Assessing variation in the potential susceptibility of fish to pharmaceuticals, considering evolutionary differences in their physiology and ecology

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Fish represent the planet’s most diverse group of vertebrates and they can be exposed to a wide range of pharmaceuticals. For practical reasons, extrapolation of pharmaceutical effects from ‘model’ species to other fish species is adopted in risk assessment. Here, we critically assess this approach. First, we show that between 65% and 86% of human drug targets are evolutionarily conserved in 12 diverse fish species. Focusing on nuclear steroid hormone receptors, we further show that the sequence of the ligand binding domain that plays a key role in drug potency is highly conserved, but there is variation between species. This variation for the oestrogen receptor, however, does not obviously account for observed differences in receptor activation. Taking the synthetic oestrogen ethinyloestradiol as a test case, and using life-table-response experiments, we demonstrate significant reductions in population growth in fathead minnow and medaka, but not zebrafish, for environmentally relevant exposures. This finding contrasts with zebrafish being ranked as more ecologically susceptible, according to two independent life-history analyses. We conclude that while most drug targets are conserved in fish, evolutionary divergence in drug-target activation, physiology, behaviour and ecological life history make it difficult to predict population-level effects. This justifies the conventional use of at least a 10-fold assessment factor in pharmaceutical risk assessment, to account for differences in species susceptibility.

1. Introduction

(a) Environmental risks associated with pharmaceuticals

Over 5000 human and veterinary pharmaceuticals are in use, or in development, and they target diverse physiological functions [1]. Many are highly potent, altering physiological processes at low therapeutic concentrations, between 0.05 and 100 µg ml⁻¹ in blood plasma in human/mammalian systems [2]. Furthermore, many drug targets are highly conserved across diverse vertebrate phyla [3–6]. Following the widespread detection of pharmaceuticals in the environment [7–9], concern has been raised over their potential impact on vertebrate wildlife health. The most notable example of an adverse effect in wildlife is for exposure to the non-steroidal anti-inflammatory drug diclofenac. This has been shown to cause population-level declines, and even localized extinctions, in three Asian vulture species (Gyps sp.) scavenging on the carcasses of treated cattle [10,11]. In another case, the contraceptive oestrogen 17α-ethinyloestradiol (EE2) has been linked directly with population-level risks in wild fish, owing to feminization in males and reduced fertility in both sexes of several fish species [12–15].
Generally, however, wildlife populations are exposed to relatively low-level environmental concentrations of pharmaceuticals and data confirming adverse effects are extremely limited. It is widely recognized that better insight and understanding of environmental risks are required concerning both newly developed drugs and older pharmaceuticals, some of which have been present in the environment for decades [16]. Owing to the large number of compounds in use, several schemes have been proposed for prioritizing testing [4,17,18], which include ‘reading-across’ plasma concentrations and therapeutic effects of human and veterinary pharmaceuticals to non-target organisms [19]. However, quantifying both interindividual and interspecies variability in drug uptake and metabolism, and extrapolating between individual physiological responses and adverse population-level effects, represent major sources of uncertainty in pharmaceutical environmental risk assessment (ERA) [16]. Fish make up half of all vertebrate species, inhabiting virtually all aquatic environments [20] and exhibiting enormous diversity in morphology, physiology, behaviour, reproductive biology and population ecology [21]. ERAs concerning the susceptibility of fish to pharmaceuticals, however, are based on studies on only a few model fish species and rarely extend to the quantification of population-level effects.

(b) Assessing the potential susceptibility of fish to adverse effects from pharmaceuticals

The susceptibility of wildlife, including fish, to adverse population-level effects from chemicals and/or pharmaceuticals, depends on their exposure, physiological responsiveness and population resilience [22].

