(Perspective)

Endocrine Disruption in Invertebrates: A survey of research progress

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Abstract

Man-made chemicals can interfere with endocrine processes and have permeated many ecosystems. Arguably, the most devastating example of endocrine disruption occurred in gastropod molluscs which led to the banning of tributyltin. The invertebrates consist of ~95% of all known animals and possess endocrine systems that can significantly differ from that of vertebrates. An expert group in the late 1990s highlighted considerable paucity in our knowledge of these endocrine systems and the limited ability to ascertain risks of endocrine disrupting chemicals (EDCs) to invertebrates. Twenty years later, we surveyed experts in this field on the current state of the science. Respondents agreed that endocrine disruption is still a significant issue and noted that there was key evidence that EDCs were impacting invertebrates groups. Respondents noted a variety of impediments to advancing the science, including inadequate funding, insufficient knowledge to develop appropriate assays, and generally low support for invertebrate studies. Several scientists highlighted that resources were being misdirected with studies that address impacts of vertebrate EDCs or using biomarkers specific to vertebrate endocrine disruption. Sadly, many of the recommendations proposed by respondents matched those made over two decades ago. Accordingly, the field has not advanced as much as one might have expected.

TOC Art

Introduction

Endocrine disruption, resulting from chemical exposure, raised concern as early as 1958 with observations that children taking vitamin pills, contaminated with estrogen during manufacture, developed gynecomastia.[1] The potential for exogenously administered estrogens, resulting in *in utero* exposure, to elicit significant adverse effects was dramatically demonstrated with the drug diethylstilbestrol (DES).[2] Subsequently, the potential for environmental chemicals to disrupt endocrine function entered the sphere of scientific awareness.[3] Much of the research on endocrine-active chemicals initially focused upon environmental estrogens and subsequently was expanded to include disruption of androgen and thyroid signaling.[4,5] This focus upon estrogen, androgen, and thyroid signaling was driven by regulatory mandates.[5] Continued research established that many endocrine signaling processes are susceptible to perturbation by environmental chemicals and accordingly warrant consideration when evaluating the safety of drugs and environmental pollutants.[6]

Just as the number of endocrine signaling processes, that are susceptible to disruption, has expanded over the years, so has the number of species. Initial emphasis and legislation focused upon the protection of human health.[7] Subsequently, the susceptibility of wildlife to environmental endocrine disruptors was realized.[8] Many compounds have been shown to pose hazard to wildlife through their action as endocrine disruptors[8], though low potency and low concentrations in the environment raises uncertainty regarding risk of hazard to wildlife. The exception being steroidal androgens and estrogens used in agriculture or in birth control pills, which are of high potency and can be present in aquatic environments at active concentrations.[9,10] Despite the growing interest in endocrine disruption in wildlife, research involving invertebrates lagged (suppl figure 1).

Endocrine disruption in invertebrates

Blaber (1971) first reported on an intersex condition in the dog-whelk, whereby females developed a penis and vas deferens.[11] This specific form of intersex was call imposex, since it is characterized by the superimposition of male sex characteristic onto a female. Gibbs and Bryan (1986) published a seminal paper that attributed the condition to tributyltin exposure.[12] Surveys revealed that tributyltin-induced imposex caused reproduction dysfunction with resulting population declines.[12,13]

This observation that an environmental chemical (tributyltin was used in anti-fouling marine paints) altered reproductive tract development led to the assertion that tributyltin was the first confirmed case of environmental endocrine disruption in an invertebrate species. Thus began the search for the mechanism by which tributyltin caused imposex. Specifically, a mechanism was sought that involved sex-steroid signaling since these hormones are largely responsible for sexual differentiation of the reproductive tract in vertebrates. Tributyltin-induced increases in testosterone levels or alterations in the androgen/estrogen ratio soon became prominent among proposed modes of action of tributyltin.[14-16] However, steroidal androgens are not involved in reproductive tract development in molluscs.[17] The discovery that tributyltin activates the retinoid X receptor (RXR) and that other RXR receptor agonists cause imposex provided compelling

evidence for a mode of action of this pollutant that did not involve steroid hormones.[18] Subsequent laboratory studies have demonstrated endocrine disruption in a variety of invertebrates[19-21] but lack of effects observed in the environment or absence of a known endocrine mechanism of effect has exemplified the high level of uncertainty that exists in this research domain.

