Learned Discourses: Timely Scientific Opinions

Timely Scientific Opinions

Introduction to the New Incarnation of *Learned Discourses*. First and foremost, the new co-editors of Learned Discourses —David DeForest, Nancy Shappell, and Guy Gilron—wish to pay tribute to our late colleague and the previous editor of *Learned Discourses*, Peter Chapman, who single-handedly shepherded this section of the *Integrated Environmental Assessment and Management* (*IEAM*) journal. Throughout his tenure with *Learned Discourses*, Peter created a vision that inspired and challenged Society of Environmental Toxicology and Chemistry (SETAC) members and other scientists in our field: to share ideas, perspectives, and commentaries on important topics of interest in the *IEAM* disciplinary sphere. This was accomplished in a unique manner: Rather than presenting in the typical "scientific method" format, the articles have a more free-form editorial-style format—short and crisp, but still focusing on science and its implications. The intent is not necessarily to arrive at well-defined conclusions, but rather to explore the thinking behind our disciplines, challenge our assumptions, ask good questions, and think creatively. Peter was known for this, and his vision lives on in this genre. We are pleased to continue co-editing this section in the spirit of Peter's vision, and we look forward to bringing more of these types of perspectives and commentaries to the *IEAM* readership.

In our inaugural issue as co-editors, we are pleased to include 3 articles that provide different perspectives related to "levels of organization" around a single theme: investigation of environmental disruption.

Landis and Fox discuss the way in which omics and biomarkers are typically used, and suggest the use of Bayesian models to improve our understanding of environmental effects;

Shappell discusses the use of fixed ratios in mixture studies, focusing on evidence of issues pertaining to in-vitro studies; and

Anderson and Winkelman probe the differences between effects on laboratory-reared versus field-caught fish.

Intent. The intent of *Learned Discourses* is to provide a forum for open discussion. These articles reflect the professional opinions of the authors regarding scientific issues—they do not represent SETAC positions or policies. Although they are subject to editorial review for clarity, consistency, and brevity, these articles are not peer reviewed.

The *Learned Discourses* date back to 1996, at which time they were part of the North America SETAC News. When that publication was replaced by the SETAC Globe, *Learned Discourses* continued there through 2005. The continued success of *Learned Discourses* depends on our contributors.

We encourage submissions that will inform and stimulate discussion. We expect that articles will address topics of interest to the SETAC community and continue to provide a venue for timely scientific opinions.

Format. All submissions must be succinct: no longer than 1000 words, at most 1 table or figure, and no more than 6 references. References are to be formatted according to journal requirements (http://www.setacjournals.org). Topics must fall within *IEAM*'s sphere of interest.

Submissions. All manuscripts should be sent via e-mail as Word attachments to learned_discourses@setac.org.

In a Nutshell. . .

ECOTOXICOLOGY

Biomarkers, omics, and the curve, by Wayne G Landis and David R Fox

A discussion of the way in which omics and biomarkers are typically used, and suggestion to use Bayesian models to improve our understanding of environmental effects.

Use of fixed ratios in mixture studies, in vitro evidence of issues, by Nancy W Shappell

Are the use of fixed ratios in mixture studies appropriate for concluding additivity of effects? Evidence of issues from in vitro studies.

Differences in vitellogenin production between laboratory-raised and wild fathead minnows: Potential consequences for understanding estrogenic exposure in wild populations, by Jordan R Anderson and Dana L Winkelman

Probing the differences between effects on laboratory-reared versus field-caught fish.

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BIOMARKERS, OMICS, AND THE CURVE

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Our point of departure is Leung (2018), "Joining the dots between omics and environmental management." The paper is a useful overview of the state of biomarkers, omics, and adverse outcome pathways and their potential applications to risk assessment and environmental management. Since the 1980s, biomarkers and now omics have been part of the toxicological literature. Adverse outcome pathways form an organizing cause–effect model and have been widely adopted. The question remains how to effectively match this body of research to current tools in ecological risk assessment, decision making, and adaptive environmental management.