Exposure of fish to pharmaceuticals is generally assumed to be via water [23] (but also see [24]), because the majority of pharmaceuticals partition to the water phase in wastewater treatment and remain in solution following discharge to surface waters. In the cases where pharmaceuticals partition to solids and/or lipids, this may trigger specific studies, simulating benthic sediment exposure and/or potential bioaccumulation in the food chain [25]. There are few data on pharmacokinetics (drug absorption, distribution, metabolism and excretion) in fish [26], but there is a wealth of human/mammalian data, which offers the potential for ‘read-across’ to fish [16]. More studies are needed to explore the extrapolation of external aqueous exposure concentrations to internal drug concentrations in blood plasma [19,27], to confirm whether expected ‘therapeutic’ effects occur in non-target organisms [28]. Drug uptake across fish gills may be controlled by a variety of membrane transporters, including multi-drug transporters [29] and/or more specific transporters such as sex hormone binding globulin (SHBG) which shows affinity across a wide dynamic range for both natural and synthetic steroids [30]. Predicting the metabolism and excretion of drugs in fish is even more challenging. While several fish species possess enzyme systems responsible for metabolism and excretion of most drugs in human/mammalian systems, limited comparative biotransformation data are available and they indicate that read-across is not straightforward. For example, in mammals, hepatic cytochrome P-450 enzymes (CYPs) are known to play a major role in xenobiotic metabolism and detoxification, with CYP1A2, 2A6, 2B6, 2C9, 2C19, 2D6, 2E1 and 3A4, in particular, mediating the metabolism of approximately 70% of pharmaceuticals [31]. However, no significant biotransformation could be measured for the known substrates of human CYP2D6, CYP2C9 or CYP3A4 for seven drugs in a study on rainbow trout (Oncorhynchus mykiss) [32]. Furthermore, the pregnane X receptor (PXR nuclear receptor subfamily 1, group I, member 2; NR1I2) which regulates many of the CYP enzymes, along with phase II metabolic enzymes and drug transporter proteins in human/mammalian systems, appears to play a role in metabolism in some fish species, but not others. PXR is active in common carp (Cyprinus carpio) [29], fathead minnow (Pimephales promelas) and zebrafish (Danio rerio) [33], but appears to be absent in cod (Gadus morhua), sea lamprey (Petromyzon marinus) and three-spined stickleback (Gasterosteus aculeatus); based on the latest gene builds for these species in Ensembl, version 74 [34]. At this time, therefore, it is difficult to predict drug metabolism and bioconcentration in fish based on the systems established for mammals, or to extrapolate between different fish species.

Physiological responsiveness of fish to pharmaceuticals is generally assumed to depend, in part, on the level of conservation of high-affinity interactions with designated drug targets (proteins) that cause the intended pharmacological action in human and veterinary drug targets, may be more responsive than species lacking orthologues. Fish underwent evolutionary divergence from other vertebrates 450 Ma [21], but they nevertheless exhibit high evolutionary conservation of human and veterinary drug targets compared with other taxonomic groups used in aquatic ERA. For example, in zebrafish and three-spined stickleback, orthologues are predicted for 86% of human drug targets [3]. These (and other) fish species used in ecotoxicology belong to the last* of the following three superclasses: (i) Agnatha, jawless fish including lampreys and hagfish (ii) Chondrichthyes, cartilaginous fish, and (iii) Osteichthyes*, bony fish. The final class is the most diverse and comprises the Actinopterygii or ray-finned fishes, and Sarcopterygii, a parathelphyletic class containing lobe-finned fishes, and all tetrapod vertebrates, including humans [21]. As Sarcopterygii are more directly related to tetrapods they may be expected to show greater conservation of human and veterinary drug targets compared with fish from other classes, particularly the more ancient superclasses Chondrichthyes and Agnatha. The influence of phylogeny on drug target conservation in fish has not yet been examined and existing orthologue predictions of drug targets have focused on only a few model ray-finned fish species [3], and/or employed simple best-match approaches [4,5]. Phylogenetically based predictions can better account for evolutionary events, including speciation and gene duplications [35], the latter being most prevalent in the ray-finned fish lineage [36]. The level of protein sequence similarity between the human drug target and the fish orthologue (especially for the ligand binding domain; LBD) may enable better predictions regarding the species responsiveness to pharmaceutical exposure [6]. However, currently, there are few experimental data comparing drug target responsiveness in fish, other than for the oestrogen receptor α (ESR1), and these indicate that LBD sequence similarity is not always predictive of receptor activation and therefore physiological response [37].

