

Journal Pre-proof

Toward a better understanding of the effects of endocrine disrupting compounds on health: Human-relevant case studies from sheep models

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PII: S0303-7207(20)30011-3

DOI: <https://doi.org/10.1016/j.mce.2020.110711>

Reference: MCE 110711

To appear in: *Molecular and Cellular Endocrinology*

Received Date: 11 June 2019

Revised Date: 9 January 2020

Accepted Date: 10 January 2020

Please cite this article as: Viguié, C., Chaillou, E., Gayrard, Vé., Picard-Hagen, N., Fowler, P.A., Toward a better understanding of the effects of endocrine disrupting compounds on health: Human-relevant case studies from sheep models, *Molecular and Cellular Endocrinology* (2020), doi: <https://doi.org/10.1016/j.mce.2020.110711>.

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1 **TITLE**

2 **Toward a better understanding of the effects of endocrine disrupting compounds on health:**
3 **human-relevant case studies from sheep models.**

4

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17 **Highlights**

- 18 • Endocrine disruption is a very complex and critical public health issue
- 19 • There is a need for human-relevant models to assess endocrine physiology and the physiology
20 of period at risks
- 21 • The sheep is a good alternative to classical rodent models to understand fetal exposure, thyroid
22 disruption and its consequences on brain development
- 23 • The sheep allows real-life exposure scenario to complex mixtures.

24

25 **Abstract:**

26

27 There are many challenges to overcome in order to properly understand both the exposure to, and
28 effects of, endocrine disruptors (EDs). This is particularly true with respect to fetal life where ED
29 exposures are a major issue requiring toxicokinetic studies of materno-fetal exchange and
30 identification of pathophysiological consequences. The sheep, a monotocous large size species is very
31 suitable for *in utero* fetal catheterization allowing a modelling approach predictive of human fetal
32 exposure. Predicting adverse effects of EDs on human health is frequently impeded by the wide
33 interspecies differences in the regulation of endocrine functions and their effect on biological
34 processes. Because of its similarity to humans as regards gestational and thyroid physiologies and
35 brain ontogeny, the sheep constitutes a highly appropriate model to move one step further on thyroid
36 disruptor hazard assessment. As a grazing animal, sheep has also been proven to be useful in the
37 evaluation of the consequences of chronic environmental exposure to “real-life” complex mixtures at
38 different stages of the reproductive life cycle.

39

40 **Key words:** endocrine disruptors – sheep model –fetal exposure- thyroid - mixture

41

42 1. INTRODUCTION

43 Many *in vitro/ex vivo* tools have recently been developed and have proved to be very useful for rapidly
44 screening and/or understanding of the mechanisms of action of endocrine disruptors (EDs). However,
45 the complexity and the diversity of the effects of EDs underline the need for integrated *in vivo* models
46 based on the appropriate use of species relevant to human for hazard assessment. This is a prerequisite
47 to minimise the levels of uncertainty when extrapolating from animal findings to human. Rodent
48 models are extensively used in toxicology, including endocrine toxicology, and are considered as the
49 reference species for regulatory toxicology purposes. These models have many advantages in terms of
50 both study duration and feasibility. These include reasonable extrapolation of some representative
51 functions to the human, complete genetic and molecular characterisation and affordable costs. Many
52 experimental studies have demonstrated that low doses of common pollutants, such as BPA (bisphenol
53 A), can disrupt endocrine function, brain development and reproduction (Heindel et al., 2015; Le
54 Magueresse-Battistoni et al., 2018; Caporossi and Papaleo, 2017), 2017; Mhaouty-Kodja et al., 2018;
55 Palanza et al., 2016; Viguié et al., 2018) in rodents exposed during intrauterine life. The rodent models
56 have, thus, been successfully used in pioneering studies establishing the proof of concept for the
57 existence of EDs and their related adverse effects on the health of individuals and/or their progeny.
58 However, the process of extrapolating experimental animal data to the human, requires an additional
59 step towards maximising the human-relevance of the different models. This step must be based on an
60 improved consideration of interspecies differences in the developmental and physiological scheme of
61 regulation of endocrine functions. The challenges and questions paving the way toward a better
62 understanding of ED effects are numerous and it is hardly conceivable that a single non-human species
63 could enable the production of a precisely human-relevant picture for every physiological system and
64 developmental stage. It would be a matter of grave concern, therefore, should the scientific community
65 and the regulatory bodies rely solely on approaches exclusively designed in and for rodents.