Current ecological risk assessment and environmental management use probabilistic tools such as Monte Carlo modeling and Bayesian approaches (Landis et al. 2017; Carriger et al. 2018). Bayesian networks and influence diagrams can incorporate a diverse array of information to estimate risk and generate management options. Also, Bayesian networks have the ability to accommodate a variety of types of data into the same model (environmental DNA [eDNA], acetylcholinesterase [AChE] inhibition, population modeling) and to innately incorporate uncertainty. It is also possible to use Bayesian networks to search for patterns in large datasets, using case-learning to derive relationships between the input and output nodes (Graham 2016). Case-learning is a type of machine learning noted by Leung (2018) as a possible application of omics.

The issue is not whether biomarkers and omics can be useful to risk assessment and environmental management. The issue is how to incorporate the entirety of this information into the estimation of risk and effective environmental management. In 2018, that answer means a probability distribution describing an exposure–effect relationship, which will result in a probability distribution describing risk to an endpoint. The risk estimate should include descriptions of uncertainty and an understanding of model sensitivity. To adequately describe exposure–effect for biomarkers or other omic applications to estimate risk, we have argued that this means that a regression or curve-fitting approach be applied to experimental design and data analysis (Fox and Landis 2016). After all, toxicity is a curve and not a *p*-value, a continuous response and not a binary effect–no effect boundary.

The difficulty is that in observations of omics papers and posters at the recent Society of Environmental Toxicology and Chemistry (SETAC) North America conference in Minneapolis, Minnesota, USA, and in surveying the literature, biomarker and omics studies often are reported using the null hypothesis significance testing approach. The results are published as a set of test concentrations, together with the mean response of a set of replicates, a standard deviation, and the *p*-value for that exposure. The *p*-values decrease as exposure increases, but not always. Using the typical design of exposure doses increasing by a factor of 10 for each treatment means that for many chemicals the transition from no effect to maximum effect will be underdescribed. This type of analysis is not a complete description of exposure–response.

In contrast, and more than a decade ago, Sandahl et al. (2005, 2007) presented examples of exposure–response curves being used to describe toxicity with a biomarker. Sandahl et al. (2005) describe the relationships between AChE activity and chlorpyrifos activity for coho salmon in a study relating AChE activity to swimming and feeding behaviors. Figure 1 in Sandahl et al. (2005) plots the relevant data sets, shows the curves, and estimates an effect concentration, in this case a benchmark concentration (BMC). Data sets are available for additional analyses. Sandahl et al. (2007) also employed curve fitting to describe the change in response of electro-olfactograms (smell response) to Cu. As in the 2005 paper, the data are made available for further analysis. In both Sandahl et al. papers, the test concentrations were appropriate to cover the entire exposure–response relationship, including points that document the portions of the curve describing the transition from no measured effect to a maximum effect. In the Sandahl et al. (2005, 2007) figures, the confidence intervals are not shown but were calculated and used in the estimation of a BMC.

Compared to computational tools of the mid-2000s, it is now routine to perform curve fitting to describe exposure–response relationships. There are numerous programs and the dose–response curve (drc) package in R Core Team (2013) is freely available for estimating the parameters of a variety of concentration–response models as well as providing measures of uncertainty. Fox and Landis (2016) have demonstrated how similarity measures can be used for categorical information to estimate exposure–response relationships. This type of tool could be used for situations in which multiple markers are being used and the measurement is an up or down regulation.

If biomarkers and omics are to be incorporated into risk assessment, decision making, and adaptive management, it is vital that we not make the error of designing experiments and reporting data for binary outcomes. It is imperative in the biomarker–omics field to design experiments and conduct analyses in a manner that describes the entire exposure–response relationship for use in the most current methods for quantifying ecological risk and evaluating management options.