Population resilience (versus susceptibility) to environmental stressors is governed by life-history traits relating to reproductive strategy, longevity, dispersal, niche specificity,
demographics and dynamic stock-recruitment [22,38,39].
A key challenge in ERA is determining whether or not physiological effects in individuals translate to adverse impacts on wild populations [16,40]. This extrapolation between effect-levels and between species has been attempted using population dynamics models, which project forward the life histories of wild populations, with and without superimposing chemical effects measured in surrogate, laboratory-exposed populations. Some models have indicated that short-lived, asynchronous spawning fish, such as the fathead minnow, may be more susceptible to population decline compared with longer-lived, seasonal spawning brook trout (Salvelinus fontinalis), following simulated multi-generation exposures to endocrine active chemicals [41]. These findings are consistent with studies on an experimental lake dosed with EE2, in order to investigate linkages between individual, laboratory-exposed populations. Some models have indicated that short-lived, asynchronous spawning fish, such as the fathead minnow, may be more susceptible to population decline compared with longer-lived, seasonal spawning brook trout (Salvelinus fontinalis), following simulated multi-generation exposures to endocrine active chemicals [41]. These findings are consistent with studies on an experimental lake dosed 

3. Methodological approach

We first examined variation in potential susceptibility in fish by describing the presence or absence of orthologues to 459 human drug targets in 12 diverse species with fully sequenced genomes and complete gene builds [34]. Then, prioritizing a subset of 45 active pharmaceutical ingredients (APIs) with potential to have direct effects on reproduction, we assessed the interspecies variation in target sequence similarity compared with the human target. The majority of the prioritized APIs mediate their pharmacological action via one or more steroid receptors, and the sequence similarity of the LBD of these receptors was assessed. Using experimental data, we then compared differences in ligand- and species-specificity of ESR1. In the next stage of our analysis, we evaluated fish life-history traits influencing environmental exposure to pharmaceuticals and population resilience (e.g. dispersal, reproductive strategy, generation time). Finally, a case study analysis was conducted for the highly potent steroidal oestrogen EE2, in order to investigate linkages between individual and population effect-levels, enabling an overall comparative assessment of risk for three model species commonly used in ERA. All fish species included in the study are listed in electronic supplementary material, table S1.

(a) Assessment of physiological responsiveness

(i) Orthologues in fish for human drug targets

Genomes were studied for 12 fully sequenced fish species with complete gene builds held in Ensembl Compara [46] (version 74, accessed January 2014): cod (G. morhua), coelacanth (Latimeria chalumnae), fugu (Takifugu rubripes), medaka (Oryzias latipes), Mexican cavefish (Astyanax mexicanus), Nile tilapia (Oreochromis niloticus), sea lamprey (P. marinus), southern platyfish (Xiphophorus maculatus), spotted gar (Lepisosteus oculatus), three-spined stickleback (G. aculeatus), tetraodon (Tetraodon nigroviridis) and zebrafish (D. rerio).

Information regarding human drugs and their targets were downloaded from DrugBank v. 3.0 [1]. Only drugs annotated in DrugBank as ‘small molecule’, ‘approved’ and with ‘humans and other mammals, as affected organisms’ were considered. Drug targets with ‘unknown pharmacological action’ were excluded from our analyses. In total, information on 978 APIs associated with 459 unique drug targets was downloaded. DrugBank previously listed over 1000 drug targets, including metabolizing enzymes and transporters, but now shows that only 459 have specific pharmacological action. These drug targets were mapped to the Ensembl database (version 74, accessed January 2014) using protein sequences from Uniprot [47]. Drug target orthologues in the 12 fully sequenced fish species and in the tree frog (Xenopus tropicalis), included as a tetrapod out-group, were then calculated (electronic supplementary material, table S2) based on the phylogenetic gene tree predictions in Ensembl Compara (version 74, accessed January 2014) [46]. A drug target was considered to be conserved in a species if it had at least one human orthologue. The associated taxonomic information was retrieved from the NCBI taxonomy database (accessed February 2014) [48].