66 The existence of windows of sensitivity to ED actions that result in potentially adverse
67 transgenerational effects is a critical issue and a challenge for the ED research field. The hormone
68 dysregulating properties of EDs are of great importance if exposure occurs during early developmental
69 stages. This is when hormones are exerting permanent effects on fetal development by interacting with

70 the *in utero* processes of biological functional programming (Catanese et al., 2015). Thus, the
71 prediction of fetal exposure to ED is a major issue that should not be neglected. It is noteworthy that
72 such considerations are now being incorporated into some research funder priorities, as for example in
73 the EU Horizon 2020 programmes around the exposome [https://ec.europa.eu/info/funding-](https://ec.europa.eu/info/funding-tenders/opportunities/portal/screen/opportunities/topic-details/sc1-bhc-28-2019)
74 [tenders/opportunities/portal/screen/opportunities/topic-details/sc1-bhc-28-2019](https://ec.europa.eu/info/funding-tenders/opportunities/portal/screen/opportunities/topic-details/sc1-bhc-28-2019). Fetal development
75 proceeds from complex multilevel interactions between the mother and the fetus. The great differences
76 in gestational physiology and, above all, endocrine function, which exists between rodents and humans
77 have led to serious concerns about extrapolating rodent data to humans (Habert et al., 2014). Sheep
78 have long been used as an animal model for studying fetal physiology and much of the available
79 information on maternal-fetal pharmacokinetic has been obtained from pregnant ewe (Szeto, 1982).
80 Together with the fact that characterising fetal exposure is a critical issue in the field of EDs, these
81 historical data prompted us to develop an *in utero* catheterised ovine fetus model (Meschia et al.,
82 1965) to determine bi-directional transplacental exchanges of bisphenol A (BPA) and its main
83 metabolite BPA-glucuronide (BPA-G). It is noteworthy that the relevance of this model goes far
84 beyond EDs since it is relevant to any contaminants for which fetal exposure is at high risk. This
85 model enables maternal and fetal compartments to be assessed separately and allows direct fetal
86 administration of EDs, thus by-passing maternal metabolism if required. We use this system to
87 develop a pharmacokinetic-based modelling approach to predict the extent of human fetal tissue
88 exposure to the active form of BPA and by comparison to BPS (Corbel et al., 2013; Gauderat et al.,
89 2017; Grandin et al., 2019, 2018).

90 One major source of uncertainty when predicting the potential adverse effects of EDs on human
91 health relies on coping with the considerable inter-species differences in the regulatory pathways and
92 feedback loops of endocrine functions and their effect on critical biological processes. It is all the more
93 worrying, therefore, that regulatory toxicological *in vivo* evaluations rely mainly on rodent studies.
94 Thyroid disruption is an emblematic example of how the relevance of rodent results to humans can be
95 debatable. We have come to the point when some of the inter-species differences in thyroid function
96 regulation are being used as an argument to refute results obtained in rodent models as not relevant to
97 humans.

98 Finally, one major challenge when investigating the toxicology of environmental contaminants
99 concerns the identification of human-relevant exposure systems/models. This is rendered even more
100 difficult by the fact that both human and animal populations are likely to be exposed to a multitude of
101 contaminants at a time and at low doses. One approach is the use of sentinel species who share the
102 human environment, as in a recent study comparing the effects of two EDs on dog and human sperm
103 but these do not necessarily address complex mixtures (Sumner et al., 2019). One other possible key
104 strategy to address this critical challenge is to expose the test animals to “real life mixture”. There is
105 some debate among the scientific community on the precise criteria for a “real life mixture” and the
106 correct approaches to address this question. Exposing animals through their “natural” environment is
107 one very interesting option that cannot be conducted in rodents. Using grazing animals that can be
108 “naturally” exposed through their environment due to their feeding behaviour has been successfully
109 developed in the sheep, notwithstanding the digestive and metabolic differences posed by the rumen in
110 the ovine.

