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1 **Comparative metabolism as a key driver of wildlife species sensitivity to human and**
2 **veterinary pharmaceuticals.**
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Abstract

Human and veterinary drug development addresses absorption, distribution, metabolism, elimination and toxicology (ADMET) of the Active Pharmaceutical Ingredient (API) in the target species. Metabolism is an important factor in controlling circulating plasma and target tissue API concentrations and in generating metabolites which are more easily eliminated in bile, faeces and urine. The essential purpose of xenobiotic metabolism is to convert lipid-soluble, non-polar and non-excretable chemicals into water soluble, polar molecules that are readily excreted. Xenobiotic metabolism is classified into Phase I enzymatic reactions (which add or expose reactive functional groups on xenobiotic molecules), Phase II reactions (resulting in xenobiotic conjugation with large water-soluble, polar molecules) and Phase III cellular efflux transport processes. The human-fish plasma model provides a useful approach to understanding the pharmacokinetics of APIs (eg diclofenac, ibuprofen and propranolol) in freshwater fish, where gill and liver metabolism of APIs have been shown to be of importance. In contrast, wildlife species with low metabolic competency may exhibit zero order metabolic (pharmacokinetic) profiles and thus high API toxicity, as in the case of diclofenac and the dramatic decline of vulture populations across the Indian subcontinent. A similar threat looms for African Cape Griffon vultures exposed to ketoprofen and meloxicam, recent studies indicating toxicity relates to zero order metabolism (suggesting P450 Phase I enzyme system or Phase II glucuronidation deficiencies). While all aspects of ADMET are important in toxicity evaluations, these observations demonstrate the importance of methods for predicting API comparative metabolism as a central part of environmental risk assessment.

[abstract word count = 249]

Keywords: medicines, environment, exposure, birds, fish, invertebrates

1.0 Introduction

Investigation of a pharmaceutical's absorption, distribution, metabolism, elimination and toxicology (ADMET) play a central role in the pre-clinical and clinical safety assessment of human medicines [1]. Likewise, Active Pharmaceutical Ingredients (APIs) used in veterinary medicine are evaluated for their ADMET profile in the species of interest (for example, poultry or ruminants) [2, 3]. Metabolism of endogenous and exogenous molecules (eg plant toxins, pesticides and pharmaceuticals) is normally classified into Phase I enzymatic reactions (which add or expose –OH, -SH, $-NH_2$ or -COOH functional groups on xenobiotics) and Phase II reactions (resulting in xenobiotic conjugation with large water-soluble, polar molecules). Additionally, lipophilic xenobiotics, or their metabolites, can be pumped out of cells by specific transporter proteins and this efflux pump activity is often termed Phase III metabolism [4]. For approximately 5-7% of human drugs, Phase I metabolism may be responsible for conversion of a prodrug into the API [5]. More broadly, many Phase I biotransformations of lipophilic xenobiotics are carried out by microsomal monooxygenases, located in the endoplasmic reticulum of the liver and other organs [6]. The haem protein cytochrome P450 provides the active centre of these enzymes and has huge diversity, with 37 cytochrome P450 families currently identified across many animal species [7]. It is hypothesized that the P450 superfamily has undergone repeated rounds of expansion by

genome duplication, whereby approximately one and a half billion years ago, the first expansion gave rise to the P450 families primarily involved in metabolising endogenous fatty acids, cholesterol and its derivatives (CYP4 and CYP11 families) which likely played a key role in maintaining the eukaryotic cell membrane integrity. A later expansion of the P450 family nine hundred million years ago may have led to several endogenous steroid-synthesizing cytochrome P450 lineages (including CYP19, CYP21 and CYP27 gene families; whereby the CYP21 family later diverged to give rise to the CYP1 and CYP2 families). A final major expansion of several P450 families involved in xenobiotic metabolism (including CYP2, CYP3, CYP4 and CYP6), began about four hundred million years ago. This most recent expansion is thought to have been driven by first the emergence of aquatic organisms onto land associated eating toxic plant allelochemicals ('animal – plant warfare'), together with exposure of terrestrial organisms to hydrocarbon-based combustion products in the atmosphere [7-11].

Much data exists on the metabolism of pharmaceuticals and other xenobiotics by the liver microsomes of mammals, birds and other species, with rates of microsomal oxidative metabolism determined across a range of vertebrates [2, 12, 13]. For example, Abass and colleagues [14] studied the metabolism of the insecticide benfuracarb by hepatic microsomes taken from seven mammalian species to investigate species-specific metabolic pathways. Benfuracarb is metabolised via sulphur-oxidation and nitrogen-sulphur bond cleavage (producing carbofuran which is further metabolised). Clearance rates for the seven species ranged from 1.4 (monkey) to 3.5 (rat), these differences being due to variability in CYP enzyme expression [14]. Among herbivorous and omnivorous mammals, there is a clear inverse correlation between the microsomal monooxygenase activity and body weight [15, 16]. When hepatic monooxygenase activities are expressed in terms of body weight, much higher values are found in small rodents than in large mammals. This observation is consistent with the concept of a co-evolutionary arms race between plants and herbivorous animals. In this context, small mammals need to consume more food per unit body weight than do large ones in order to maintain body temperature due to their high surface area to volume ratios. In contrast to the mammalian species studied by Walker and colleagues, the carnivorous (piscivorous or raptorial) species showed distinctly lower microsomal monooxygenase activities than did herbivorous or omnivorous birds (an observation also explicable in terms of 'animal-plant arms race' theory). Predatory mammals (eg cats) and birds (eg raptors) eat very little, if any, plant material and therefore do not incur major pressure to drive the evolution of enzymes to metabolise plant toxins [16-19]. Interestingly, zebrafish (a widely used model in pharmaceutical research) show a dramatic increase in Phase I and II enzyme activity at the juvenile life stage in association with being fed plant based diets [20].

In contrast to terrestrial vertebrates, Phase I enzyme activities in fish are generally lower and there is only a weak correlation with body weight (whereas individual avian species show a correlation between body weight and hepatic microsomal monooxygenase activity across species) [12]. For fish, this has been explained on the grounds that they can excrete many xenobiotics by diffusion across gills into the large volume of ambient water and it has been argued that there has not been a strong pressure for the evolution of highly active detoxification enzymes as seen in mammals [13, 21]. A similar situation is thought to apply to aquatic invertebrates [22-24]. Nonetheless, as molecular and biochemical methods have advanced there growing evidence of both Phase I and II enzyme activity in fish [20, 25, 26]

and recent studies have addressed how dietary and trophic variables may affect enzyme activity in fish [27]. There are also a growing number of studies on the metabolism of pharmaceuticals in fish [28-38] but to far lesser extent invertebrates [39]. Veterinary pharmaceuticals have also been studied from a comparative metabolism perspective [40,41]. Table 1 summarizes Phase I pathways of pharmaceutical and xenobiotic metabolism in mammals and other vertebrates, adapted from [42, 43] and updated with examples from the DrugBank on-line database http://www.drugbank.ca/ established by Wishart *et al*. [44].

Inset Table 1.