(ii) Assessment of sequence similarities

Sequence similarities were calculated for drug targets (21) associated with APIs (45) with anatomical therapeutic chemical classification codes suggesting direct effects on reproduction (prostaglandins (A02BB), oxytocics/uterus-contracting agents (G02A), contraceptives for topical use (G02B), sex hormones and modulators of the genital system (G03) and endocrine agents used in the treatment of neoplastic diseases (L02)). The sequence similarity was estimated from the multiple alignments available in Ensembl (version 74, accessed January 2014) [34]. To reduce the effects of erroneously aligned gene regions, produced by the large evolutionary distance between the species, only aligned amino acids were considered in the estimates. All sequence similarities are presented in electronic supplementary material, table S3. LBDs were annotated using the position-specific scoring matrices from the Conserved Domain database [49]. The sequence similarities of LBDs were calculated in an analogous manner to the sequence similarities of complete drug targets.

(iii) Assessment of amino acid sequence alignments with the LDB for human ESR1

Multiple alignment of amino acid sequences of the LBD of ESR1 was assessed with the following sequences: human ENSP00000405330; tree frog ENSXETG00000012364; cavefish ENSAMXG00000006267; cod ENSGMOG00000014898; common carp BAP99812; fathead minnow AA41373; fugu...
ENSTRUG00000018219; medaka ENSORLG00000014514; platyfish ENSXMAG00000010384; rainbow trout P16058; roach BAD91035; stickleback ENSGACG0000008571; tetraodon ENSTNG00000012264; tilapia ENSONIG00000013354; sea lamprey ENSPMAG0000005727; zebrafish ENSDARG00000044111. The amino acid residues in the human ESR1 that have been shown to have direct contact with the co-crystallized ligands 17β-oestradiol (E2) and diethylstilboestrol (DES) according to pocketome.org [50] (accessed in April 2014) were highlighted, and the alignment is presented in electronic supplementary material, table S4. The ESR1 in the coelacanth was found to be erroneous and was therefore excluded from the alignment analysis.

(iv) Assessment of interactions of pharmaceutical oestrogens with ESR1
Interactions with ESR1 of oestrogenic pharmaceuticals E2, EE2, the equine oestrogen equinul (used in hormone replacement therapy) and DES were compared across six different fish species (common carp, fathead minnow, medaka, roach (Rutilus rutilus), three-spined stickleback and zebrafish). Full-coding regions for ESR1 in each species were cloned and transfected into separate HEK293 cell lines and ESR1 receptor trans-activation assays were conducted as described in [37].

(b) Assessment of exposure potential and population resilience
(i) Life-history trait analysis
Using the databases FishBase (version 12/2013) [51] and FishTraits (version 2) [52], and the available scientific literature, life-history data were obtained for a broad selection of fish species in which physiological responsiveness to pharmaceuticals had been assessed. Rainbow trout and the Chinese rare minnow were also included as an alternative model species used in ecotoxicology and ERA. Searches focused on the compilation of qualitative and quantitative trait data (electronic supplementary material, table S5) required to calculate susceptibility indices incorporating indicators of exposure potential (e.g. dispersal, niche specificity, lifespan) and population resilience (e.g. spawning frequency, parental care, recruitment). Two alternative indices were used, the population survivorship index [38] and the ecological vulnerability index [39].

(ii) Life-table-response experiments integrating life cycle assessments of individual and population-level effects of EE2
Published life cycle data (F1 embryo to F1 adult to F2 embryo) quantifying the effects of the oestrogen receptor (ESR1, ESR2) agonist EE2 in three model freshwater fish species, fathead minnow, medaka and zebrafish, under standard flow-through conditions and test temperatures (25–28°C), were compiled and scrutinized according to established quality/reliability criteria [53]. Those studies incorporating measurement of a range of effect levels (molecular, physiological, behavioural, population-relevant effects) and confirmation of exposure concentrations by chemical analysis were used to determine the lowest observed effect concentration (LOEC) for population-relevant endpoints in each species: zebrafish LOEC = 0.5–1 ng l⁻¹ EE2 [54,55]; fathead minnow and medaka LOEC = 1 ng l⁻¹ EE2 [43,56,57]. Endpoint values (vital rates: proportion of viable fertilized eggs; proportion of females; female fecundity (eggs per female per day); survival) were tabulated as mean values ± standard error of the mean (electronic supplementary material, table S6). A lower LOEC of 0.2 ng l⁻¹ EE2 was reported for the Chinese rare minnow [45]; however, insufficient life-table data were available to permit life-table-response experiments (LTREs) for this species.