111 Considering the most human-relevant strategies to evaluate the health and developmental risks of
112 EDs, our goal here is to show, using the sheep model as a proof of concept, how a non-rodent animal
113 model can complete and, in some instances, validate classical regulatory rodent studies. We chose
114 three well documented examples from ovine ED studies to demonstrate how using non-rodent models
115 can be very useful to answer to some of the main scientific challenges in the ED research field. More
116 specifically, we addressed the questions of the characterisation and/or prediction of exposure during
117 fetal life one of the most sensitive life stages, the relevance of the model with respect to endocrine
118 regulation through the thyroid function and the ability to set up long-term “real life” exposure studies-

119

120 **2. MODELING MATERNO-FETAL EXCHANGES OF TOXICANTS**

121 **2.1. Fetal sheep to predict human fetal exposure to an endocrine disruptor from semi-** 122 **physiological toxicokinetic models: application to the assessment of fetal exposure to** 123 **bisphenols**

124 Exposure to chemicals during prenatal life is a major concern for toxicologists. Fetal exposure to
125 endocrine disruptors (EDs) during critical windows of development has repeatedly been suggested to

126 be involved in the onset of a variety of adverse biological effects, later in life, even when exposure
127 occurs at low doses (Vandenberg et al., 2012).

128 A sound interpretation of human health implications of ED exposure, based on toxicological
129 studies carried out in animal models, requires to be able to determine the human blood and/or urinary
130 concentrations of biologically active compounds. These can be compared with the concentrations
131 measured in test species that have responded adversely to exposure through the mother. Indeed, it is
132 typically acknowledged that any adverse systemic effect of a compound is directly related to the
133 plasma concentrations of its biologically active form/s. Such relationships more often follow a
134 classical linear or monotonic pattern, but it should be noted that non-monotonic dose responses, which
135 are very difficult to characterise, have been reported for many EDs. The biomonitoring data regarding
136 the concentrations in human maternal, fetal, and neonatal fluids are of the utmost importance to
137 provide a reliable foundation for the extrapolations of risks, from animal studies to humans. However,
138 measuring fetal chemical concentrations is difficult in humans and very few measurements of EDs in
139 the human fetal circulation have ever been made. The quantification of low levels of xenobiotics is
140 hampered when the molecule of interest is ubiquitous in the environment and/or in tissue/sample
141 collection, processing and analytical devices as is the case for BPA (Ye et al., 2013). Hence, the high
142 values of cord blood BPA concentrations (in the ng/ml range) reported in some human biomonitoring
143 studies (Zhang et al., 2014) should not be included in the risk assessment of BPA due to possible
144 contaminations specifically related to exposure during delivery or to post-exposure sample
145 contamination (Dekant and Völkel, 2008).

146 The glucuronidation of drugs constitutes a major pathway of elimination and glucuronide
147 metabolites have been detected in fetal plasma following maternal drug administration, as it is the case
148 for BPA. Hence, toxicokinetic studies have shown that BPA glucuronide (BPAG) accumulates in the
149 plasma of fetal sheep and monkeys following maternal administration (Corbel et al., 2013; Patterson et
150 al., 2013; Viguié et al., 2013; Vom Saal et al., 2014). Due to their lack of estrogenicity (Matthews et
151 al., 2001; Skledar et al., 2016), the systemic exposure to bisphenol conjugated metabolites is not taken
152 into account for risk assessment purposes. However, *in vitro* studies suggest that BPA glucuronide
153 may exert biological activities similar to (Boucher et al., 2015) or different from those of the parent

154 compound (Viñas et al., 2013). This highlights the need for experimental data to elucidate the
155 maternal-fetal toxicokinetic behaviour of bisphenols and to provide precise time-dependent
156 measurements of the plasma concentrations of both the parent and glucuronidated metabolites. These
157 data are essential for interpreting human biomonitoring data and for the development of toxicokinetic
158 models to predict the extent of human fetal internal exposure, under an appropriate scheme of dietary
159 and environmental exposures.

160

161 **2.2. Choice of a relevant animal model: the sheep for studying maternal-fetal** 162 **pharmacokinetics**

163 Pregnant ewes have been used extensively for translational research and have contributed to very
164 important advances in prenatal human medicine (Morrison et al., 2018). Due to the large size of these
165 animals, catheters can be surgically inserted and maintained in both the maternal and fetal vasculature.
166 Hence the chronically catheterised fetal sheep provides a unique model that permits the serial
167 sampling of maternal and fetal blood, as well as other fluids, after maternal or fetal administration
168 (Rurak et al., 1991). This animal model has already been applied to evaluate the disposition of several
169 drugs during the prenatal period (Kumar et al., 1997; Ngamprasertwong et al., 2016). Furthermore,
170 considerable insight regarding nutrient profiles has been gained from investigations of nutrient
171 exchange in pregnant sheep (Barry and Anthony, 2008) highlighting important physiological
172 similarities between the sheep and human placental functions despite structural differences. Indeed,
173 although the sheep synepitheliochorial placenta differs histologically from the human hemochorial
174 placenta, the microarchitecture of the ovine cotyledon is structurally analogous to that of the human
175 placenta (Barry and Anthony, 2008; Leiser et al., 1997; Morrison et al., 2018).

