when.

And I believe some of the molecular regulation that's -- that's been uncoupled so far supports a lot of similarities between the zebrafish mammalian systems. So it's a combination of transcytosis and tight junction regulation that dictates what size molecules can get through and when.

So there's a -- there's a literature on that. I'm not personally an expert in it, but it is out there. So I think you could -- you could definitely approach questions of barrier penetrance and the zebrafish, if you have the right timing, and knew where -- knew where to look.

DR. PADILLA: My understanding is it's controlled by many of the same molecular mechanisms too.

DR. WAGNER: Yeah.

CHAIRPERSON LUDERER: Yeah. Thank you for that.

Dr. Woodruff.

COMMITTEE MEMBER WOODRUFF: Just let me -- I just want to make sure I got that -- I understood that, that you said that the blood-brain barrier formed 3 days post-fertilization, is that what you said? That wasn't my question, but then when you said that, I wanted to make sure I understood that.

DR. WAGNER: I think it's 3 to 5 days, but I'd

have to check to be perfectly --

COMMITTEE MEMBER WOODRUFF: But it's after fertilization some day -- number of days.

DR. WAGNER: Yeah.

COMMITTEE MEMBER WOODRUFF: Okay. That's. Thank you. I just wanted to follow up on Stephanie's point, and I just -- going back to, you know, the ability of the zebrafish to -- or exposures in the zebrafish to be predictive of what we might see in other species. And I thought your presentation on chlorpyrifos and BPA were pretty illustrative. Those are two chemicals that we've already declared as a committee as developmental reproductive toxicants, and they seem to be well conserved in your model. I wonder if you could comment on that or -- in that, I feel that -- think -- I mean, I agree about the exposures, but I also think that we -- thinking about chronic exposures is really important for us, because that's typically what we are looking at in our -- in the population of humans is not one-time exposures. That can occur with some of these chemicals, but for many of the chemicals that we're evaluating, it's a chronic exposure.

And I think that the -- also, the information you purpose -- presented on concordance in general with other zebrafish models is also very useful for us, because I

think that zebra -- I'm just going to say, I think zebrafish is an underutilized tool for us for evaluating toxicity of hazards. And I think just thinking about all the way to the beginning presentation about conservation of development across species is generally true or majority or super majority true, I think helps us -- I think moves -- I think zebrafish should be, you know, placed more in our wheelhouse for evidence that we're evaluating for toxicity and hazard.

DR. PADILLA: So I actually don't really remember collecting the BPA data. All I know is it was positive.

COMMITTEE MEMBER WOODRUFF: Oh, you showed some on the -- on the slides.

DR. PADILLA: I know. I know.

COMMITTEE MEMBER WOODRUFF: Yeah.

DR. PADILLA: But as I said, I mean, we -- we collect most of our data in a blinded fashion. We -- the -- for the chlorpyrifos, chlorpyrifos is a chemical that -- that I studied in many different ways and I had a lot of it, so -- and it tends to be very stable in DMSO. And so we decided to use it as our positive control. And it's very interesting, because we were checking these plates every day. And I always would get sucked into thinking that the chlorpyrifos positive control wasn't working, because the fish would look completely normal up

until about day 5, which is about the time that the liver comes on board. And so as soon as the liver comes on board, and the Chlorpyrifos gets converted to the chlorpyrifos oxon, then the fish begins to go down hill developmentally. And by day 6, we always see either death or malformation in the -- in the chlorpyrifos animals.

And we've -- we've also run other people's chlorpyrifos. We've also run chlorpyrifos oxon and so it is extremely toxic very early in development. And so it appears to be behaving. We also use it -- we haven't talked about this, but I also use it as a positive control for my behavioral studies in zebrafish. So even when we dose at levels that don't cause any developmental effects in the zebrafish, we still see behavioral effects. And the behavioral effects, if we keep them until they're 14 days old, are even more pronounced at 14 days than they were at 6 days.

So it seems to be a chemical that is behaving as you would predict it from the human and mammalian laboratory animal tests in the zebrafish. But, I mean, that's one example.

CHAIRPERSON LUDERER: Thank you.

Any -- any other thoughts or questions on that topic or other topics? We can think about it while I ask whether we have any public comments. Did we have any

comments from members of the public or questions?

**PUBLIC COMMENT**

MR. LEICHTY: If a member of the public would like to make a comment, please use the Zoom function to raise your hand.