Species-specific vital rates for control (non-exposed) and EE2 (1 ng l⁻¹) exposed fish were then input with specific life-table data for wild populations of fathead minnow, medaka, zebrafish (electronic supplementary material, table S7) to stage-based Leslie matrix population models (electronic supplementary material, figure S1), constructed for each species using POPTOOLS (v. 3.2.5) [58]. Population projections were based on females, because female fecundity limits population numbers. Populations were assumed to be closed with no immigration or emigration, and spawning was assumed to be asynchronous and protracted in all three species [59–61]. Finite, geometric population growth rate (λ) was then projected from n = 100 replicate LTREs [62] for each species, by resampling vital rates from their means and standard deviations, assuming normal distributions for each vital rate [60]. The statistical significance of the effect of EE2 (1 ng l⁻¹) exposure on projected population growth rate (λE) versus control population growth rate (λC) was assessed on ranked projections using the non-parametric Kruskal–Wallis test, owing to inequality of variances between exposed and control populations (according to Levene’s test). These stochastic projections of treatment effects at the population-level enabled an integrated assessment of ecotoxicological and ecological population susceptibility. Determining the mean sensitivities (control + treatment/2) of each vital rate in each LTRE also enabled decomposition analysis to quantify the relative contribution and importance of these vital rates (performed according to Caswell [62]).

3. Results and discussion
(a) Assessment of physiological responsiveness
(i) Conservation of human drug targets
More than 80% of the 459 human drug targets had orthologues in all the investigated bony fish, while the sea lamprey (a jawless fish) had orthologues to 65% of the targets. These results suggest that responses in sea lamprey may differ frequently and substantially from those in bony fish (figure 1). Information regarding the APIs, the drug targets and their orthologues and co-orthologues in each species is presented in electronic supplementary material, table S2.

Although the vast majority of the APIs had at least one orthologous drug target in fish, this does not necessarily mean all these APIs will have a functional drug interaction and invoke a physiological response in fish, or that this response will resemble that occurring in humans. Even between mammalian species (human–mouse), there are numerous examples of orthologues that have diverged functionally [63]. The general sequence similarity between orthologous proteins may potentially give more information, but the data should be interpreted with caution, because orthologous proteins with similar function can have significant domains that are missing or differ substantially. The LBD and especially the specific amino acids that interact with the ligands are, however, generally highly conserved and could provide additional information regarding potential drug interactions [6].
(ii) Variation in sequence similarity

Alignments of the full protein sequence and LBD of the nuclear steroid hormone receptors, specifically, ESR1, oestrogen receptor β (ESR2), progesterone receptor (PGR), androgen receptor (AR), glucocorticoid receptor (NR3C1) and mineralocorticoid receptor (NR3C2), showed that the LBDs had higher sequence similarity to the corresponding human drug target than the full proteins (figure 2). Furthermore, in line with the established phylogenetics of fish evolution, the lobe-finned fish had the highest sequence similarities (75–81%) with the LBDs of the human steroid receptors, whereas the more primitive sea lamprey had the lowest LBD sequence similarities (52–63%; figure 2). This finding, however, does not necessarily mean that sea lamprey is less susceptible to drugs targeting these receptors, because it is also important to consider gene duplication and function. As an illustration of this, the human glucocorticoid- and mineralocorticoid-receptors. There are also very few data concerning the promiscuity, responsiveness and function of the PGR, ESR1 and ESR2 in sea lamprey. Nevertheless, plasma progesterone and E2 concentrations have been shown to vary between the sexes and reproductive stages, suggesting links with sexual function in this species [65]. Here, we show that sea lamprey has orthologues to both the human ESR1 and ESR2 and their LBDs are conserved to a similar degree in sea lamprey as in the ray-finned fishes (63–71%).