176 Drug concentrations in fetal plasma result from a complex interplay between bidirectional placental
177 clearances and fetal metabolism. For many drugs, in particular the most lipophilic and the low
178 molecular ionized ones, the most important mechanism of transfer across the placenta is the passive
179 diffusion. In this case, the extent of drug transport is dependent upon not only the physicochemical
180 properties of the drugs but also maternal and fetal blood flows (Rurak et al., 1991). Relative to the fetal
181 body weight, the blood flow in the umbilical vein and artery is of the same order of magnitude in sheep

182 (12 L/h.kg; Faber and Green, 1972; Rudolph, 1985) as in humans (7 L/kg.h, (Clavero et al., 1973;
183 Haugen et al., 2004), indicating that the flow-limited transfer of drugs across the placenta in sheep and
184 humans should be comparable. Accordingly, uteroplacental blood flow at the end of pregnancy is quite
185 well preserved between rabbit, rodents, macaque, sheep and human, when normalized by the placenta
186 weight (Mourier et al., 2017). By contrast, there are important interspecies differences in active
187 transport of molecules (Walker et al., 2017).

188 It has been shown that fetal sheep, compared to adults, possess low levels of some UDP-
189 glucuronosyltransferases (UGT) genes and thus likely lower activity (Pretheeban et al., 2011), similar
190 to humans (Burchell et al., 1989), suggesting a lower capacity of the metabolic pathways in which
191 these enzymes are involved in fetuses. In agreement with this observation, the intrinsic clearance of
192 BPA glucuronidation in near-term fetal sheep (120–135 days of gestation) is about 2-fold lower than
193 that of adults, the latter being of the same order as in adult humans (Corbel et al., 2015). Since a
194 significant fraction of venous umbilical flow, about 75% in humans and 50% in sheep, passes through
195 the fetal liver before reaching the systemic circulation, some pre-systemic conjugation can occur
196 between the maternal and fetal circulations in both the sheep and human fetus (Edelstone et al., 1978;
197 Haugen et al., 2004).

198

199 **2.3. Toxicokinetic modelling of fetal exposure to bisphenol A**

200 Toxicokinetic studies of BPA have been performed using the chronically catheterised fetal sheep
201 model to evaluate the time-course of BPA and BPAG concentrations in maternal and fetal plasma
202 following maternal and fetal BPA or BPAG intravenous dosing (Corbel et al., 2014; Gauderat et al.,
203 2016). This approach was applied to determine the relative contributions of the different pathways
204 controlling fetal exposure to BPA. The parameters estimated by the model are summarized in fig. 1
205 right-hand panel. At the end of pregnancy, about 6% of the maternal BPA dose enters the fetal
206 circulation, *i.e.* a dose relative to body weight equivalent to the maternal dose. The closeness of this
207 value with that estimated at 3.1% in human, using an *ex-vivo* model of perfused human placental
208 cotyledon (Corbel et al., 2014), emphasizes the relevance of the sheep model for studying placental
209 drug transfer. Most of the BPA entering the fetal circulation was rapidly eliminated, mainly through

210 the direct clearance of BPA from the fetus to the mother. This represented approximately 74% of the
211 fetal clearance of BPA. The remaining part of fetal BPA clearance (26%) was accounted for by the
212 fetal metabolism of BPA into BPAG. Although not the predominant mechanisms of BPA elimination,
213 the fetal conjugation clearance of BPA was higher than in the adult, despite the lower fetal expression
214 of the UGTs involved in fetal BPA glucuronidation (Pretheeban et al., 2011). This high fetal
215 conjugation clearance of BPA was responsible for a significant first-pass conjugation of BPA
216 transferred from the mother to the fetus. The limited fetal to maternal transfer accounted for about only
217 17% of the total BPAG output. Therefore, the elimination of BPAG trapped in the fetal compartment
218 mainly involved BPAG hydrolysis (83%).