The assumption that vertebrate hormones have similar function in invertebrates permeated early research on endocrine disruption in invertebrates.[9,22,23] Further, the general lack of knowledge of the endocrine system of invertebrates precluded the evaluation of the susceptibility of specific endocrine targets to disruption resulting from chemical exposure. All told, research on environmental endocrine disruption in invertebrates was hampered by misconceptions on the endocrinology of invertebrates and a general lack of knowledge of the endocrine system of invertebrates.

The Society of Environmental Toxicology and Chemistry (SETAC) sponsored a workshop held in The Netherlands in 1998 to address the issue of endocrine disruption in invertebrates. Proceedings of the workshop were subsequently published.[24] Two goals of the workshop were to evaluate what was known of the endocrine system along with chemical-induced endocrine disruption of various invertebrate groups. Insects and crustaceans dominated the knowledge base with respect to both endocrinology and endocrine toxicology. Workshop participants agreed on the need for more research on basic endocrinology and endocrine toxicology, particularly among non-arthropod groups.

Twenty-years later, SETAC's Endocrine Disrupter Testing and Risk Assessment Interest Group (EDTRA IG) held a meeting at the SETAC Europe annual meeting in Helsinki Finland (2019). Preceding this meeting, the authors of this paper surveyed experts in the field of endocrine disruption for their views on the status of endocrine disruptor research in invertebrates. Results of the survey were presented at the meeting and are summarized below.[25]

Survey Design and Questions

The anonymous survey was designed to determine whether the participants believed endocrine disruption in invertebrates was still an issue of concern, how strong the evidence based was that invertebrates were being adversely impacted and how far the field had progressed. Respondents were given the opportunity to highlight any impediments to the field progressing and were able to make free text comments to expand on their opinions. Detailed survey results can be found in Appendix 2. In total there were 46 respondents to the survey working in 12 different countries and representing 15 nationalities. All respondents were active in the field of ecotoxicology. Forty-seven percent identified themselves as male and 51% females plus 2% prefer not to say. The age distribution of respondents was: 4.5% (age 18-24), 40.9% (age 25-39), 45.5% (age 40-59), and 9.1% (age 60+). The majority of the respondents worked in academia (71%) and the rest from government, regulatory authorities and NGOs. Half of the respondents identified themselves as primarily working on vertebrates whilst approximately 40% worked on invertebrates (primarily molluscs, crustaceans and annelids) and 10% in regulatory/risk assessment fields.

Survey Results and Discussion

When participants were asked whether they believe that ED is a significant problem in non-target invertebrates (Q1) the majority replied yes (71.7%) and a smaller proportion either replying No (10.9%) or Don't Know (17.4%). A significantly larger proportion of the researchers studying vertebrates (100%) said yes to this question compared to those studying invertebrates (73.3%) (Fisher's exact test, $X = 5.462$, $df = 1$, $p = 0.033$).

Question 1	**Do you believe that endocrine disruption is a significant problem in non-target invertebrates**		
	Yes 71.7%	No 10.9%	Don't Know 17.4%

A slight majority considered there to be very or fairly strong evidence (~59%) for endocrine disruption in invertebrates (Q2), however interestingly, very few selected 'very strong' (13%) amongst the available options rather opting for 'fairly strong' (45.7%) evidence for endocrine disruption in invertebrates. Approximately 37% of respondents considered the evidence for endocrine disruption in invertebrates fairly or very weak and a further 4% responded 'Don't Know'. There was no significant difference in the responses of those studying vertebrates or invertebrates in answering this question (Chi-square $p > 0.05$). We deliberately asked respondents to exclude imposex in gastropod snails from their deliberations. This suggests that whilst the majority of people believe endocrine disruption is a current issue impacting invertebrates groups, the evidence based to support this view could be improved.