CHAIRPERSON LUDERER: Have we -- we haven't received any in the chat, I don't think, or -- I guess they -- or -- or through email.

MR. LEICHTY: We do have one hand raised.

CHAIRPERSON LUDERER: Let's see. I'm not -- let's see, I'm not seeing that.

MR. LEICHTY: Okay. Well, I -- I can allow that -- oh, well, it looks like they just lowered their hand.

CHAIRPERSON LUDERER: Okay. All right. And we don't have any to read either, is that right?

MR. LEICHTY: Okay. And they've raised their hand again, so I'll let that person speak.

CHAIRPERSON LUDERER: Great.

MR. LEICHTY: You have five minutes.

MS. BURGESS: Thank you for this -- all these presentations. They were really wonderful. My name is Sean Burgess. I am at the University of California, Davis. And I'm also a colleague of Dr. Draper who spoke this morning.

I just want one comment about the chronic exposure versus the kind of more immediate exposure that was -- that was just mentioned. You know, I think that one -- one point is is that, you know, during development or during human development, there will always be some stage where they're being exposed to whatever they're being exposed to, and it can have an effect. So I don't think -- I think that, you know, thinking about chronic exposure is important, but I also think you can think about how you can have kind of acute exposure just because, you know, we develop and experience all stages of, you know, embryogenesis. Any one of those could be sensitive to one of those chemicals. So I was wondering if anyone could comment on that.

CHAIRPERSON LUDERER: Thank you for that comment.

Did any of the -- I think you mentioned one of the speakers. Do any of the speakers want to comment on that?

I mean, certainly I don't think we should discount acute exposures. You know, obviously, they -- you can have different -- different information based on the exposure paradigms and that obviously is something to think about what are the -- what are the exposure paradigms that maybe would be most useful in this type of an application. And I think there was an argument made

for -- for chronic exposures, but certainly as we saw from some of our speakers, acute exposures can also have very pronounced effects. And I see that Dr. Padilla has her hand raised.

DR. PADILLA: Yes. So, I mean, one of the things that we've often thought about, sort of like what Sean is talking about, is one day in the life of a zebrafish embryo is a very long time when you think of it compared to a human embryo or -- I mean, so it's -- it's hard to quantify those differences, and to -- and to equate them. So, I mean, so much happens in a very, very short time for zebrafish development that -- that, you know, an entire human trimester could be a day and a half. And so it's -- it's -- it is -- it's going to be hard to equate those and decide how you feel about them.

Thank you.

**COMMITTEE DISCUSSION**

CHAIRPERSON LUDERER: Thank you. Any other thoughts from other panel members or speakers?

Dr. Allard

COMMITTEE MEMBER ALLARD: Yeah. I guess I'm going to react to some of the comments that I heard earlier, and forgive me if my comments are not necessarily well put together, but I heard the comment moving away from the point of interest, and, you know, thinking about

the concordance sometimes between rodent species, between a mouse and a rat, or, you know, thinking about the lagomorphs as well, like bringing in the rabbit. And the problem is is between each one of them and -- and humans, those concordances are sometimes not great either. And using an evolutionary approach where we look at multiple species of the same time to really identify the critical chemicals that seem to alter the same developmental pathways or similar developmental pathways across phyla is actually to me a much more powerful tool to really identify something that will be potentially potent in -- in humans as well.

Yeah. The other thing I think that's -- to me that's also important to think about is that I think we're all kind of making, or some of us, including me, I think we're making comments about, you know, replicability between laboratories and whether that's concerning or not. But this problem for replicability, at least I'm -- I'm sort of heartened to see that -- that, you know, several labs look at the data, analyze the data together using the same tools, which is often lacking in vertebrate data, which also suffers from replicable -- replicable -- the lack of repetition -- I cannot say it, but you understand what I'm trying to say. And we've seen that on this panel before, right? We look at this data and we have X number

of studies showing a positive outcome and X number of studies showing a negative outcome and then we use weight of evidence.

It's kind of part of the -- the process of weighing those things. But at least with zebrafish data, what I'm again heartened to see, and I'll repeat myself, is the fact that then you can take large data streams where people have screened large amounts of chemicals, large number of chemicals and then use similar methodologies to -- to analyze this data and compare it. And I -- I think this is actually very powerful.

CHAIRPERSON LUDERER: Thank you, Dr. Allard.