2.0 ***In Vitro*** **&** ***In Silico*** **Methods To Understand Comparative Metabolism**

In vitro systems are widely used for the investigation of xenobiotic metabolism in mammals [1], birds [45] and fish [36, 38]. Techniques include: (a) whole liver tissue slices which retain an accurate, structural framework of the liver; (b) whole isolated hepatocytes where the endoplasmic reticulum bound and cytosolic enzymes are present the structural integrity of liver network lost; (c) after centrifugation at 9000g, the S9 fraction supernatant from liver (or other tissue) homogenate contains both cytosolic (predominantly Phase II) and microsomal (predominantly Phase I) enzymes; and (d) microsomes comprising of endoplasmic reticulum bound enzymes that have been separated from cytosolic enzymes (P450 enzymes are concentrated in this subcellular fraction). These methods are routinely used to determine the rate and extent of metabolism and mass-spectroscopic analysis of specific metabolites. Results for clearance rates obtained from *in vitro* metabolism experiments can then be extrapolated to the *in vivo* situation using scaling factors (e.g. number of hepatocytes per liver; weight of microsomal protein per gram of liver, etc). Allometric methods can also be used to scale *in vitro* results between different species (used in drug development for scaling from preclinical species to man). Where such values are known for wildlife species, this may allow for approximations between different species [15, 16] and form a basis for models to aid in environmental risk assessment using fish [29,46,47], invertebrates [39] and plants [48].

Novel *in silico* tools may also be useful to predict metabolism, this approach tending to focus on the semi-quantitative prediction of potential metabolites and identification of the specific enzymes responsible for the metabolism. Prediction of metabolic rates of drug metabolism remains a key challenge, especially with regard to identification of potential metabolites (which may be associated with specific toxicities) and identification of the enzymes responsible (combined with knowledge of different enzyme expression in different species). Kirchmair *et al*. [49] provide an overview of *in silico* tools for predicting key factors associated with metabolism (including sites of metabolism (SOM) within a molecule; potential metabolites; cytochrome P450 (CYP) binding affinity / inhibition; and prediction of CYP induction). Table 2 shows a representative software tool for each of these categories, however, many other tools are available [49].

156 Insert Table 2.

157

158 *In silico* tools have a number of potential advantages and provide complementary
159 techniques to *in vitro* methods. One area where information from both fields can be
160 combined to build improved predictions is in physiologically-based pharmacokinetic (PBPK)
161 modelling. In this method an organism is divided into a sequence of physiological
162 compartments (e.g. brain, liver, lungs, etc.). The models integrate compound-specific data
163 (e.g. physico-chemical properties, such as log P, pK_a or solubility, these values may be
164 measured or predicted using *in silico* techniques) and species (or even subject)-specific data
165 (e.g. physiological factors such as body or organ weights, volumes, or blood flow rates).
166 Subject to validation, these models are potentially of high value in predicting concentration-
167 time profiles for pharmaceuticals in wildlife species [29,34,46]. Understanding inter-species
168 differences in metabolism is essential for reliable PBPK models, especially in non-
169 mammalian species. For example, Ohyama *et al.* [45] studied methoxychlor (MXC)
170 metabolism in rat, mouse, Japanese quail and rainbow trout using liver slices. Each species
171 showed differences in metabolism, considered due to substrate specificity of CYP450s
172 involved. MXC was metabolised to bis-OH-MXC which was then glucuronidated (with only
173 rats producing the bis-OH-MXC 4 O-sulphate 4-O- glucuronide). In mice and Japanese quail,
174 mono-OH-MXC (and glucuronide conjugate) were the main metabolites and little bis-OH-
175 MXC glucuronide was formed (dechlorinated mono-OH-MXC glucuronide was found only in
176 mice). Rainbow trout liver slices formed similar amounts of both metabolites. In conclusion,
177 rat and trout livers slices were able to metabolise both MXC and mono-OH MXC, whereas
178 only MXC could be metabolised in mouse and Japanese quail [45].

179

180 *3.0 In Vivo* Approaches in Studying Comparative Metabolism

181

182 The overall effect a xenobiotic has on any organism is ultimately the result of it intrinsic
183 activity and its concentration at the target site. Concentration at a given target site is
184 determined by the ADME properties of the compound. The history of studying the time
185 course and concentration of xenobiotics at different sites within the body has been
186 developed predominantly within the pharmaceutical industry, with respect to drug effects
187 on humans. However the techniques are applicable to diverse chemical space and across
188 diverse species. *In vivo* measurements determining the pharmacokinetic profiles of
189 xenobiotics in environmental species are largely unavailable, hence extrapolation and
190 predictive models (combining *in silico* and *in vitro* methods) become essential tools in
191 determining organ-level concentrations [50]. Metabolism is one of the key factors to
192 consider when modelling the time course of a xenobiotic within an organism, not only as it
193 can determine the overall period of exposure, but also because the metabolite(s), rather
194 than the parent drug, may be responsible for the toxic effect [1,51]. In the non-mammalian
195 area where much less is known about metabolic profiles of drugs in animals, *in vivo*
196 experiments still have a major role to play to derive reliable environmental risk assessments
197 [for case studies with freshwater fish see references 33,34,52] and also in wildlife forensic
198 studies (see following case study on birds).

199

200

201

202 **4.0 Case Study - Vulture toxicity to NSAIDs (A Process of Zero-Order Metabolism)**
203
204 The dramatic impact of diclofenac (a non-steroidal anti-inflammatory drug or 'NSAID') on
205 Asian vulture populations represents one of the most serious ecological catastrophes of
206 recent times. In just over a decade, diclofenac has been responsible for the deaths of
207 millions of vultures of the Asian White-backed (*Gyps bengalensis)*, Long-Billed (*G. indicus*),
208 Slender-billed (*G. tenuirostris*), Egyptian (*Neophron percnopterus*) and red-headed
209 (*Sarcogyps calvus*) vulture species across the Indian subcontinent [53,54]. In addition to the
210 scale of the toxicity, the exposure route to the product was probably highly unconventional
211 as these birds were inadvertently being poisoned by the oral route even though diclofenac
212 was only available as an injectable cattle formulation. Whereas previous veterinary
213 medicines and pesticides had caused their negative effects by ending up in the water, soil or
214 general environment of the species affected, these vultures were being exposed to this
215 product as residues in the meat of the dead cattle carcasses upon which they fed. This
216 unique mode of exposure was linked to cultural and religious practices in the region
217 whereby sick and old cattle were routinely treated in a palliative manner with diclofenac, a
218 cheap and effective NSAID. The net effect of this practice was an unfortunate high
219 occurrence of diclofenac residues in the tissues of recently dead cattle.
220
221 In the vulture, diclofenac is highly toxic with acute death resulting from a single meal of 1kg
222 of meat rich in residue, with an estimated LD_{50} of 0.1 to 0.2 mg/kg [55]. Toxicity following
223 exposure is also fairly predictable with birds showing signs of depression and head drooping
224 as early as 24 hours post exposure. Death is the typical end-point with birds literally being
225 described as falling dead from their perches. Based on the results from controlled toxicity
226 studies, it has been shown that death after a single exposure consistently resulted within 48
227 hours of exposure, with related massive increases in plasma uric acid and potassium
228 concentrations and increased alkaline phosphatase (ALP) activity. Necropsies are also very
229 typical with signs of severe nephrosis, dehydration and accompanying diffuse visceral and
230 articular gout. Histopathology indicated toxicity was characterised by necrosis of
231 hepatocytes and the renal tubular epithelial cells (RTE) of the proximal convoluted tubules
232 with associated uric acid tophi accumulation. While the mechanism of toxicity of diclofenac
233 remains incompletely described, toxicity has been linked to RTE cell damage in a time
234 related manner, subsequent accumulation of uric acid, acidosis and terminal hyperkalaemia
235 [56]. Results from various pharmacokinetics studies of diclofenac in different bird species,
236 and the pharmacokinetic profiles of ketoprofen and meloxicam in the vulture, clearly
237 indicate that toxicity is related to the drug's pharmacokinetics (Figure 1).
238
239 Insert Figure 1
240
241 For the first of these studies, the pharmacokinetics of diclofenac was evaluated in the Cape
242 Griffon Vulture (*Gyps coprotheres*) [57]. While environmental toxicity has not been seen in
243 this vulture, the species was specifically validated as a suitable model for further
244 mechanistic studies on the toxicity of diclofenac and other NSAIDs. The choice of this
245 species was two-fold, firstly the easier availability to the study site as well as being less
246 endangered than the Indian vulture species. From this controlled acute toxicity study, the
247 Cape Griffon was shown to be equally susceptible to diclofenac as the Oriental White-
248 backed at 0.8 mg/kg i.v. with exactly the same clinical signs, clinical pathological and