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USE OF FIXED RATIOS IN MIXTURE STUDIES, IN VITRO EVIDENCE OF ISSUES

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In attempts to investigate the effects of chemicals in a way that more accurately reflects the reality of environmental exposure, scientists have moved from evaluating single chemical exposure to mixtures. The logic behind this approach is undeniable, given that species of concern are rarely exposed to only a single compound with potential detrimental effects. As our instrumentation has gotten more advanced, and our list of contaminants of emerging concern expands, our mixture experiments have included a greater number of test chemicals. A standard approach in mixture experiments has been to prepare a mix of the chemicals, each present at a fixed concentration, typically based on their biological activity relative to one another, and in a few instances formulations have been based on measured environmental concentrations. These mixtures are then "dosed" at various dilutions of the mixture. Results of these experiments are typically used to support the theory of chemical additivity, although this design truly assesses only the effects of increasing mixture concentrations.

Before attempting to assess chemical mixtures, compounds should be investigated two at a time, holding the concentration of 1 chemical constant while varying the concentration of the other chemical. Starting at the receptor level, we know at a minimum that ligand binding to a receptor can be competitive or noncompetitive, acting as either an agonist or antagonist. Although a ligand's affinity for its receptor is often considered, the environment does not typically provide ligands to the receptor on an equimolar basis. Therefore, it is critical to take the environmental concentrations of the 2 chemicals into account, at the site of specific concern. In the case of natural estrogens present in livestock waste, the most potent natural estrogen, 17ß-estradiol (17ßE2), can be found at 11× to 141× lower concentration than the less potent estrogen estrone (E1, Table 1). Bermudez et al. (2012) tested the estrogenicity of a mixture of various natural estrogens at concentrations found in various animal wastes, using the in vitro transcriptional assay KBluc. Transcriptional activation of the estrogen receptor was assessed using dilutions of the fixed ratio mixtures, comparing predicted to measured estradiol equivalents (EEQ). Laboratory-prepared mixtures representing measured dairy and swine waste estrogen concentrations resulted in lower than predicted estrogenicity. Diminished responses were also observed with a mixture that included ethinyl estradiol, the most potent synthetic estrogen. In in-vivo experiments on fathead minnows, fish were exposed to an estrogen mixture containing each individual estrogen at its EC50 (Brian et al. 2005). Fish were exposed to dilutions of the fixed ratio mixture for 21 d, and induction of plasma vitellogenin (an egg protein) was measured. Though induction results were similar to those predicted, individual responses were highly variable. But of primary concern is that testing of dilutions of fixed ratio mixtures does not establish chemical additivity, but instead provides only a dose response of the specific mixture used.

In our laboratory, a contradiction to the currently accepted theory of chemical additivity of natural estrogens was serendipitously exposed in an in vitro proliferation assay used to assess estrogenicity (E-Screen, human mammary epithelial cells [Soto et al. 1995; Shappell 2006]). In order to ascribe the estrogenic activity of a sample to specific estrogens, the same samples were also analyzed by liquid chromatography tandem mass spectrometry (LC-MSMS). Although extraction losses are typically accounted for by fortification with deuterated internal standards prior to solid-phase extraction, this approach was inappropriate because deuterated standards have the same biological activity as the native compounds. Alternatively, mass spectral analysis of samples was compared ± preextraction fortification with E1, which has 1% to 2% the EEQ of estrogenic activity of 17ßE2 by E-Screen. Results of the mass spectral analysis were as expected, that is, fortification with E1 was reflected by an increase of E1 concentration. But addition of E1 to the samples repeatedly resulted in a decrease, rather than an increase, in the estrogenicity measured by E-Screen.