Let's see, I just that someone else had their hand raised, but I think -- okay. I think they lowered their hand.

Any other comments from other Committee members or from any of the panel members?

Dr. Baskin.

COMMITTEE MEMBER BASKIN: So, you know, looking at all the zebrafish data and then getting, you know, incredible lectures today, it seems like it's going to be a very important model. And I was kind of just asking staff of OEHHA, it -- would the future possibly be that this would be kind of our first line of screening tool and then if there was a question, we would still go to, you

know, mouse/rodent models, or do we think that this is going to replace the use of mouse and rats?

CHAIRPERSON LUDERER: So you're ask -- are you asking for the staff to respond to that?

COMMITTEE MEMBER BASKIN: Or whoever would like to comment on it, because, you know, it's -- I just -- the zebrafish is still a zebrafish.

CHAIRPERSON LUDERER: Dr. Sandy, did you have a comment?

DR. SANDY: Yes. For OEHHA, and others can jump in, but I'd just say that we're not proposing that we would use this as a screen or first step, but we're just acknowledging that there are more studies out there in the literature when we do a search for DART effects on a particular -- with a particular chemical. We're going to -- we're running into -- we already have been presenting it over the last few years data on zebrafish. And we anticipate that we'll see more and more data in zebrafish, so we thought it was useful to delve into this topic in more detail today.

CHAIRPERSON LUDERER: Thank you.

Dr. Woodruff.

COMMITTEE MEMBER WOODRUFF: Yeah, I would hope that we see zebrafish presented as another stream of evidence along with our human and mammalian evidence. I

mean, I -- the data presented today -- I guess I would not say just screening, but it's a line of evidence along with what the other pieces of evidence that we have, and the presenters, including using it to interrogate developmental biology that's relevant to humans, though it feels that this is important sorts of information for us to be evaluating as we're deliberating on our decisions.

CHAIRPERSON LUDERER: Yeah. Thank you. And, of course, we're also evaluating mechanistic data streams too --

COMMITTEE MEMBER WOODRUFF: Right.

CHAIRPERSON LUDERER: -- with culture models and putting it all together.

COMMITTEE MEMBER WOODRUFF: And there's mechanistic data in the zebrafish data.

CHAIRPERSON LUDERER: In the zebrafish data, yes.

Yeah. Dr. Padilla.

DR. PADILLA: So I just want -- sorry to talk so much, but I'm really enthusiastic about this. And so one of the things that we haven't talked about today is there is a whole literature on using zebrafish to discover the underpinnings for human disease. And there's been very successful -- there's a whole literature about tank to bedside, where there's -- there's very little laboratory mammals in between. And that's also very interesting and

may give you more faith in some of the decisions that you want to make.

And then also, I wanted to respond to Dr. Baskin's remark. As somebody who's been trying to convince the regulatory community of the usefulness as zebrafish, one of the things -- and you sort of alluded to this. The first thing is sort of a screening of prioritization, right? We have 100 chemicals, which ones are the worst -- which ones are the worst actors? And so, you know, can we identify those and then test them in our more familiar laboratory animals?

And then as the Europeans are beginning to move to, they -- you know, actually regulating on human toxicity using Zebrafish data. But for that, of course, you need to understand exposure. So these are very common problems that many, many other regulatory agencies are trying to struggle with.

And in my experience with the EPA, 10, 15 years ago, they really weren't too much interested in zebrafish data. But now, because there is so much out there, as -- as you all were talking about, you do a search and all of a sudden you've got all these zebrafish papers. There's so much out there, that it kind of behooves people to figure out how it is useful, which is I know what you all are struggling with, but everybody else is struggling with

it too.

But there is a whole literature out there using zebrafish to -- to determine mechanisms and mechanisms of disease and identifying chemicals that could possibly treat disease that we haven't even touched on.

CHAIRPERSON LUDERER: Thank you.

Dr. Hertz-Picciotto.

COMMITTEE MEMBER HERTZ-PICCIOTTO: All right. Yeah, I think several people have already, you know, hit on some of the main -- the main issues, and just somewhat reiterating and maybe putting a little different light on my earlier comments. You know, I find the -- what -- you know, what Stephanie just said to be really interesting that, I mean, obviously we're focused on developmental, reproductive, that's -- that's the -- that's this Committee's charge. But the use of zebrafish widely now in a lot of diseases, I think, you know -- and I should be looking at that too -- does -- does increase, you know, some confidence in -- in utilizing these data.