(iii) Variation in target-ligand binding and activation of ESR1

Assessment of pharmaceutical oestrogen interactions with fish ESR1 in trans-activation assays showed that DES was the most potent ligand and 10 times more potent than the natural oestrogen E2. EE2 was approximately twice the potency of E2, and equilin was around 10 times less potent (in all species) compared with E2. The effective concentration corresponding with 50% of maximum ESR1 transactivation (EC$_{50}$) showed little variation for E2 between the different fish species, ranging 2.4-fold between 0.18 and 0.43 nM (zebrafish and stickleback, respectively, figure 3). For the pharmaceutical oestrogens, however, there were somewhat larger differences between the different species.

![Species tree illustrating the taxonomic relationships (based on NCBI taxonomy) between all species included in this study. The total number (no.) of conserved human drug targets for each of the 12 fish species with fully sequenced genomes and complete gene builds is presented, together with the number of active pharmaceutical ingredients (API) with at least one drug target orthologue.](http://rstb.royalsocietypublishing.org/)
fish species. For EE2, the EC\textsubscript{50} ranged 4.3-fold between 0.07 and 0.30 nM (zebrafish and stickleback, respectively) and for DES, the EC\textsubscript{50} ranged 5.7-fold between 0.007 and 0.30 nM (zebrafish and stickleback, respectively) and 0.07 and 0.30 nM (coelacanth and southern platyfish, respectively). The greater interspecies variation in EC\textsubscript{50}s and fold-activation in all bony fish (electronic supplementary material, table S4). Known to have direct contact with E2 or DES were identical range of tissues, functions and processes in fish [66,67]. Curiously, 14 of the 15 amino acid residues of the human ESR1 known to have direct contact with E2 or DES were identical in all bony fish (electronic supplementary material, table S4). The greater interspecies variation in EC\textsubscript{50}s and fold-activation for DES (up to 5.7-fold variation) could therefore not easily be explained by the variations in sequence similarity in the LBD (electronic supplementary material, table S4). Instead, the influence of cofactors, differences in promoter sequences and amino acid residues in other domains, such as the DNA binding domain [37,68,69], may better account for the observed differences in ESR1 activation.

(b) Variation in exposure potential and population resilience

(i) Life-history trait analysis

According to two alternative susceptibility indices, based on a range of life-history traits, species with longer generation times and lower spawning frequency, fecundity and recruitment (coelacanth, sea lamprey and fugu) were ranked as the most susceptible to environmental stressors (figure 4). The higher ranking of these species is consistent with their higher conservation status, compared with other species analysed (electronic supplementary material, table S1). The rapidly reproducing southern platyfish was ranked as the least susceptible to

Figure 2. Sequence similarities (%) between six human nuclear steroid hormone receptors and their corresponding orthologues in 13 species. Sequence similarities of the full sequences are presented on the left and similarities of the ligand binding domains are presented on the right. AR, androgen receptor; PGR, progesterone receptor; ESR1, oestrogen receptor 1 (α); ESR2, oestrogen receptor 2 (β); NR3C1, nuclear receptor subfamily 3, group C, member 1 (glucocorticoid receptor); NR3C2, nuclear receptor subfamily 3, group C, member 2 (mineralocorticoid receptor). Percentage similarities are emphasized using a coloured heat map. Empty cells indicate that evidence for an orthologue is lacking in current genome versions and gene builds. Asterisk denotes missing value owing to errors in current gene build. (Online version in colour.)
environmental stressors (figure 4). The ‘population survivorship index’, specifically tailored for fish and amphibians [38], provided greater differentiation between the most and least susceptible species, indicating a threefold difference, compared with a 1.6-fold difference indicated by the more general ‘ecological vulnerability index’ [39]. Both these ranges are within the 10-fold assessment factor traditionally used in pharmaceutical ERA to account for variation in species susceptibility [25].