219 A non-linear mixed effect model (NLMEM) was used to simultaneously analyse these
220 toxicokinetic data and build a semi-physiologically-based toxicokinetic model of fetomaternal BPA
221 exchange (Gauderat et al., 2017). The semi-physiologically-based toxicokinetic model of fetomaternal
222 BPA toxicokinetic developed in sheep was then humanised (Gauderat et al., 2016), using
223 toxicokinetic data obtained in adult humans via ingestion of a BPA (d6-BPA) labelled cookie. This
224 enabled the prediction of BPA and BPAG concentration-time profiles in human maternal and fetal
225 plasma. After a pattern of repeated maternal exposure to BPA that mimics dietary exposure to the
226 average dose estimated by EFSA ((EFSA, 2015), 11.05 nmol/d for 70 kg body weight), the
227 toxicokinetic model predicted human plasma concentrations of non-conjugated BPA fluctuating
228 between 14 and 140 pg/L in the adult and between 11 and 23 pg/L in the fetus, *i.e.* well below some of
229 the very high concentrations (several hundred ng/L) of unconjugated BPA in cord blood reported in
230 some human biomonitoring studies (Gerona et al., 2013; Veiga-Lopez et al., 2015). The maternal
231 plasma BPAG concentrations predicted by the toxicokinetic model fluctuated considerably (between
232 1.6 and 40 ng/L) depending on the delay between measurements and ingestion of BPA, while the
233 mean BPAG-predicted concentrations in the plasma of the human fetus were relatively stable and in
234 the order of 40 ng/L. The consistency of these predictions with measurements of BPAG (median
235 values of 120 and 960 ng/L, (Gerona et al., 2013; Veiga-Lopez et al., 2015) or total BPA (mean value
236 of 46 ng/L; (Yamamoto et al., 2016) in human cord blood again emphasises the relevance of the
237 pregnant sheep model to predict human fetomaternal toxicokinetics of BPA.

238

239 **2.4. Toxicokinetics of bisphenol S**

240 Owing to serious concerns regarding BPA the adverse effects of BPA on human health, it has been
241 replaced by structural analogues, mainly bisphenol S (BPS, (Liao et al., 2012). The high prevalence of
242 BPS in urine from a cohort of US adults (Ye et al., 2015), and its detection in human cord blood
243 (Kolatorova et al., 2018), gives rise to the question of the level of risk for human health associated
244 with prenatal exposure to this BPA analogue. Indeed, *in vitro* studies have shown that BPS binds the
245 estrogen receptors (ER) α and β (Molina-Molina et al., 2013) and acts as an ER agonist with a weaker
246 or equivalent potency to that of BPA (Kojima et al., 2019; Kuruto-Niwa et al., 2005; Rosenmai et al.,
247 2014). Although limited in number, *in vivo* studies have shown that prenatal exposure of mice to BPS
248 alters mammary gland development (Tucker et al., 2018). There is currently a critical gap in
249 knowledge about the extent to which the human, especially the fetus, is internally exposed to emerging
250 bisphenols. The only available toxicokinetic study of BPS performed in adult humans (Oh et al., 2018)
251 suggests that the toxicokinetic behaviour of BPS may differ from that of BPA and highlights the need
252 for animal studies of BPS toxicokinetic to identify the underlying mechanisms of gestational exposure
253 to BPS.

254 The main metabolite of BPS formed *in vivo* is BPS glucuronide (BPSG, Le Fol et al., 2015).
255 Toxicokinetic studies of BPS and its major metabolite, were carried out using a chronically
256 catheterised fetal sheep model to evaluate the time-course of BPS and BPSG concentrations in
257 maternal and fetal plasma following maternal and fetal BPS or BPSG intravenous dosing (Grandin et
258 al., 2018). It was striking that the fraction of the maternal BPS dose transferred from mother to fetus
259 (0.40%) was about ten times lower than that of BPA (Gauderat et al., 2017; fig.1). A limited placental
260 passage of BPS has also been revealed using an *ex-vivo* model of the perfused human placental
261 cotyledon model (Grandin et al., 2019). In this model, the materno-fetal clearance index (0.0852) was
262 about 10-fold lower than that of BPA. In sheep, only 26% of the BPS dose entering the fetal blood
263 against 74% for BPA (fig.1) were rapidly eliminated through its transfer into maternal blood. This
264 result is in agreement with the higher efficiency of fetal to maternal transfer as compared to its
265 maternal to fetal counterpart evidenced in the perfused human placenta (Grandin et al., 2019). About