And, you know, I continue to -- to be curious, not just about dose but routes of exposures and that entire, you know, unknown, and is there uncertainty really about how -- how to -- how to compare across routes of exposure, which -- and, in fact, I, you know -- really some of the questions of -- for some of these exposures,

how are -- do we -- what data do we have about, you know, low level human exposures. You have to kind of look at it chemical by chemical. And there's still -- we have a lot on some chemicals and maybe a lot less on some others, including, you know, domoic -- domoic acid, it seems.

But, you know, with all of those caveats, the basic question of does -- does this particular exposure pose a potential -- does it have the potential to cause reproductive or developmental harm? It still stands as -- as valid evidence in living systems, including organisms that share quite a bit with the humans, and 70 percent of our -- of our genes, so -- and the genes regulate our responses to -- to, you know, exogenous insults.

So from that perspective, you know, I think, you know, obviously it requires looking at all the mechanisms, looking at exposures, looking at routes, looking at -- and the endpoints and how, you know, what -- do those -- the -- do the -- what was it, the Mauthner -- Mathen -- I've already forgotten the name of the cells.

DR. PANLILIO: Mauthner

COMMITTEE MEMBER HERTZ-PICCIOTTO: Yeah. Are -- do the correlates respond similarly to similar types of -- of triggers? And, you know, those -- those are -- those are all answerable questions, you know, with -- with maybe some certain kinds of experiments that are a little dif --

just, you know, slight perturbations of your -- of current sorts of experiments, which, I mean, that's the beauty of experimental science, that, you know -- as an epidemiologist, I can boast that, oh, it's relevant for the people we're trying to protect directly, but boy, do I get envious when I hear some of these presentations where you can, you know, have a very specific question, you can answer that question, then take that result, and that raises the next question of, okay, then does this -- you know, the next step in the pathway.

So, yeah, it's -- it's -- it's compelling in many ways and I -- and I really appreciate the presentations and the -- and today really opening up that -- that -- that issue for us on this panel.

CHAIRPERSON LUDERER: Thank you.

Dr. Allard.

COMMITTEE MEMBER ALLARD: Sorry. I know I talk too much. I guess I would just like to reiterate the point made earlier is that as the number of studies are growing again exponentially if you look at PubMed, and we're going to -- probably going to review a lot more of that data in the future, I think it's very important to have expertise related to noncanonical or alternative animal models including zebrafish especially, both on the staff side and perhaps on this panel as well, so that we

can really easily tell apart a high-quality study, used sound methods for people in the field as opposed to the opposite, not sound methods. So being able to tease those things apart will require expertise. And I'm not sure if we right it now. It would be great to know whether we do or not. But if we don't, then we definitely need to have that in-house.

CHAIRPERSON LUDERER: Thank you.

I'm just looking to see if there are any additional comments from panel members or from any of the presenters.

Certainly, we've heard a lot of really great presentations today about the -- the -- the utility of using zebrafish to -- both to understand mechanisms and to -- potentially for -- and also for -- for screening for toxicity. And I -- as we -- I think we've heard from several panel members too that the -- that the -- given the increase in the number of studies in zebrafish and also just the utility of looking at various different model systems, and multiple streams of evidence from mammalian systems and the fish models as well as potentially other models, and of -- the utility of comparing those systems and especially if there is -- if there are similar signals that come out in multiple model systems how that strengthens the evidence for reproductive

and developmental toxicity since that is our focus.

So are -- if there are no other comments that -- and I don't see any additional raised hands, then I think that we can check one more time with Julian whether there are any additional public comments. And if not, I think we can move on to the next item.

MR. LEICHTY: I'm not seeing any at this time.

CHAIRPERSON LUDERER: All right. Great. Thank you very much and I'd like to thank all of our speakers again too.

## III. CONSENT ITEM - UPDATE OF THE CALIFORNIA CODE OF REGULATIONS TITLE 27 SECTION 27000 LIST OF CHEMICALS WHICH HAVE NOT BEEN ADEQUATELY TESTED AS REQUIRED

CHAIRPERSON LUDERER: And now we're going to move on to the consent item, which is an update on the California Code of Regulations Title 27, Section 27000, list of chemicals which have not been adequately tested as required. So we are now ready to take up this consent item. The Committee is being asked to affirm changes in response to submissions from the Department of Pesticide Regulation. The U.S. EPA has indicated that there are no changes. This is a ministerial duty of the Committee and that we rely on the information provided to OEHHA by the Department of Pesticide Regulation and U.S. EPA in order

to identify the chemicals that need to be added to or removed from the Section 27000 list.