249 histopathological changes. Non-compartmental analysis revealed a half-life of elimination
250 ($T_{1/2}$) of 12.24 ± 0.99 hours, area under curve to the last quantifiable time point (AUC_{last}) of
251 80.28 ± 51.26 µg/ml/hour, a mean residence time (MRT) of 15.11 ± 4.13 hours. To evaluate
252 the importance of the obtained pharmacokinetic profile obtained, parameters were
253 previously compared by [58] to that published for other bird species (Figure 2). This included
254 the African-white backed vulture (*G. africanus*), the Pied crow (*Corvus albus*), the turkey
255 vulture (*Cathartes aura*) and the domestic chicken (*Gallus domesticus*). For these studies no
256 mortalities were reported for the Pied Crow (0.8 and 10mg/kg), Turkey Vulture (8 and 25
257 mg/kg) and the domestic chicken (0.8 mg/kg), while toxicity was reported in the Cape
258 Griffon (0.8 mg/kg), the African white-back (0.8 mg/kg) and one chicken at a higher dose (5
259 mg/kg). An important finding from these comparisons was a tentative link between the $T_{1/2}$
260 and the occurrence of toxicity with a $T_{1/2}$ above 12 hours being associated with death.
261 Furthermore zero order metabolism was seen as a feature of toxicity as the $T_{1/2}$ was
262 increased in the one chicken that died, from 0.89 hours at 0.8 mg/kg to 14.34 hours at 5
263 mg/kg.
264
265 Insert Figure 2.
266
267 While diclofenac has received wide attention in published literature as a result of its
268 environmental toxic effect it is not, however, the only NSAID evaluated in vultures in terms
269 of safety and pharmacokinetics. In an attempt to have diclofenac removed from the Indian
270 veterinary market, a replacement for the drug needed to be found for use in cattle, as
271 diclofenac was of valuable cultural benefit to the sick cattle being treated. Following an
272 international survey, meloxicam and ketoprofen were identified as potentially replacement
273 i.e. they were effective in cattle with some evidence of safety in captive vulture species
274 [59,60]. Subsequently both these drugs were evaluated in extensive safety studies including
275 full characterisation of their pharmacokinetics once again in Cape Griffon as the model, with
276 vastly contrasting results.
277
278 In the first ketoprofen study Cape Griffon vultures treated at 1 mg/kg showed no indications
279 of toxicity on both clinical and clinical pathological evaluations [61]. However, when a
280 second group of vultures were treated at increased dose of 5 mg/kg, the study resulted in
281 mortalities in seven of the 11 birds treated with the characteristic signs of toxicity seen in
282 the diclofenac treated birds. The most interesting finding for this study was a difference in
283 the $T_{1/2}$ between these two dose levels but also between the birds that died or survived at
284 the 5 mg/kg dose. At 1 mg/kg the half-life was 2.66 ± 0.46 hours. In the four birds that
285 survived at 5 mg/kg the half-life was marginally higher at 3.24 ± 1.59 hours. For the birds
286 that died at the 5 mg/kg dose, the half-life had increased to 7.38 ± 1.72 hours. With regards
287 to AUC_{last}, the four birds that survived had an AUC_{last} five-fold higher as expected for the 5-
288 fold increase in dose (9.79 ± 3.23 µg/ml/hour versus 50.31 ± 17.71 µg/ml/hour, respectively).
289 However, the birds that died at 5 mg/kg had an increased AUC_{last} of 156.51 ± 33.14
290 µg/ml/hour and Cmax of 21.0 ± 1.88 µg/ml in comparison to 10.77 ± 3.26 µg/ml to the birds
291 that survived. This once again supported previous findings that toxicity is related to zero
292 order metabolism. In addition, the increase in the AUC_{last} and Cmax also indicated that
293 toxicity resulted in saturation of presystemic elimination pathways [61].
294

295 In the last of the described pharmacokinetic studies, meloxicam was administered to Cape
296 Griffon vultures in a two-way cross over study at a dose of 2 mg/kg by either oral or
297 intramuscular route, without any signs of toxicity or changes in the monitored clinical
298 pathology parameters [59]. Meloxicam was characterised by a short half-life of elimination
299 of 0.33 ± 0.167 hours and 0.42 ± 0.11 hours for the oral and intramuscular routes
300 respectively. This study further attempted to characterise the metabolites produced via LC-
301 MSMS analysis. Two CYP metabolites, hydroxymethyl meloxicam (87%) and an unknown
302 hydroxylated metabolite (7%), and one glucuronide (0.56%) metabolite were identified
303 (Figure 3). Based on literature in laboratory animals, it is suspected that the CYP most likely
304 involved in metabolism was predominantly CYP2C9.
305
306 Insert Figure 3.
307
308 While the metabolic pathway for diclofenac in the vulture is yet to be evaluated, the current
309 pharmacokinetic information available allows for some conclusions to be drawn. The first of
310 these is that toxicity is clearly linked to zero order kinetics. For the NSAIDs, this deficiency
311 could be at the level of the Phase I enzyme (CYP) system or Phase II glucuronidation, both of
312 which have been previously described. Decreased CYP2C9 activity in people has been
313 associated with resultant longer half-life of metabolised NSAIDs, while the absence of
314 glucuronidation (UGT1A6) has been described as an important mechanism in the toxicity of
315 paracetamol in the cat [18]. Limited glucuronide activity has also been described in people
316 in association with aspirin toxicity. Based on the presence of a glucuronide metabolite for
317 meloxicam, it is likely that toxicity in humans is not due to a complete absence of Phase II
318 processes as in the cat. In addition, it is also doubtful that limited glucuronidation plays a
319 role in human toxicity [62]. As a result, the rate limiting step in avian metabolism is most
320 likely at the level of cytochrome P450 enzyme system. From medical literature, meloxicam is
321 metabolised predominantly by the CYP2C9 and, to a much lower extent CYP3A4); diclofenac
322 predominantly by the CYP2C9, with some metabolism by CYP3A4 and CYP2C8 [63,64]; and
323 ketoprofen by CYP2C9 [65]. When the half-life of elimination of diclofenac, ketoprofen and
324 meloxicam in people is compared to the vulture, an important difference is present. In
325 humans the half-life of elimination of diclofenac, ketoprofen and meloxicam is typically 1-2
326 hours, 2 hours and 15-20 hours, respectively [66], while as reported above this is ±14 hours,
327 ±3 hours and 0.33 hours, respectively for the vulture, with the metabolism of ketoprofen in
328 vultures also being zero order. With the CYP2C9 being the one common enzyme in
329 metabolism, it is most probably that this is the rate limiting enzyme. With the rapid
330 metabolism of meloxicam in vultures in contrast to humans, it may even be possible that
331 the vulture is reliant on a Phase I system other than CYP2C9 for metabolism (in vultures
332 CYP3A4 seem a possibility). If this is the case, then the extreme sensitivity of the vulture to
333 NSAID toxicity may be associated with the hepatotoxicity of diclofenac in humans which is
334 tentatively linked to CYP3A4 metabolism [67].
335