Subsequent experiments were conducted to ascertain the effect of increasing concentrations of E1 or 17α-estradiol (17αE2), in the presence of constant 17ßE2 on cellular proliferation. As illustrated in Figure 1, the proliferative response to 17ßE2 (Panel A) is reduced in the presence of E1 and 17αE2 (Panels B and C, respectively). The presence of weaker estrogens, at one-tenth the concentration of 17ßE2, reduced the cellular proliferative response to 17ßE2. Therefore, rather than chemical additivity, the presence of weaker estrogens

Table 1. Estrogen composition in agricultural waste[a]

Livestock[c]		Dairy 1° lagoon		Sows 2° lagoon		Finisher swine		Poultry 1° lagoon		Poultry 2° lagoon	
Estrogen		ng/L	Ratio X/ßE2[b]	ng/L	Ratio X/ßE2[b]	ng/L	Ratio X/ßE2[b]	ng/L	Ratio X/ßE2[b]	ng/L	Ratio X/ßE2[b]
ßE2		27	1	962	1	152	1	64	1	21	1
E1		1816	67	45379	47	1640	11	1570	25	2970	141
αE2		100	4	5189	5	184	1	408	6	131	6
E3		0	—	6789	7	1540	10	489	8	190	9

[a]Gall et al. 2014.
[b]Ratios are of X estrogen concentration to 17ß-estradiol (17ßE2).
[c]Image credits: Cow and nursing sow were released by the Agricultural Research Service (the research agency of the US Department of Agriculture) to Wikipedia Commons. The chicken photos are Creative Commons photos, without attribution.

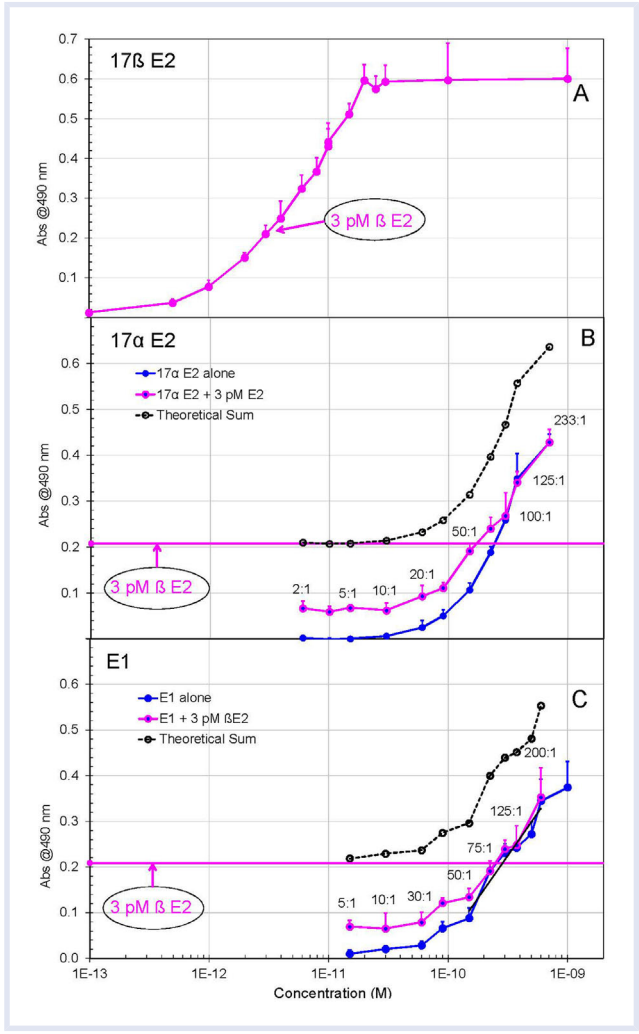

Figure 1. Effect of estrone (E1) or 17α-estradiol (17αE2) on cellular response to 17ß-estradiol (17ßE2). The effect of coincubation of estrogens was tested, holding 17ßE2 concentration constant (3 pM), while varying the concentration of less potent estrogens (E1 from 15 to 375 pM, and 17αE2 from 6 to 300 pM). Standard curves were run concurrently. Molar ratios of E1 or 17αE2 to 17ßE2 are indicated by values on graph. Data are means ± SD of 5 wells from a representative experiment.

actually decreased the effect of the most potent natural estrogen. The explanation for the reduction of proliferation could be as simple as competition for receptor binding, or more complicated, possibly involving a feedback response.