I'd like to introduce OEHHA Special Assistant for Programs and Legislation Julian Leichty to give the staff present on this item.

Julian.

MR. LEICHTY: Thank you, Dr. Luderer.

So Proposition 65 requires the State to publish and update annually a list of chemicals that are required to be tested under federal or State law for carcinogenicity or reproductive toxicity and have not yet been adequately tested as required. This list can be found in Title 27, Section 27000 of the California Code of Regulations and is commonly referred to this -- to -- referred to as the Section 27000 list.

It's separate and distinct from the Proposition 65 list of chemicals known to cause cancer or reproductive toxicity. Section 27000 list has no regulatory impact. It does not require that any testing be done. Rather, it's a source of information concerning chemicals that need further testing pursuant to State or federal law.

To update the list, OEHHA requests information from the California Department of Pesticide Regulation and the U.S. Environmental Protection Agency's Office of Pollution Prevention and Toxics, and Office of Pesticide

Programs each year.

This year, OEHHA staff reviewed these responses and identified one recommended change to the Section 27000 list, removal of bromadialone. Based on information received from DPR, data requirements for this compound have been fulfilled and further carcinogenicity and reproductive toxicity testing are not required.

The letter from DPR along with additional background, response letters from U.S. EPA, a mock up of the proposed change are available in the staff report provided to the Committee and posted online on September 30th. The proposed change is also shown on this slide.

As Dr. Luderer mentioned, this is a consent item and ministerial duty of the Committee, and that the DARTIC and CIC committees use the information provided by DPR and U.S. EPA to identify the chemicals that need to be added to or removed from the Section 27000 list.

We ask the Committee members to vote in favor of the proposed change, so we can update the list. Unless you have any questions, I will now turn it back to Dr. Luderer.

CHAIRPERSON LUDERER: Thank you, Julian. So again, are there any questions from the -- any panel members?

Okay. I'm not seeing any raised hands. The --

then I will read the question. So the question we're voting on is should Section 27000 of Title 27 of the California Code of Regulations be amended as indicated in the staff report? And I will now call your names and ask you to vote yes, no, or abstain on this question.

Dr. Allard?

COMMITTEE MEMBER ALLARD: Yes.

CHAIRPERSON LUDERER: Dr. Auyeung-Kim?

She may have --

COMMITTEE MEMBER AUYEUNG-KIM: Yes.

CHAIRPERSON LUDERER: Yes. Okay.

Dr. Baskin?

COMMITTEE MEMBER BASKIN: Yes.

CHAIRPERSON LUDERER: Dr. Carmichael?

COMMITTEE MEMBER CARMICHAEL: Yes.

CHAIRPERSON LUDERER: Dr. Hertz-Picciotto?

COMMITTEE MEMBER HERTZ-PICCIOTTO: Yes.

CHAIRPERSON LUDERER: Dr. Pessah?

COMMITTEE MEMBER PESSAH: Yes.

CHAIRPERSON LUDERER: Dr. Plopper?

COMMITTEE MEMBER PLOPPER: Yes.

CHAIRPERSON LUDERER: Dr. Woodruff?

COMMITTEE MEMBER WOODRUFF: Yes.

CHAIRPERSON LUDERER: Okay. And I also vote yes. So I affirm that we have the, I guess, the

required -- we have a unanimous yes vote, and I think six yes votes are required to affirm the change. So based on that, we have more than six votes.

## IV. STAFF UPDATES

CHAIRPERSON LUDERER: All right. And next, we will move on to the staff updates. We'll close out this meeting with these updates on the Proposition 65 listings regulations and litigation that have taken place since our last meeting. So I will again ask Julian to present on the chemical listings and safe harbor levels.

MR. LEICHTY: Thanks again, Dr. Luderer.

(Thereupon a slide presentation.) .

MR. LEICHTY: So we are providing you with an update on important Proposition 65 developments since the last DARTIC Committee meeting. I'll start by going over the chemicals or endpoints added to the Proposition 65 list or under consideration for potential listing as well as data call-ins requesting information on chemical toxicity. Then I'll discuss adopted and proposed safe harbor levels.