Question 2	**Apart from the well-known know cases of imposex in gastropod molluscs, how strong do you feel the evidence is for endocrine disruption in invertebrates**				
	Very Strong 13%	Fairly Strong 45.7%	Fairly Weak 26.1%	Very Weak 10.9%	Don't Know 4.3%

We followed up this with a question on whether the participants agreed with the statement "*Research on Endocrine disruption in invertebrates has advanced substantially over the past decade*". The opinions were very much split with ~54% agreeing, 35% disagreeing and ~11% neither agreeing or disagreeing. There was no significant difference in the responses of those studying vertebrates or invertebrates in answering this question (Chi-square $p > 0.05$). Free text opinions (see suppl. 2) were also permissible and of the 21 statements submitted the overwhelming majority were from respondents to clearly felt that science hadn't advanced or hadn't advanced as much as they would have expected. Where a limited number had felt there was advancement was in the 'omic' led technologies which were able to not only identify some homologous gene pathways but also link to some plausible adverse outcomes. Conversely,

others felt that ‘omics’ as a tool was only useful when the underpinning well characterised endocrine systems and pathways.

Reasons given for the lack of advancement were insufficient funding, and scientists focusing on the wrong endpoints as the field was driven by known vertebrate endocrine-disrupting chemicals rather than chemicals that might interact with invertebrate endocrine systems. Several scientists suggested the need for basic mechanistic endocrinology from which to fully understand endocrine disruption. It was noted that scientists were ‘trying to run before they could walk’ and that endocrine disruption was often being confused with reproductive toxicology which may or may not be linked to specific endocrine pathways. Some scientists mentioned that we still do not fully understand the population level impacts of endocrine disruption in invertebrates whilst another mentioned that they ‘could not think of a clear case of endocrine disruption in invertebrates apart from imposex in gastropod snails’.

Question 3	**Do you agree with this statement: "Research on Endocrine disruption in invertebrates has advanced substantially over the past decade"**				
	Strongly agree 15.2%%	Mildly agree 39.1%	Mildly disagree 19.6%	Strongly Disagree 15.2%	Neither agree or disagree 10.9%

Participants were asked to state how significant various potential impediments to endocrine disruption research in invertebrates were (see suppl 2). The impediment that accumulated the most number of votes was an insufficient knowledge of invertebrate endocrinology with 65.2% thinking this was highly significant and a further 26.1% considering this moderately significant. However, pooled responses of ‘moderately’ and ‘highly’ significant for each of the options given highly hugely outnumbered those which disagreed with any of the statements. For example, over 90% of respondents considered low regard for invertebrates and 85% considered low regard amongst regulators amongst the reasons for lack of progress with this topic.

Question 4	How significant do you consider the following potential impediments to endocrine disruption research in invertebrates?			
	Insignificant	Moderately significant	Highly significant	Don't Know
Lack of Funding	0% (0/46)	41.3% (19/46)	41.3% (19/46)	17.4% (8/46)
Insufficient Knowledge of Invertebrate Endocrinology	2.2% (1/46)	26.1% (12/46)	65.2% (30/46)	6.5% (3/46)
Lack of interest amongst researchers	15.2% (7/46)	47.8% (22/46)	28.3% (13/46)	8.7% (4/46)
Low regard of invertebrates amongst the general public	10.9% (5/46)	41.3% (19/46)	50% (23/46)	0% (0/46)
Low regard of invertebrates amongst regulatory agencies	15.2% (7/46)	34.8% (16/46)	50% (23/46)	2.2% (1/46)
Other impediments (please comment on next question)	15.2% (7/46)	8.7% (4/46)	13% (6/46)	56.5% (26/46)

Future Perspectives

There is little doubt that studies of endocrine disruption in vertebrate wildlife advanced primarily because the endocrine systems of many wildlife species are closely conserved with that of humans. Secondly, vertebrate wildlife are more appealing to the public, both aesthetically and as indicators of potential health threats to humans. This survey revealed that research in invertebrates suffers from a low regard for invertebrates amongst the general public and therefore also with regulatory authorities. The role of charismatic species in conservation has been well documented[26-28], so perhaps more efforts around environmental literacy surrounding the importance of invertebrates is required to push their importance up the policy agenda.