336 5.0 Conclusions

337
338 Pharmaceuticals provide many important health and economic benefits in the context of
339 their capacity to generate desired and specific therapeutic effects in the target species
340 (namely humans or in some cases, domestic animals and companion animals). In some
341 cases, however, environmental exposures of wildlife to pharmaceutical residues can have

dramatic consequences on non-mammalian species, as seen in the case of diclofenac and vultures [54,55] or fish populations in ecosystems exposed to synthetic oestrogens [68]. These notable examples, together with evidence of the widespread presence of pharmaceuticals in the environment, have been widely recognized to support the need for predictive environmental risk assessments [69-72] and consider API residues in cattle and other livestock species [73].

A fundamental aspect of this challenge relates to the need to consider comparative metabolism for a range of non-mammalian species. Specifically, it is clear that there remain major knowledge gaps regarding the comparative metabolisms of human and veterinary pharmaceuticals in non-mammalian species and this situation needs to be addressed in order to develop reliable environmental risk assessments for these important groups of medicines. It is proposed that this knowledge gap could be addressed in an efficient and ethical manner through the use of *in vitro* methods to define metabolism of reference APIs (selected from Table 1) in hepatocytes from carnivorous birds compared with omnivorous bird species, for example cormorants *Phalacrocorax auritus* and chickens *Gallus domesticus*, respectively [74,75]. For fish, the same approach is feasible using *in vitro* hepatocyte assays for mainly carnivorous salmonid species such as rainbow trout (*Oncorhynchus mykiss*) versus the mainly herbivorous cyprinid species such as zebrafish (*Danio rerio*) or carp (*Cyprinus carpio*) [20, 25]. For invertebrates, an *in vivo* approach would seem the best option and should be extended to both freshwater and marine species as part of an Adverse Outcome Pathways approach [39, 76-78]. Subsequently, the *in vitro* avian and fish metabolic data and the *in vivo* invertebrate data for reference APIs could be used to develop and validate *in silico* tools to better predict which enzymes are responsible for API metabolism. If the measured or predicted metabolism of a human or veterinary drug in mammalian or non-mammalian wildlife species raised concerns, further work could be done to evaluate the *in vitro* metabolites data through computational toxicology or metabolic pathway analysis [50, 79, 80].

In the wider context, where predicted regional increases in drug use occur or measurements of APIs in the environment raise concerns, the availability of validated *in silico* and *in vitro* methods to predict comparative metabolism will be of immense use in conducting environmental risk assessments. Specifically, together with prioritisation through the Predicted Exposure Concentration (PEC) approach, an understanding of ADMET can play an important role in defining Predicting No-Observed Effect Concentrations (PNECs) for freshwater, terrestrial and other environmental compartments, including predators [70,71,81]. In addition to this predictive aspect of pharmaceutical risk assessment, an understanding of ADMET can provide an important role for targeted monitoring of wildlife species of concern (eg vultures and other ultra-carnivorous species) [72,77].

[word count = 4269]

384 **References**

385 [1] Yengi LG, Leung L, Kao J. 2007 The evolving role of drug metabolism in drug discovery
386 and development. Pharm. Res. **24**, 842–858

387 [2] Ping PH, Fouts JR. 1979 Drug metabolism in birds. Pharmacol. **19**, 289–293

388 [3] Juskevich JC. 1987 Comparative metabolism in food producing animals: programs
389 sponsored by the Center for Veterinary Medicines. Drug Metab. Rev. **18**, 345-362

390 [4] Xu C, Li CY, Kong AN, 2005 Induction of phase I, II and III drug metabolism/transport by
391 xenobiotics. Archives of Pharm. Res. **28**, 249–268

392 [5] Rautio J., Kumpulainen, H., Heimbach, T., Oliyai, R., Oh, D., Järvinen, T., Savolainen, J.
393 2008. Prodrugs: design and clinical applications. Nature Reviews Drug Discovery **7**, 255-
394 270

395 [6] Lewis DFV. 1996 Cytochromes P450: Structure, Function and Mechanism. London:
396 Taylor and Francis. pp 348

397 [7] Nelson DR, Goldstone JV, Stegeman JJ. 2013 The cytochrome P450 genesis locus: the
398 origin and evolution of animal cytochrome P450s. Phil. Trans. R. Soc. B **368**, 20120474

399 [8] Ehrlich PR, Raven PH. 1964 Butterflies and plants: a study in coevolution. Evol. **18**, 586-
400 608

401 [9] Gonzalez FJ, Nebert DW. 1990 Evolution of the P450 gene superfamily: animal-plant
402 'warfare', molecular drive and human genetic differences in drug oxidation. Trends
403 Genet. **6**, 182-186

404 [10] Harborne JB. 1993 Introduction to Ecological Biochemistry 4th Ed London: Taylor and
405 Francis

406 [11] Nelson DR. 1998 Metazoan cytochrome P450 evolution. Comp. Biochem. Physiol. **121C**,
407 15-22

408 [12] Ronis MJJ, Walker CH. 1989 The microsomal monooxygenases of birds. Rev. Biochem.
409 Toxicol. **10**, 301-384

410 [13] Walker CH, Hopkin SP, Sibly RM, Peakall DB. 2012 Principles of Ecotoxicology 4th Edition.
411 Taylor and Francis.

412 [14] Abass K, Reponen P, Mattila S, Rautio A, Pelkonen O. 2014 Comparative metabolism of
413 benfuracarb in *in vitro* mammalian hepatic microsomes and its implications for chemical
414 risk assessment. Toxicol. Lett. **224,** 290-299

415 [15] Walker CH. 1978 Species differences in microsomal monooxygenase activity and their
416 relationship to biological half-lives. Drug Metab. Rev. **7**, 295–323

417 [16] Walker CH. 1980 Species differences in some hepatic microsomal enzymes that
418 metabolise xenobiotics. Prog. Drug Metab. **5**, 118-164

419 [17] Walker CH. 1981 The correlation between *in vivo* metabolism and *in vitro* metabolism in
420 vertebrates. Prog. Pesticide Biochem. 1 (Eds, Hutson DH, Roberts TR), pp 247-285

421 [18] Shrestha B, Reed JM, Starks PT, Kaufman GE, Goldstone JV, Roelke ME, O'Brien SJ,
422 Koepfli K, Frank LG, Court MH. 2011 Evolution of a major drug metabolizing enzyme
423 defect in the domestic cat and other felidae: phylogenetic timing and the role of
424 hypercarnivory. PLoS One **6**, e18046

425 [19] Court MH. 2013 Feline drug metabolism and disposition: pharmacokinetic evidence for
426 species differences and molecular mechanisms. Veterinary Clinics of North America:
427 Small Animal Practice **43**, 1039-1054