266 half of the remaining BPS (46%) was metabolised by the fetus into BPSG. The elimination of this
267 BPSG from the fetal compartment required its hydrolysis, like that of most of the BPAG, due to a
268 limited placental transfer. Our compartmental toxicokinetic model predicted that, despite a lower
269 materno-fetal passage of BPS compared to BPA, the higher persistency of BPS in the fetal
270 compartment leads to expected BPS concentrations in fetal plasma of the same order than that of BPA
271 (Grandin et al., 2018).

272

273 **3. INTERSPECIES DIFFERENCES IN ENDOCRINE REGULATION: THE** 274 **EXAMPLE OF THE THYROID FUNCTION**

275 Although the basic mechanisms of endocrine regulation, in particular feedback mechanisms, are well
276 preserved among vertebrates, there are interspecies differences in the pathways mediating those
277 mechanisms. This raises concern about the relevance of animal models for human endocrinology and
278 our capacity to extrapolate results from animal to human when dealing with EDs. The sheep is a human-
279 relevant model to elucidate the regulation of several endocrine systems, including the somatotropic,
280 corticotropic, reproductive and thyroid systems in terms of both physiological and exposed conditions
281 (Dutour et al., 1997; Evans et al., 2016; Karsch and Battaglia, 2002; Puttabyatappa et al., 2019).
282 Unfortunately, in sheep as in other species, the ED research field is, to a large part, limited to the
283 reproductive, metabolic and thyroid functions. The interest in thyroid function is justified by the
284 relatively high incidence of thyroid dysfunctions in humans. Overall in Europe, there are around 200
285 new cases of thyroid disease/100,000/year. This is highest for hypothyroidism at 127 new
286 cases/100,000/year (for review see Garmendia Madariaga et al., 2014). Furthermore, epidemiological
287 and meta-analysis studies indicate a high incidence of undiagnosed thyroid function abnormalities
288 (Garmendia Madariaga et al., 2014), including in pregnant women (Andersen, 2019).

289 The fact that neural development and associated behavioural and cognitive capacities can be altered
290 in response to even mild thyroid disruption has reinforced the increasing ED research focus on thyroid
291 disruption. A prevailing hypothesis is that exposure to chemical contaminants targeting thyroid function,
292 during critical windows of sensitivity in terms of thyroid and/or neural development, might play a
293 substantial role in the development of such disorders (Zoeller et al., 2002). The regulatory evaluation of

294 developmental toxicity of chemicals is based on specific guidelines. In mammals, despite ongoing
295 debate on the relevance of rodent models toward human thyroid physiology, the only *in vivo* proposed
296 OECD guidelines for thyroid are rodents screening assays (OECD series on testing & assessment, 2012,
297 n°150, 524). Thyroid investigations in those tests are limited and are not specifically designed to
298 identify thyroid disruption. They are included in male and female pubertal assays, described by US EPA
299 (US EPA OPPTS 890 1450-1500), and other repeated doses toxicity assays (OECD TG 407, 416, 443).
300 These are based on amphibian studies or, in mammals, on thyroid hormones (TH) and/or Thyroid
301 Stimulating Hormone (TSH) measurement in rodents at a given time point, usually at termination with,
302 in the best case, histological evaluation of the thyroid gland. The OECD guidelines for 28 and 90 day
303 repeated adult rodent exposure studies stipulate: “although in the international evaluation of endocrine
304 related endpoint a clear advantage for the determination of TH or TSH could not be demonstrated, it
305 may be helpful to retain to measure T3, T4 and TSH if there is an indication for an effect on the
306 pituitary-thyroid axis. Definitive identification of thyroid-active chemicals is more reliable by
307 histopathology analysis” [https://www.oecd-ilibrary.org/environment/test-no-407-repeated-dose-28-day-](https://www.oecd-ilibrary.org/environment/test-no-407-repeated-dose-28-day-oral-toxicity-study-in-rodents_9789264070684-en)
308 [oral-toxicity-study-in-rodents_9789264070684-en](https://www.oecd-ilibrary.org/environment/test-no-407-repeated-dose-28-day-oral-toxicity-study-in-rodents_9789264070684-en).