Despite various efforts to develop invertebrate specific biomarkers and assays for endocrine disruption, international funding programs have favored research on endocrine disruption in vertebrates. Where research was developed for invertebrates it often focused on translating vertebrate specific biomarkers of endocrine disruption or focused on vertebrate specific endocrine disrupting chemicals. For example, very few studies have been able to demonstrate upregulation in vitellogenin biomarkers even in highly feminized crustaceans.[29-31] Ford proposed that biomarkers of de-masculinisation might be more informative than those of feminisation in crustaceans.[32] However, comprehensive analysis of gene expression in parasite induced and non-parasite induced intersex amphipods highlighted that these specimens had many 'female' genes upregulated and very few 'male' expressed genes downregulated.[33] This led the authors to conclude that crustacean intersexuality is the result of broad feminization without any large scale demasculinization contradicting the original hypothesis when looking at all the gene expression holistically. What this process did do however was underscore the need for invertebrate-specific biomarker development to fully answer questions relating to endocrine disruption in invertebrates.

A number of recommendations came from the 1998 SETAC workshop on endocrine disruption in invertebrates [24] which are paraphrased below:

- There is a requirement to conduct basic research on invertebrate endocrinology to remedy our lack of knowledge in mechanisms of action, physiological control and hormone structure and function.
- Research is needed to test endocrine disrupting compounds using a variety of invertebrate bioassays with particular reference to non-vertebrate types of endocrine disrupting compounds.
- There is a requirement for field assessments and surveys informed through valid invertebrate specific biomarkers.
- Standard toxicity tests with invertebrates should be modified to include endocrine-related endpoints.

Sadly, these key recommendations are as valid now as they were 20 years ago and results from this survey indicate that the science has not advanced as vehemently as scientists would have liked. However, research over the last 20 years with vertebrates have revealed some research directions that are relevant to future endocrine disruption research in invertebrates.

- <u>Field Investigations</u> All documented cases of environmental endocrine disruption in vertebrates originated with field observations. Greater emphasis needs to be placed on the evaluation of field populations of invertebrates with sensitivity towards population disruptions that may be associated with putative endocrine disrupting chemicals.
- <u>Biological Target Discovery</u> The identification and understanding of targets of endocrine disruption in various invertebrate phyla requires continued expansion. Evolutionary studies of similarities and divergences among invertebrate endocrine systems would facilitate the identification of common targets of endocrine disruption among invertebrate phyla along with unique targets that may render some phyla particularly susceptible to

some endocrine disrupting chemicals. Biomarkers of specific interactions between endocrine disrupting chemicals and molecular targets in invertebrates must be developed. Such biomarkers could be used as monitors of exposure and predictors of effects.

- Adverse Outcome Pathway Construction Plausible mechanisms of disruption by invertebrate endocrine-disrupting chemicals must be established along with the identification of effects on individuals that could lead to loss of population sustainability.
- Laboratory Corroboration of Field Observations Laboratory studies are required with endocrine disrupting chemicals to investigate adverse outcomes that are relevant to field observations at exposure concentrations that are environmentally relevant.

Like any area of science, research on endocrine disrupting compounds competes for a finite amount of resources. The topic areas of interest within ecotoxicology can be transient based on government and public concerns of the time.[34] Results of this survey indicate that endocrine disruption in invertebrates is a significant environmental issue that continues to be understudied due to lack of resources. Perhaps, this deficiency will be remedied over the next 20 years.

Supporting Information. Number of endocrine disruptor citations in fish and invereberates by decade (SI Figure 1) and free text reponses to Questions 3 and 4 (SI Tables 1 & 2)

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Ethical Approval

This survey was approved by the University of Portsmouth ethic committee.

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Biography

Professor Alex Ford is a Professor of Biology and Deputy Director for the Centre of Blue Governance at the University of Portsmouth (UK). His interests and expertise lie within invertebrate biology, parasitology and environmental toxicology. He is a long serving member of the Society of Environmental Toxicology and Chemistry (SETAC) and has a passion for cross-disciplinary research and scientific communication.