428 [20] Wiegand C, Pflugmacher S, Oberemm A, Steinberg C. 2000 Activity development of
429 selected detoxication enzymes during the ontogenesis of the zebrafish (*Danio rerio*). Int.
430 Rev. Hydrobiol. **85**, 413-422

431 [21] Randall DJ, Connell DW, Yang R, Wu SS. 1998 Concentrations of persistent lipophilic
432 compounds in fish are determined by exchange across the gills, not through the food
433 chain. Chemosphere **37**, 1263-1270

434 [22] Livingstone DR, Kirchin MA, Wiseman A. 1989 Cytochrome P450 and oxidative
435 metabolism in molluscs. Xenobiotica **19**, 1041-1062

436 [23] Livingstone DR. 1998 The fate of organic xenobiotics in aquatic ecosystems: quantitative
437 and qualitative differences in biotransformation by invertebrates and fish. Comp.
438 Biochem. Physiol. A **120**, 43-49

439 [24] Snyder MJ. 2000 Cytochrome P450 enzymes in aquatic invertebrates: recent advances
440 and future directions. Aquat. Toxicol. **48**, 529–547
441
442 [25] Buhler DR, Wang-Buhler JL. 1998 Rainbow trout cytochrome P450s: purification,
443 molecular aspects, metabolic activity, induction and role in environmental monitoring.
444 Comp. Biochem. Physiol. **121**, 107–137
445

446 [26] Celander M, Leaver MJ, George SG, Forlin L. 1993 Induction of cytochrome P450 1A1
447 and conjugating enzymes in rainbow trout (*Oncorhynchus mykiss*) liver: a time course
448 study. Comp. Biochem. Physiol. A **106**, 343–349

449 [27] Solé M, Rodríguez S, Papiol V, Maynou F, Cartes JE. 2009 Xenobiotic metabolism
450 markers in marine fish with different trophic strategies and their relationship to
451 ecological variables. Comp. Biochem. Physiol. C **149**, 83–89
452

453 [28] Arnot JA, Mackay D, Parkerton T, Bonnell M, 2008 A database of fish biotransformation
454 rates for organic chemicals. Environ. Toxicol. Chem. **27**, 2263–2270
455

456 [29] Gomez CF, Constantine L, Huggett DB. 2010 The influence of gill and liver metabolism
457 on the predicted bioconcentration of three pharmaceuticals in fish. Chemosphere **81**,
458 1189-1195

459 [30] Hasselberg L, Grøsvik BE, Goksøyr A, Celander MC. 2005 Interactions between
460 xenoestrogens and ketoconazole on hepatic CYP1A and CYP3A, in juvenile Atlantic cod
461 (*Gadus morhua*). Comp. Hepatol. **4**, 2-8
462
463 [31] Hasselberg L, Westerberg S, Wassmur L, Celander MC. 2008 Ketoconazole, an antifungal
464 imidazole, increases the sensitivity of rainbow trout to 17α-ethynylestradiol exposure.
465 Aquat. Toxicol. **86**, 256–264
466
467 [32] Hegelund T, Ottosson K, Rådinger M, Tomberg P, Celander MC. 2004 Effects of the
468 antifungal imidazole ketoconazole on CYP1A and CYP3A in rainbow trout and killifish.
469 Environ. Toxicol. Chem. **23**, 1326–1334
470
471 [33] Jones HS, Trollope HT, Hutchinson TH, Panter GH, Chipman JK. 2009 Assessment of
472 ibuprofen metabolism by zebrafish larvae larvae, using liquid chromatography–mass
473 spectrometry (LC–MS). Toxicol. **262**, 14-16

474 [34] Owen SF, Huggett DB, Hutchinson TH, Hetheridge MJ, Kinter LB, Ericson JF, Sumpter JP.
475 2009 Uptake of propranolol, a cardiovascular pharmaceutical, from water into fish
476 plasma and its effects on growth and organ biometry. Aquat. Toxicol. **93**, 217-224

477 [35] Smith EM, Chu S, Paterson G, Metcalfe CD, Wilson JY. 2010 Cross-species comparison of
478 fluoxetine metabolism with fish liver microsomes. Chemosphere **79**, 26–32
479
480 [36] Thibaut R, Porte C. 2008 Effects of fibrates, anti-inflammatory drugs and
481 antidepressants in the fish hepatoma cell line PLHC-1: Cytotoxicity and interactions with
482 cytochrome P450 1A. Toxicol. in Vitro **22**, 1128-1135

483 [37] Bartram AE, Winter MJ, Huggett DB, McCormack P, Constantine LA, Hetheridge MJ,
484 Hutchinson TH, Kinter LB, Ericson JF, Sumpter JP, Owen SF. 2012 *In vivo* and *in vitro* liver
485 and gill EROD activity in rainbow trout exposed to the beta-blocker propranolol. Environ.
486 Toxicol. Chem. **27**, 573-582

487 [38] Wassmur B, Gräns J, Norström E, Wallin M, Celander MC. 2013 Interactions of
488 pharmaceuticals and other xenobiotics on key detoxification mechanisms and
489 cytoskeleton in *Poeciliopsis lucida* hepatocellular carcinoma*,* PLHC-1 cell line. Toxicol. in
490 Vitro **27**, 111-120

491 [39] Jeon J, Kurth D, Hollender J. 2013 Biotransformation pathways of biocides and
492 pharmaceuticals in freshwater crustaceans based on structure elucidation of
493 metabolites using high resolution mass spectrometry. Chem. Res. Toxicol. **26**, 313−324

494 [40] Canga AG, Sahagún Prieto AM, Liébana MJD, Martínez NF, Vega MS, García Vieitez JI.
495 2009 The pharmacokinetics and metabolism of ivermectin in domestic animal species.
496 The Veterinary Journal, **179**, 25-37

497 [41] Carlsson G, Patring J, Kreuger J, Norrgren L, Oskarsson A. 2013 Toxicity of 15 veterinary
498 pharmaceuticals in zebrafish (*Danio rerio*) embryos. Aquat. Toxicol. **126**, 30-41

499 [42] Parkinson A. 1996. Biotransformation of xenobiotics. In Klaassen CD, ed, Casarett and
500 Doull's Toxicology, The Basic Science of Poisons, Unit 2, Chapter 6, McGraw-Hill, New
501 York, USA, pp 133-186.