309

310 **3.1. TH metabolisms and specific binding proteins**

311 All levels of regulation of thyroid function are potential targets for environmental contaminants (for
312 review see: (Howdeshell, 2002; Zoeller, 2010). However, one of the most frequently reported
313 mechanisms of action concerns the hepatic metabolism of thyroid hormones (TH, fig. 2). This is
314 essentially based on extensive experimental data in rodents. In animals as in humans, hepatic catabolism
315 of TH is mainly via glucuronidation catalysed by hepatic microsomal enzymes from the UDP-
316 glucuronyltransferase (UGT) and or cytoplasmic sulphotransferase (SULT) families. TH can also be
317 metabolised by deiodinase enzymes, leading to either inactivation as for example the transformation of
318 T3 to the inactive metabolite rT3 or bioactivation of T4 into T3. About 80% of circulating T3 comes
319 from hepatic deiodination of T4. Both conjugations and deiodination pathways can be targets of EDs but
320 conjugation pathways are the most frequently investigated.

321 Among factors contributing to the inter-specific differences in hepatic TH metabolism, plasma
322 transport proteins of high specificity and affinity for TH namely thyroxin binding globulin (TBG) and
323 transthyretin (TTR), are at the heart of the controversy over the relevance of rodent models to humans.
324 These proteins are common targets of several EDs. For example, data from biosensor assays have
325 indicated the potent binding by hydroxylated metabolites of the polybrominated diphenyl ethers
326 (PBDEs), BDE47, 49 and 99 to human TBG (Marchesini et al., 2008). The same biosensor assay
327 confirmed the binding capacity of TTR for hydroxylated metabolites of PCBs and PBDEs, halogenated
328 BPA and genistein. The expression profiles of these proteins differ markedly between the adult rodent
329 and humans. The main difference lies in the level of expression of TBG, the protein with the highest
330 affinity and specificity for thyroxine. It is indeed strongly expressed in humans, although subject to
331 some variations in expression according to the physiological status. TBG is only expressed at very low
332 levels in the adult rat, but at higher levels in very young animals or aged individuals (Larsson et al.,
333 1985; Mendel et al., 1987; Savu et al., 1991). The differences in half-life and/or clearance of thyroxine
334 are frequently attributed to the expression profile of TBG, which is thought to be involved in decreasing
335 the accessibility of circulating TH to hepatocytes, thereby limiting the catabolism of these hormones.
336 These differences in the pattern of plasma TH binding proteins often lead to the assumption that rats are
337 more sensitive than humans to thyroid disruption proceeding from an increased hepatic catabolism of
338 TH and are used as an argument to refute the results obtained in rodents. For example, the effects of
339 both the insecticide fipronil (European Food Safety Authority (European Food Safety Authority
340 (EFSA), 2006) and the no-phthalate alternative plasticizer 1,2-Cyclohexane Dicarboxylic Acid, Di-
341 Isononyl Ester (DINCH, (Bhat et al., 2014) on thyroid function were considered as not relevant to
342 human during the course of their regulatory evaluation based on the interspecies difference in TBG
343 expression. Interestingly, the profile of expression and pharmacodynamics properties of these proteins
344 are very similar between human and sheep (fig. 3).

345 It is noteworthy, that, at the level of the EU the revised guidance for the identification of EDs states
346 that, “so long as not demonstrated as totally irrelevant to human, all results in rodents showing an effect
347 of a xenobiotic on TH metabolism should be considered as relevant to humans” (European Chemical
348 Agency (ECHA) and European Food Safety Authority (EFSA) with the technical support of the Joint

349 Research Centre (JRC) et al., 2018). It remains true anyway that, to date, it is unclear to what extent
350 inducible UGT/SULT expression and activity can mediate disturbances of TH catabolism in humans.
351 The ECHA recommendation for a better consideration of rodent data for the evaluation of potential
352 thyroid disruptors can thus be viewed like a precautionary measure in face of the uncertainties related to
353 the human-relevance of animal models for this particular mechanism.