After that, I will turn it over to our Chief Counsel, Carolyn Rowan, who will provide an update on other regulatory -- other regulatory actions and significant Proposition -- Proposition 65 litigation.

Next slide, please

NEXT SLIDE.

MR. LEICHTY: Since the Committee's last meeting five chemicals have been added to the Proposition 65 list, PFNA and its salts, trimethylolpropane triacrylate, technical grade, tetrahydrofuran, methyl acrylate, and 2-ethylhexyl acrylate.

Additionally, the cancer endpoint has been added for two chemicals previously listed for reproductive toxicity, PFOS and its salts and transformation and degradation precursors, and PFOA.

Next slide, please

NEXT SLIDE

MR. LEICHTY: Two potential cancer listings are under consideration. At the December meeting of the Carcinogen Identification Committee, the Committee will consider whether to list bisphenol A as causing cancer. This chemical was first added to the Proposition 65 list by the DARTIC in 2015.

Finally, antimony and trivalent compounds is under consideration for listing administratively under the Labor Code mechanism.

Since the last DARTIC meeting, OEHHA has issued a data calling on bisphenol S to request information related to its reproductive toxicity. This information may be used in the preparation -- preparation of a hazard

identification document. The data call-in ended last April. BPS was one of the chemicals prioritized by the DARTIC in 2020.

Next slide, please.

NEXT SLIDE

MR. LEICHTY: Since the Committee's last meeting, safe harbor levels have been adopted in regulation for carcinogens. A no significant risk levels -- no significant risk levels were adopted for Oral and inhalation exposures to 1,3-dichloropropene, 1,3-D, and became effective October 1st, 2022.

We also proposed a safe harbor level for antimony trioxide and are reviewing comments received on the proposal. And now I will turn things over to Carolyn.

NEXT SLIDE

CHIEF COUNSEL ROWAN: Thanks, Julian.

So in the first Slide here, we have other Prop 65 regulatory actions from the past year. The first one on the list is safe harbor warning for cannabis smoke and Delta-9-THC exposure. The regs provide non-mandatory specific safe harbor exposure warning methods and content for weed sale products that can expose consumers to cannabis smoke or delta-T -- delta-9-THC via inhalation, ingestion, or dermal application and for environmental exposures to cannabis smoke and delta-9-THC businesses --

where smoking of cannabis, or vaping, or dabbing delta-9-THC occurs. So this regulation became effective on October 1st, 2022.

We also have the safe harbor warning for glyphosate. And this regulation provides safe harbor guidance for businesses that cause exposures to glyphosate from consumer products that require a warning. And this regulation was recently adopted and will become effective on January 1st, 2023.

The next step is the safe harbor warning for acrylamide in food. This regulation also provides safe harbor warning content. This one is for businesses that cause exposures to Prop 65 listed chemicals in food and beverages that require warnings. And we submitted it to OAL last month and we expect to hear back on this proposed reg by October 28th.

And finally, we are actually working on exposures to acrylamide in cooked and heat processed foods. On October 6th, we noticed a second modification of proposed text and documents and information to the rulemaking file. So this regulation is out for comment at the moment.

And litigation, I think is on the next slide.

NEXT SLIDE

CHIEF COUNSEL ROWAN: Thanks, Julian. We have four cases that are currently active that I can provide

updates on. One is the Physicians Committee for Responsible Medicine, or PCRM, versus Newsom. And that case is a challenge to OEHHA's decision not to list processed meats. We're in the discovery stage right now, so we haven't reached any merits, briefings, or hearings. And so that's the update there.

We also have the National Association of Wheat Growers versus Bonta case. This one involves a First Amendment challenge to the glyphosate warning requirement. The challenge centers on the argument that because only the IARC has identified that chemical as a carcinogen, and other agencies including U.S. EPA have said it is unlikely to be a human carcinogen, there can be no warning that will be misleading. The district court determined that required warnings for glyphosate exposures violated First Amendment limits on compelled speech and the AG appealed to the Ninth Circuit. And the case was on hold while OEHHA was preparing its new glyphosate warning reg. Now, that that new regulation has been approved, the case will become active again. The parties are going to file one last brief in the Ninth Circuit and the court will then decide whether to send the matter back to the lower court or proceed to oral argument.