It is known that even receptor binding assays can exhibit nonadditivity. Proliferative responses of screening assays of mixtures, as well as in vivo exposures, should always consider the potential for homeostatic responses. Although Ankley et al. (2017) elegantly demonstrated the capacity of fathead minnows to convert E1 to 17ßE2, it remains unknown as to how an environmentally relevant mixture of E1 and 17ßE2 would affect the in vivo production of 17ßE2, or its conversion from E1. The overarching goal of our experimental investigations is to predict organismal responses to endocrine disruptors. Although this discourse addresses investigations of mixtures and whether they reflect additivity of action, scientists, whether using in vivo or in vitro assays, need to consider an organism's potential for homeostatic responses.

Assays measuring receptor binding or short exposure transcriptional activation do not reflect an organism's capacity for up or down regulation of receptors, transcription, translation, or …. The issue with fixed ratio mixtures was investigated using estrogenic endocrine disruptors, but it may also prove relevant to evaluate the use of fixed ratio mixtures in the study of metal effects on biological systems.

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DIFFERENCES IN VITELLOGENIN PRODUCTION BETWEEN LABORATORY-RAISED AND WILD FATHEAD MINNOWS: POTENTIAL CONSEQUENCES FOR UNDERSTANDING ESTROGENIC EXPOSURE IN WILD POPULATIONS

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Endocrine-active compounds (EACs) are among many chemicals of concern for fish and wildlife populations because they can cause reproductive malfunction and decreased survival on an individual basis, and may also have population-level consequences (Kidd et al. 2007; Vajda et al. 2008; Schwindt et al. 2014; Schwindt and Winkelman 2016). However, estrogenic EACs can be challenging to study because they are difficult to measure at environmentally relevant concentrations. Difficulty in directly quantifying estrogenic EACs results in the frequent use of biomarkers and bioassays to determine whether EACs are present within aquatic habitats. One frequently used biomarker to indicate estrogen exposure is the production of the egg yolk precursor protein, vitellogenin (VTG), in male fish (Flick et al. 2014).

One potential challenge with the use of biomarkers is that they are often assessed using model test organisms, such as fathead minnows (FHM), *Pimephales promelas*, from laboratory-cultured populations. Although little is known about the consequences of rearing test organisms in controlled environments, it has been documented that hatchery-raised species are not exposed to natural selection pressures and have reduced fitness when released into natural environments (e.g., Christie et al. 2014). Moreover, laboratory populations can have less genetic variation than do their wild counterparts (Coe et al. 2009), potentially resulting in important differences in their responses to environmental stressors. If laboratory-raised FHM differ from their wild counterparts, then inferences made from laboratory experiments may not reflect the responses of wild fish to estrogenic EACs and other compounds found in wastewater effluent.

One goal of our research has been to evaluate wastewater treatment facilities (WWTFs) in several locations in the South Platte River drainage in northern Colorado, USA, for evidence of estrogenic chemicals in their effluents. To accomplish this goal, we caged laboratory-raised male FHM at a number of WWTFs for 7 d and then performed real-time quantitative polymerase chain reaction (qPCR) to determine if they were producing VTG messenger RNA (mRNA). The results we are presenting here are from 1 effluent that consistently induced VTG mRNA in laboratory-raised male FHM. At this site, we also measured VTG mRNA in wild FHM. In another experiment conducted at the study site, we caged wild FHM alongside laboratory-raised FHM to restrict the potential movement of wild FHM. Comparisons of VTG expression between laboratory-raised FHM with wild FHM illustrated 3 important trends:

1) Laboratory-raised FHM caged downstream of the WWTF effluent showed consistent elevated expression of VTG mRNA compared with FHM caged upstream (Figure 1),
2) wild FHM caught in the effluent near the downstream caging sites yielded low levels of VTG mRNA expression (Figure 1), and
3) wild FHM caged in the effluent next to laboratory-raised FHM also showed significantly lower VTG expression than did laboratory-raised FHM; however, they had higher VTG expression than did wild fish caged upstream of the effluent (Figure 1).