Figure 3. Fish oestrogen receptor (ESR1) responses in transactivation assays induced by oestrogenic pharmaceuticals. (a) 17β-oestradiol (E2), (b) 17α-ethinylestradiol (EE2), (c) diethylstilboestrol (DES), (d) equilin. Receptor transactivation data are shown in terms of fold-activation (i) and EC50 (ii). Fold-activation represents the difference in receptor activity (non versus pharmaceutical exposure) of ESR1 from six selected fish species transfected into human HEK293 cells [32]. Receptor activity was quantified using a dual luciferase reporter assay. EC50 is the effective concentration (nM) corresponding with 50% of maximum ESR1 transactivation. Data are presented as mean ± s.e.m. from three independent assays, each consisting of three technical replicates per concentration tested. (Online version in colour.)
Figure 4. Potential susceptibility of fish populations to environmental (chemical) stress. Population susceptibility index (1 − survivorship index, Sprombing & Birge [38]) was calculated using a scoring system based on spawning frequency, parental care, lifespan, recruitment, niche specificity. Ecological vulnerability index (De Lange et al. [39]) was based on broader life-history data. Data were obtained mainly from www.fish.base.org/ and www.fishtraits.info/ (see electronic supplementary material, table S5). (Online version in colour.)

(ii) Life-table-response experiments

Available life-table data and full life-cycle studies for EE2 enabled integrated assessments of physiological susceptibility and population resilience for three model freshwater species commonly used in pharmaceutical ERA, specifically, fathead minnow, medaka and zebrafish.

Beginning with an analysis of life-cycle effects data, the LOECs for EE2 were: zebrafish LOEC = 0.5 ng l\(^{-1}\) [54]; fathead minnow and medaka LOEC = 1 ng l\(^{-1}\) [43,55–57]. While there were no effects on survival at these concentrations, there were significant effects on other population-relevant endpoints (vital rates), and their magnitudes of effect differed between species. There was a female-biased sex ratio in both zebrafish (57%) [55] and fathead minnow (65%) [43], but there was no female bias in medaka (50%) [56–57]. Plasticity in sexual differentiation is typical in some species, such as zebrafish, that are sometimes referred to as juvenile hermaphrodites. Alternatively, in gonochorists, such as fathead minnow and medaka, sex is determined around fertilization and feminization of males typically results in ovo-testes, as occurred in both species following exposure to 1 ng l\(^{-1}\) EE2 [43,56]. Female-bias appeared to be ‘compensated’ for by reductions in fecundity and fertilization success in fathead minnows. Greater reduction in fertilization success occurred in zebrafish, which may be linked to their broadcast spawning strategy, reduced likelihood of fertilization and oophagy, as opposed to substrate spawning and egg guarding displayed by fathead minnows. Fertilization success was affected least in medaka, whose females produced and brooded fewer eggs. EE2 exposure concentrations of between 0.5 and 2 ng l\(^{-1}\) resulted in no significant alteration in male courtship behaviour in zebrafish [54,71]. In contrast, male courtship was reduced in medaka [53,54]. Overall, there were reductions in fertilization/hatching success in all three species compared with controls: 25 ± 5% reduction in medaka [57]; 35 ± 30% in fathead minnows [43]; 54.5 ± 15% in zebrafish [44]. Female fecundity also showed declining (but non-significant) trends for all three species. Reciprocal pair-breeding of control (non-exposed) and EE2-exposed fish revealed that reproductive impairment occurred in both sexes, but was generally greater in males in both zebrafish [54] and medaka [57].

The integration of life-cycle effects data and ecological life-history data for fathead minnow, medaka and zebrafish, in separate LTREs, showed that finite population growth (\(\lambda\)) following exposure to 1 ng l\(^{-1}\) EE2 was more variable compared to controls (\(\lambda\)C), but was reduced significantly in fathead minnow (\(\lambda\)C = 1.12, \(\lambda\)E = 0.90, −20%; Kruskal–Wallis \(H = 64.82, d.f. = 1, p < 0.001\)) and medaka (\(\lambda\)C = 3.59, \(\lambda\)E = 2.97, −17%; Kruskal–Wallis \(H = 43.55, d.f. = 1, p < 0.001\)), but not in zebrafish (\(\lambda\)C = 2.30, \(\lambda\)E = 2.17, −6%; Kruskal–Wallis \(H = 2.88, d.f. = 1, p = 0.089\)) (figure 5). Proportional reductions in population growth rate (fathead minnow > medaka > zebrafish) contrasted with the susceptibility indices derived from
finite population growth in exposed populations versus non-exposed (control) populations of model fish species. (Online version in colour.)