502 [43] Ogu CC, Maxa JL. 2000 Drug interactions due to cytochrome P450. Baylor University
503 Medical Center Proceedings **13**, 421-423

504 [44] Wishart DS, Knox C, Guo AC, Shrivastava S, Hassanali M, Stothard P, Chang Z, Woolsey J.
505 2006 DrugBank: a comprehensive resource for *in silico* drug discovery and exploration.
506 Nucleic Acids Res. **34**, D668–D672

507 [45] Ohyama K, Maki S, Sato K, Kato Y. 2004 *In vitro* metabolism of [14C]methoxychlor in rat,
508 mouse, Japanese quail and rainbow trout in precision-cut liver slices. Xenobiotica **34**,
509 741-754

510 [46] Huggett DB, Cook JC, Ericson JE, Williams RT. 2003. A theoretical model for utilizing
511 mammalian pharmacology and safety data to prioritize potential impacts of human
512 pharmaceuticals to fish. J. Human Ecol. Risk. Assess. **9**, 1789-1799

513 [47] Schreiber R, Gündel U, Franz S, Küster A, Rechenberg B, Altenburger R. 2011 Using the
514 fish plasma model for comparative hazard identification for pharmaceuticals in the
515 environment by extrapolation from human therapeutic data. Reg. Toxicol. Pharmacol.
516 **61**, 261-275

517 [48] Huber C, Bartha B, Schröder P. 2012 Metabolism of diclofenac in plants in plants –
518 hydroxylation is followed by glucose conjugation. J. Hazardous Materials **243**, 250-256
519
520 [49] Kirchmair J, Williamson MJ, Tyzack JD, Tan L, Bond PJ, Bender A, Glen RC. 2012
521 Computational prediction of metabolism: sites, products, SAR, P450 enzyme dynamics,
522 and mechanisms. J. Chem. Inf. Model. **52**, 617-648

523 [50] Obach RS, Baxter JG, Liston TE, Silber M, Jones BC, MacIntyre F, Rance DJ, Wastall P.
524 1997 The prediction of human pharmacokinetics parameters from preclinical and *in*
525 *vitro* metabolism data. J. Pharmacol. Exp. Ther. **283**, 46-58

526 [51] Barton HA, Pastoor TP, Baetcke K, Chambers JE, Diliberto J, Doerrer NG, Driver JH,
527 Hastings JH, Iyengar S, Krieger R, Stahl B, Timchalk C. 2006. The acquisition and
528 application of absorption, distribution, metabolism and excretion (ADME) data in
529 agricultural chemical safety assessments. Crit. Rev. Toxicol. **36**, 9-35
530
531 [52] Nichols JW, McKim JM, Andersen ME, Gargas ML, Clewell HJ III, Erickson RJ. 1990. A
532 physiologically based toxicokinetic model for the uptake and disposition of waterborne
533 organic chemicals in fish. Toxicol. Appl. Pharmacol. **106**, 433-447

534 [53] Schultz S, Baral HS, Charman S, Cunningham AA, Das D, Ghalsasi GR, Goudar MS, Green
535 RE, Jones A, Nighot P, Pain DJ, Prakash V. (2004) Diclofenac poisoning is widespread in
536 declining vulture populations across the Indian subcontinent. Proc. Royal Soc. B:
537 Biological Sciences **271**, S458-S460

538 [54] Cuthbert R, Green DR, Ranada S, Saravanan S, Pain D, Prakash V, Cunningham AA. 2006
539 Rapid population declines of Egyptian vulture (*Neophron percnopterus*) and red-headed
540 vulture (*Sarcogyps calvus*) in India. Animal Consservation **9**, 349-354

541 [55] Swan GE, Cuthbert R, Quevedo M, Green RE, Pain DJ, Bartels P, Cunningham AA,
542 Duncan N, Meharg A, Oaks JL, Parry-Jones J, Schultz S, Taggart MA, Verdoorn GH,
543 Wolter K. 2006 Toxicity of diclofenac in Gyps vultures. Biol. Lett. **2**, 1-4
544

545 [56] Naidoo V, Duncan N, Bekker L, Swan G. 2007 Validating the domestic fowl as a model to
546 investigate the pathophysiology of diclofenac in Gyps vultures. Environ. Toxicol.
547 Pharmacol. **24**, 260-266

548 [57] Naidoo V, Wolter K, Cuthbert R, Duncan N. 2009 Veterinary diclofenac threatens
549 Africa's endangered vulture species. Reg. Toxicol. Pharmacol. **53**, 205-208

550 [58] Naidoo V, Mompati KF, Duncan N, Taggart MA. 2011 The pied crow (*Corvus albus*) is
551 insensitive to diclofenac at concentrations present in carrion. J. Wildlife Dis. **47**, 936-944

552 [59]Naidoo V, Wolter K, Cromarty AD, Bartels P, Bekker L, McGaw L, Taggart MA, Cuthbert R,
553 Swan GE. 2008 The pharmacokinetics of meloxicam in vultures. J. Veterinary Pharmacol.
554 Therap. **31**, 128-134

555 [60] Naidoo V, Wolter K, Cromarty D, Diekmann M, Duncan N, Meharg AA, Taggart MA,
556 Venter L, Cuthbert R. 2010 Toxicity of non-steroidal anti-inflammatory drugs to Gyps
557 vultures: a new threat from ketoprofen. Biol. Lett. **6**, 339-334

558 [61] Naidoo V, Venter L, Wolter K, Taggart M, Cuthbert R. 2010 The toxicokinetics of
559 ketoprofen in *Gyps coprotheres*: toxicity due to zero-order metabolism. Arch. Toxicol.
560 **84**, 761-766

561 [62] Walsh CT, Schwartz-Bloom RD. 2004 Levine's Pharmacology: Drug Actions and
562 Reactions. CRC Press, 7th edition, pp 561

563 [63] Yan Z, Li J, Huebert N, Caldwell GW, Du Y, Zhong H. 2005 Detection of a novel reactive
564 metabolite of diclofenac: evidence for CYP2C9-mediated bioactivation via arene oxides.
565 Drug Metab. Disp. **33**, 706-713

566 [64] Dorado P, Cavaco I, Caceres MC, Piedade R, Ribeiro V, LLerana A. 2008 Relationship
567 between CYP2C8 genotypes and diclofenac 5-hydroxylation in healthy Spanish
568 volunteers. Eur. J. Clin. Pharmacol. **64**, 967-970

569 [65] Zhou SF, Zhou ZW, Yang LP, Cai JP. 2009 Substrates, inducers, inhibitors and structure-
570 activity relationships of human cytochrome P450 2C9 and implications in drug
571 development. Curr. Med. Chem. **16**, 3480-3675

572 [66] Grosser T, Smyth E, FitzGerald GA. 2011 Anti-inflammatory, Antipyretic and Analgesic
573 Agents; Pharmacotherapy of Gout in Brunton L, Chabner B and Knollman B (eds)
574 Goodman and Gilman's The Pharmacological Basis of Therapeutics 11th edition, Chapter
575 **34**, 959-1004

576 [67] Bort R, Macé K, Boobis A, Gómez-Lechón MJ, Pfeifer A, Castell J. 1999 Hepatic
577 metabolism of diclofenac; role of human CYP in the minor oxidative pathways. Biochem.
578 Pharmacol. **58**, 787-796

579 [68] Kidd KA, Blanchfield PJ, Mills KH, Palace VP, Evans RE, Lazorchak JM, Flick RW. 2007
580 Collapse of a fish population after exposure to a synthetic estrogen. Proc. Nat. Acad. Sci.
581 **104**, 8897–8901

582 [69] Daughton CG, Ternes TA. 1999 Pharmaceuticals and personal care products in the
583 environment: agents of subtle change? Environ. Hlth. Perspect. Suppl. **107 (S6)**, 907–
584 938

585 [70] Schmitt H, Boucard T, Garric J, Jensen J, Parrott J, Péry A, Römbke J, Straub JO,
586 Hutchinson TH, Sánchez-Argüello P, Wennmalm A, Duis K. 2010 Recommendations on
587 the environmental risk assessment of pharmaceuticals: Effect characterization. Int.
588 Environ. Assess. Manag. **6**, 588-602
589