We also have Cal Chamber verus Bonta. This case involves another First Amendment challenge. Here, it's a

challenge to the safe harbor warning for acrylamide. And the district court previously granted a preliminary injunction and the Ninth Circuit affirmed. The case is back with the trial court, although there has been very little activity since the new judge was assigned.

And finally, there is Council for Education and Research on Toxics versus Starbucks. In this case, CERT challenged the OEHHA regulation on coffee as part of a long-running enforcement action. As you may recall, OEHHA adopted a reg essentially saying that chemicals formed in coffee from the roasting and brewing don't require a warning under Prop 65. The coffee reg was used as a defensive in the case and the trial court upheld the regulation and entered judgment for the coffee companies. CERT appealed and the court heard oral arguments in September, this last month, and we are awaiting a decision.

So that's the status of litigation at the moment.

CHAIRPERSON LUDERER: All right. Thank you very much, Carolyn.

Our last item, I'd like to ask Director -- OEHHA Director Lauren Zeise to summarize the Committee actions.

COMMITTEE MEMBER HERTZ-PICCIOTTO: I have a quest --

CHAIRPERSON LUDERER: It looks like Dr. -- yes.

COMMITTEE MEMBER HERTZ-PICCIOTTO: I just have a question. I just had a question. Can you go back one slide. I was trying to figure out did you -- did I hear this -- did you say that the safe harbor warnings for cannabis were not required, the others were, in this -- on this slide or did I mishear?

CHIEF COUNSEL ROWAN: No, the safe harbor warning for cannabis are final and effective now. They were effective October 1st.

COMMITTEE MEMBER HERTZ-PICCIOTTO: Okay. I -- I just misheard something.

CHIEF COUNSEL ROWAN: Yeah, no problem.

## V. SUMMARY OF COMMITTEE ACTIONS

CHAIRPERSON LUDERER: Okay. Thank you. And I'd like to turn over the -- to Dr. Lauren Zeise, Director of OEHHA to summarize committee actions.

DIRECTOR ZEISE: Great. Thanks, Ulrike.

Okay. So we had a fascinating set of presentations and also a really rich discussion on zebrafish. And I think we heard a lot enthusiasm for continuing to present this stream of evidence, this -- this -- the committee this stream of evidence. And you know, I think we'll reflect on the discussions of the Committee and speakers as we prepare the material for the hazard identification documents. And we'll really look

forward to future engagement on present -- presenting this evidence and also evidence on other NAMS. So thank you so much. It was a very full day and a lot of rich discussion. So I thank the Committee and the speakers for that, as well as the staff for all the work to put this session together, and a special call-out to Marlissa Campbell. So thank you so much.

When we -- also, a decision -- a unanimous decision to remove bromadialone from the section 2700[SIC] list. So that was a unanimous finding by the committee. And so -- and the Committee also heard the updates.

So with that, I think we'll wrap it up and I'll turn it back over to Ulrike. I -- again, I just want to thank the Committee for a really rich discussion and all the participation. Very helpful to us and I hope it was -- it's going to be helpful to you as you consider these new data, especially the zebrafish, and other new data streams.

I'd like to thank the audience and also -- and also staff from all the work to put together this meeting. It definitely takes a village for this, so thank you so much.

Back to you, Ulrike.

CHAIRPERSON LUDERER: Yeah. Thank you. Yeah, I'd like to echo Dr. Zeise's comments and thank everyone

that -- the speakers, the staff, the Committee members, and adjourn the meeting.

So thank you, everyone.

Goodbye.

(Thereupon the Developmental and Reproductive Toxicant Identification Committee adjourned at 4:07 p.m.)

CERTIFICATE OF REPORTER

I, JAMES F. PETERS, a Certified Shorthand Reporter of the State of California, do hereby certify:

That I am a disinterested person herein; that the foregoing California Office of Environmental Health Hazard Assessment, Developmental and Reproductive Toxicant Identification Committee was reported in shorthand by me, James F. Peters, a Certified Shorthand Reporter of the State of California, and thereafter transcribed under my direction, by computer-assisted transcription.

I further certify that I am not of counsel or attorney for any of the parties to said meeting nor in any way interested in the outcome of said meeting.

IN WITNESS WHEREOF, I have hereunto set my hand this 30th day of October, 2022.

JAMES F. PETERS, CSR, RPR
Certified Shorthand Reporter
License No. 10063