**compared with control populations with no chemical exposure. (ERA. This approach can help to identify which levels of factors for consideration when extrapolating between fish species and effects endpoints in pharmaceutical ERA. This approach can help to identify which levels of mechanistic, individual-based models [40,61], may be better able to discern effects of EE2 on male reproductive fitness, including those relating to effects on behaviour.

**Figure 5. (a) Mean (s.e.m.) projected finite population growth in model fish species following lifetime exposure (embryo to adult) to 1 ng l^{-1} ethinylestradiol (EE2) compared with control populations with no chemical exposure. (b) Contribution of each vital rate to reductions in finite population growth rate. Mean of n = 100 stochastic matrix projections and standard error of the mean (SEM) shown. **p < 0.001 according to the Kruskal–Wallis test comparing ranked projections of finite population growth in EE2 exposed populations versus non-exposed (control) populations of model fish species. (Online version in colour.)

ecological life-history data for these species. Absolute population growth rate for fathead minnows was also projected to fall below $\lambda = 1$ under EE2 exposure, indicating population decline.

Decomposition analysis revealed that reduction in age 0+ fecundity (fecundity in the first year of life), although highly variable, was most influential on reducing population growth in all three model species under EE2 exposure. These results highlight potential drawbacks of traditional statistical evaluation of individual endpoints such as fecundity, which are often shown to be highly variable and ‘statistically insignificant’ [43,44,57]. Alternatively, integrative modelling approaches can use stochastic variation in multiple endpoints and, by extrapolating population-level effects, can indicate ‘ecological significance’. The second most influential parameter affecting population growth was age 0 viability (proportion of viable female eggs × proportion fertilized), in which the proportion of eggs fertilized is influenced by the effects of EE2 on reducing male fertility. Reduction in fertilization success, that can act directly to reduce population growth, was greatest in zebrafish, but this was compensated by female-biased sex ratios and high fecundity in this species. Other modelling approaches, including mechanistic, individual-based models [40,61], may be better able to discern effects of EE2 on male reproductive fitness, including those relating to effects on behaviour.

4. Conclusion

Here, we identify factors for consideration when extrapolating between fish species and effects endpoints in pharmaceutical ERA. This approach can help to identify which levels of biological organization, from the molecular to the population-level, are most likely to account for interspecies variation. Drug bioavailability and biotransformation in fish are likely to be further sources for interspecies variation in responsiveness to drugs, which we have not explored fully because data are currently sparse.

We illustrate that although fish generally show high conservation of human drug targets across diverse taxonomic groups, there is some interspecies variation, for example in the LBD sequences for nuclear hormone receptors. Greater variation (up to 5.7-fold) was identified in the physiological responsiveness of different fish to drugs targeting these receptors. Additional analysis of ecological life-history data indicated further interspecies variation (up to threefold) in potential susceptibility to population decline. Ultimately, we integrated ecological life-table data with life-cycle effects data for EE2 and showed distinct differences in proportional reductions in population growth between fathead minnow, medaka and zebrafish (around threefold). These results, however, were not entirely consistent with the physiological and ecological susceptibilities indicated by the preceding analyses. This work illustrates that extrapolating from individual-level effects in laboratory tests to population effects in wild fish is challenging. Furthermore, while small fish models offer considerable utility for pharmaceutical ERA, they are not necessarily protective of all fish owing to wide ranging evolutionary divergence of physiologies, behaviours and ecological life histories. Nevertheless, based on our analyses on the data available, the use of at least a 10× assessment factor to account for interspecies differences in ERA seems appropriate for application to pharmaceuticals.
References


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