We currently propose 3 working hypotheses that could explain the differences in VTG expression between wild and laboratory-raised FHM. First, the elevated VTG expression observed in caged wild FHM compared with unconfined wild FHM indicates that movement and avoidance of effluents could be occurring. The difference in VTG

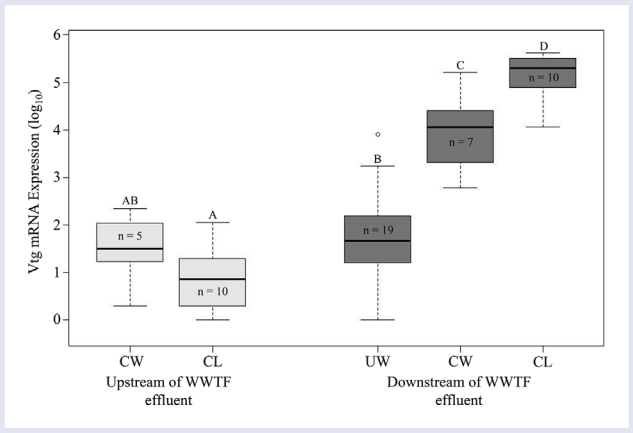

Figure 1. Vitellogenin mRNA expression for caged FHM both upstream and downstream of the WWTF effluent. All data were log10-transformed to meet the assumptions of equal variance and analyzed using ANOVA. Different letters above each box indicate significant differences at $p < 0.05$ (Tukey's HSD test adjusted for multiple comparisons). CL = caged laboratory FHM; CW = caged wild FHM; FHM = fathead minnow; HSD = honest significant difference; mRNA = messenger RNA; UW = unconfined wild FHM; VTG = vitellogenin; WWTF = wastewater treatment facility.

expression between wild FHM caged upstream and downstream of the effluent may indicate that wild FHM can avoid exposure by moving upstream of the effluent. There may also be refugia from effluent exposure for wild FHM downstream; however, it is important to reiterate that the resident wild FHM in our study were restricted to an effluent discharge ditch. The ditch comprised more than 80% effluent during our caging events, and it seems unlikely that wild FHM could entirely avoid exposure. Second, wild FHM may have become physiologically more tolerant compared with laboratory-raised FHM due to chronic exposure, which we refer to as "acclimation." Third, wild FHM may have genetically adapted to chronic exposure through natural selection. The latter 2 hypotheses are supported by differences in VTG expression of wild and laboratory-raised FHM caged in the effluent and the low VTG expression of unconfined wild FHM. Currently, we consider all 3 hypotheses to be viable explanations and we are designing experiments to address them.

Our results have 2 consequences regarding inferences about estrogenic exposure and suggest that using observations from both wild and laboratory-raised populations is sensible. First, we never measured significant VTG mRNA upregulation in unconfined wild fish at our site. Had we relied solely on collection and analysis of unconfined wild FHM, we would have incorrectly concluded that there was no evidence of estrogen exposure at our study site. Second, differences in VTG expression between unconfined wild and caged laboratory-raised FHM indicate that laboratory-raised FHM may not reflect responses of unconfined wild FHM. Currently, population-level analyses and models rely on data derived from experiments with laboratory FHM and may bias conclusions regarding how wild FHM are responding to effluent exposure. Both consequences represent valid concerns, regardless of the mechanism responsible for the differences in VTG expression. Our findings suggest that inferences regarding the responses of wild fish made from experiments with laboratory populations should be viewed with caution; more research is needed to address these concerns. On the other hand, inferences regarding exposure made with unconfined wild fish may underestimate exposure and should also be regarded with caution. In addition to potential consequences regarding inference, it is important to note that wild FHM in many rivers and streams face chronic exposure to effluent and may be acclimating or adapting to effluent-dominated environments. Understanding the long-term consequences of exposure will be critical to population-level assessments.

Disclaimer—Any use of trade, firm, or product names is for descriptive purposes only and does not imply endorsement by the US Government. This study was performed under the auspices of Colorado State University protocol #15-5883.

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