590 [71] Küster A, Alder A, Escher B, Duis K, Fenner K, Garric J, Hutchinson TH, Lapen D, Duprey A,
591 Römbke J, Snape J, Ternes T, Topp E, Wehrman A, Knacker T. 2010 Environmental risk
592 assessment of human pharmaceuticals in the European Union - a case study with the
593 beta-blocker atenolol. Int. Environ. Assess. Manag. **6**, 514-523
594

595 [72] Boxall A, Rudd MA, Brooks BW, Caldwell DJ, Choi K, Hickmann S, Innes E, Ostapyk K,
596 Staveley JP, Verslycke T, Ankley GT, Beazley KF, Belanger SE, Berninger JP,
597 Carriquiriborde P, Coors A, Deleo PC, Dyer SD, Ericson JF, Gagné F, Giesy JP, Gouin T,
598 Hallstrom L, Karlsson MV, Larsson DG, Lazorchak JM, Mastrocco F, McLaughlin A,
599 McMaster ME, Meyerhoff RD, Moore R, Parrott JL, Snape JR, Murray-Smith R, Servos
600 MR, Sibley PK, Straub JO, Szabo ND, Topp E, Tetreault GR, Trudeau VL, Van Der Kraak G.
601 2012 Pharmaceuticals and personal care products in the environment: what are the big
602 questions? Environ. Hlth. Perspect. **120**, 1221-1229
603

604 [73] Taggart MA, Senacha KR, Green RE, Jhala YV, Raghaven B, Rahmani AR, Cuthbert R,
605 Pain DJ, Meharg AA. 2007 Diclofenac residues in carcasses of domestic ungulates
606 available to vultures in India. Environ. Int. **33**, 759-765

607 [74] Verbrugge LA, Giesy JP, Verbrugge DA, Woodin BR, Stegeman JJ. 2001 Catalytic and
608 immunochemical properties of hepatic cytochrome P450 1A in three avian species
609 treated with beta-naphthoflavone or isosafrole. Comp. Biochem. Physiol. C **130**, 67–83

610 [75] Kubota A, Kim EY, Iwata H. 2009 Alkoxyresorufin (methoxy-, ethoxy-, pentoxy- and
611 benzyloxyresorufin) O-dealkylase activities by *in vitro*-expressed cytochrome P450 1A4
612 and 1A5 from common cormorant (*Phalacrocorax carbo*). Comp. Biochem. Physiol. C
613 **149**, 544–551

614 [76] David P, Dauphin-Villemant C, Mesneau A, Meyran C. 2003 Molecular approach to
615 aquatic environmental bioreporting: differential response to environmental inducers of
616 cytochrome P450 monooxygenase genes in the detritivorous subalpine planktonic
617 Crustacea, *Daphnia pulex*. Mol. Ecol. **12**, 2473–2481

618 [77] Hutchinson TH, Lyons BP, Thain J, Law RJ. 2013 Evaluating legacy contaminants and
619 emerging chemicals in marine environments using adverse outcome pathways and
620 biological effects-directed analysis. Mar.Poll. Bull. **74**, 517-525

621 [78] Celander M, Goldstone J, Denslow N, Iguchi T, Kille P, Meyerhoff R, Smith B, Hutchinson
622 TH, Wheeler JR. 2011 Species extrapolation for the 21st century. Environ. Toxicol.
623 Chem. **30**, 52-63

624 [79]Kolanczyk RC, Schmieder P, Jones WJ, Mekenyan OG, Chapkanov A, Temelkov A, Kotov S,
625 Velikova M, Kamenska V, Vasilev K, Veith GD. 2012 MetaPath: An electronic knowledge
626 base for collating, exchanging and analyzing case studies of xenobiotic metabolism.
627 Reg. Toxicol. Pharmacol. **63**, 84-96

628 [80] Li S, Pozhitkov A, Ryan RA, Manning CS, Brown-Peterson N, Brouwer M. 2010
629 Constructing a fish metabolic network model. Genome Biol. **11**, R115

630 [81] Murray-Smith RJ, Coombe VT, Haag Grönlund M, Waern F, Baird JA. 2012 Managing
631 emissions of active pharmaceutical ingredients from manufacturing facilities: an
632 environmental quality standard approach. Integ. Environ. Assess. Manag. **8**, 320–330

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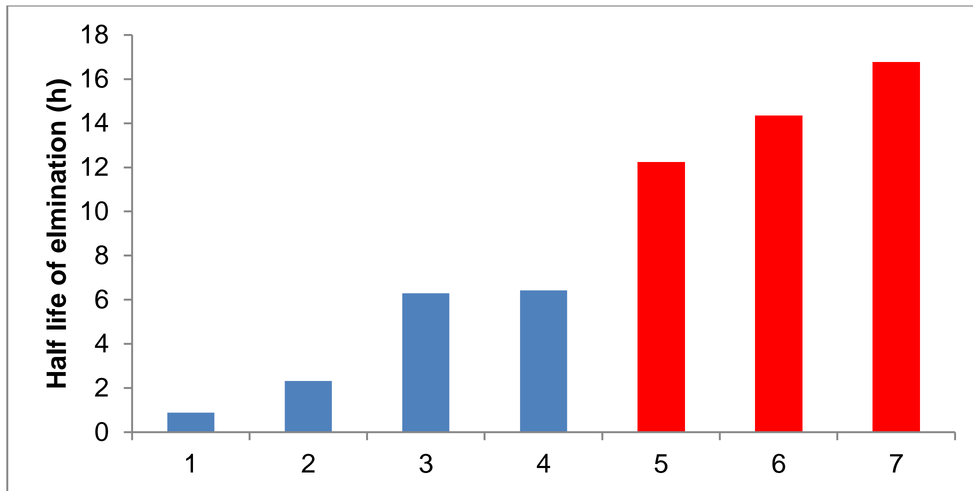

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638 **Figure 1.** Estimated half-life of elimination for various avian species dosed with diclofenac in
639 controlled toxicity studies. The half-lives have be ranked from fastest to slowest and
640 represent; 1- *Gallus domesticus* (0.8 mg/kg); 2- *Corvus albus* (10 mg/kg); 3- *Cathartes aura*
641 (25 mg/kg); 4- *Cathartes aura* (8 mg/kg); 5- *Gyps coprotheres* (0.8 mg/kg); 6- *Gallus*
642 *domesticus* (5mg/kg); 7- *Gyps africanus* (0.8 mg/kg). The red bars, indicate those doses
643 associated with mortality.

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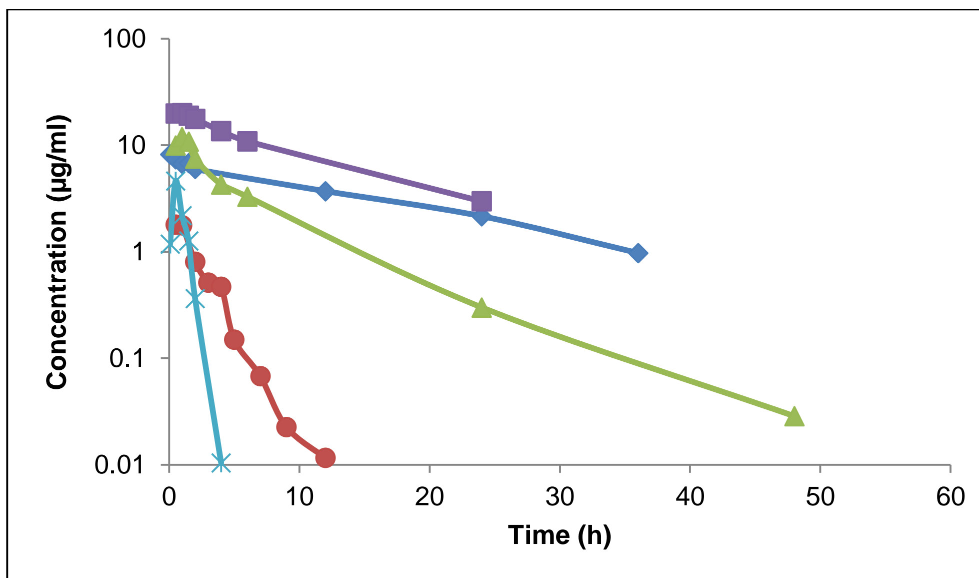

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650 **Figure 2.** Plasma versus time profiles for diclofenac at 0.8 mg/kg in the *G. coprotheres*
651 (Rhomboid); ketoprofen at 5 mg/kg for the *G. coprotheres* that died (Square); ketoprofen at
652 5 mg/kg in *G. coprotheres* that survived (Triangle); Diclofenac in chickens at 0.8 mg/kg (circle)
653 and meloxicam in *G. coprotheres* at 2 mg/kg (cross).
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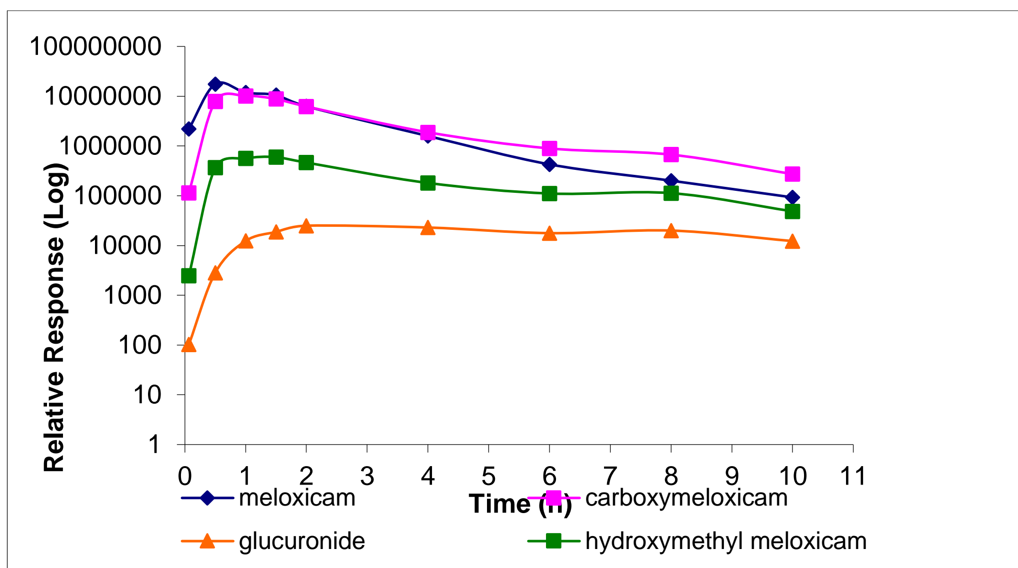

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658 **Figure 3.** Relative response (AUC per peak on LC-MSMS chromatograms) versus time
659 profiles for parent meloxicam and its three metabolites hydroxymethyl meloxicam; an
660 unidentified hydroxymethyl metabolite carboxymeloxicam and the glucuronide metabolite
661 following treatment at 2 mg/kg in *G. coprotheres*.
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667 Table 1. Summary of vertebrate metabolic pathways with examples of pharmaceutical and
668 xenobiotic substrates and inhibitors.

Enzyme	Localization	Substrate	Inhibitor
Phase I – Hydrolysis Reactions:			
Esterase	Microsomes, cytosol	trandolapril	tamoxifen
Peptidase	Lysosomes	-	alogliptin
Epoxide hydrolase	Microsomes, cytosol	Diazepam	valproate
Phase I – Reduction Reactions:			
Azo- and nitro-reduction	Microsomes, cytosol	Prontosil	clofibrate
Carbonyl reduction	Microsomes, cytosol	Loxoprofen	befunolol
Disulphide reduction	Cytosol	captopril	-
Sulphoxide reduction	Cytosol	-	dimethylsulfoxide
Quinone reduction	Microsomes, cytosol	Trenimon	warfarin
Reductive dehalogenation	Microsomes	Chloramphenicol	-
Phase I – Oxidation Reactions:			
Alcohol dehydrogenase	Cytosol	ethanol	fomepizole
Aldehyde dehydrogenase	Mitochondria, cytosol	acetaldehyde	disulfiram
Aldehyde oxidase	Cytosol	Aldehyde	raloxifene
Xanthine oxidase	Cytosol	xanthine	allopurinol
Monoamine oxidase	Mitochondria	Monoamine	moclobemide
Diamine oxidase	Cytosol	diamine	phenformin
Prostaglandin H synthase	Microsomes	arachidonic acid	ibuprofen
Flavin-monooxygenases	Microsomes	riboflavin	nitric oxide
Cytochrome P450:	Microsomes	-	-
CYP1A1	Microsomes	7-ethoyxyresorufin	galangin
CYP1A2	Microsomes	clozapine propranolol	cimetidine citalopram
CYP2C19	Microsomes	citalopram diazepam	fluoxetine ketoconazole
CYP2C9	Microsomes	diclofenac ibuprofen	fluconazole fluoxetine
CYP2D6	Microsomes	metoprolol tramadol	fluoxetine sertraline
CYP2E1	Microsomes	acetaminophen ethanol	disulfiram water cress
CYP3A4	Microsomes	carbamazepine simvastatin	flavonoids ketoconazole
Phase II – Enzyme Reactions:			
Glucuronide conjugation	Microsomes	Phase I metabolites	valproic acid
Sulphate conjugation	Cytostol	Phase I metabolites	harmol
Glutathione conjugation	Microsomes, cytosol	Phase I metabolites	tannic acid
Amino acid conjugation	Microsome	Phase I metabolites	kinetin
Acetylation	Mitochondria, cytosol	Phase I metabolites	garcinol
Methylation	Microsomes, cytosol	Phase I metabolites	5-A-2’deoxycytidine

669

670 Table 1 = 220 words

671 Table 2. Representative examples of computational tools for predicting factors associated
672 with mammalian metabolism (note - programs may have additional capabilities).

Factor Predicted	**Software**	**Summary of method**	**Website or key citation**
(i) Site of metabolism	Metaprint2D	Predicts sites of Phase I metabolism in dog, human and rat through data-mining and statistical analysis of published metabolic transformations.	http://www-metaprint2d.ch.cam.ac.uk/metaprint2d
(ii) Potential Metabolites	Meteor Nexus	Uses expert knowledge rules for metabolism to predict metabolites which are presented in metabolic trees	http://www.lhasalimited.org/products/meteor-nexus.htm
(iii) CYP binding affinity / inhibition	isoCYP	Predicts the predominant human cytochrome P450 isoform by which a compound is metabolised	http://www.molecular-networks.com/products/isocyp
(iv) CYP induction	VirtualToxLab	Predicts binding affinities to Aryl hydrocarbon receptor (and other targets) using flexible docking and quantitative structure-activity relationships	http://www.biograf.ch/index.php?id=projects&subid=virtualtoxlab

673
674 Table 2 = 121 words
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