354 The induction of enzyme pathways involved in thyroid metabolism is often mediated through the
355 activation of Xenobiotic Nuclear receptors (XNRs; (Kretschmer and Baldwin, 2005) and is not strictly
356 specific of thyroid hormones. Most of these receptors exhibit wide interspecies differences in their
357 pharmacological properties (Gibson et al., 2002; Jones et al., 2000). It is therefore very difficult to
358 predict the hepatic inductive properties of xenobiotics in humans on the basis of data collected in
359 rodents. Our work on thyroid disruption in response to fipronil provides a good example of how
360 interspecies differences render dealing with the issue of the relevance of animal models toward human
361 so difficult when looking at TH hepatic catabolism. The effect of the insecticide fipronil and/or its much
362 more persistent metabolite, fipronil sulphone, on thyroid function has been well established in the rat.
363 Both fipronil and its main metabolite fipronil sulphone treatments increase T4 clearance jointly to liver
364 UGT activity in the rat (Leghait et al., 2009; Roques et al., 2012). In the sheep, a species expressing a
365 very similar TBG to that of humans, the effect of fipronil exposure on thyroid homeostasis is much
366 more limited (Leghait et al., 2010). However, it cannot be assumed at this point that these differences
367 are solely related to different patterns of TBG expression. Pertinently, the rate of metabolic conversion
368 of fipronil into its bioactive persistent metabolite, fipronil sulphone, differs markedly between the two
369 species (Leghait et al., 2010) indicating differential effects on the induction of the hepatic cytochrome
370 enzymes involved in fipronil metabolism. Fipronil sulphone is much more persistent than fipronil itself
371 and at least as active as fipronil to increase TH hepatic catabolism. In the rat, fipronil treatment led to
372 the upregulation of several genes involved in the metabolism of xenobiotics and or TH, including the
373 cytochrome P450 Cyp2b1, Cyp2b2 and Cyp3a1, the carboxylesterases Ces2 and Ces6, the phase II
374 enzymes Ugt1a1, Sult1b1 and Gsta2, and the membrane transporters Abcc2, Abcc3, Abcg5, Abcg8,
375 Slco1a1 and Slco1a4. Our data in CAR- or PXR-invalidated mouse indicate that PXR and CAR are
376 mediating, at least in part, the fipronil-induced upregulation of the expression of enzyme/transporter

377 genes (Roques et al., 2013) which, in rats, may contribute to increase thyroid hormone clearance. Given
378 the wide interspecies differences in XNRs pharmacology, it is impossible at this point to determine if
379 the difference in response to fipronil between the two species, in terms of thyroid disruption, is due to
380 differential expression of TBG or to differences in XNR receptor affinities for fipronil or its metabolite
381 or both. Although the sheep can be considered much more relevant to human than rodent from the
382 standpoint of specific TH binding proteins, the relevance of this model to human in terms of the effect
383 of fipronil on thyroid homeostasis is not currently certain.

384

385 **3.2. Thyroid-mediated alterations of brain development**

386 While the mode of action of EDs on thyroid function is much debated as regards hepatic initiating
387 events, the evaluation of the adverse effects of subtle thyroid disruption is a real matter of complexity.
388 One of the most, if not the most, challenging and worrying adverse effect of thyroid disruptors is the
389 potential alteration of the development of the central nervous system (CNS) and resulting behavioural
390 and cognitive deficiencies even with moderate thyroid disruption as with isolated hypothyroxinemia
391 (Bellanger et al., 2015; Ghassabian et al., 2014; Gilbert et al., 2012a; Howdeshell, 2002). The proof of
392 concept for linking subtle perinatal thyroid disruption, alterations of the CNS and related-cognitive
393 deficiencies has been efficiently established in rodents, but the process still needs to be taken one step
394 further, with integrated complementary models more relevant than rodents as regards human
395 physiology. The period of gestation is a window of high vulnerability to perturbed thyroid function
396 and, consequently, for developing tissues and functions which are under the control of TH. During this
397 period, the mothers have to face increased TH requirements due to the fact that their own metabolic
398 rate is increased to maintain a normal gestation. In addition, the needs of the fetus, particularly at early
399 developmental stages when the fetal thyroid system is not yet functional, adds to this burden. Thus,
400 pregnancy involves profound changes in almost all aspects of thyroid regulation. For example, the
401 increase in TH production rate to satisfy gestational demand is estimated at about 50% of the basal
402 rate. The maintenance of normal thyroid function is strictly-dependent upon iodine supply and/or
403 turnover. During gestation, urinary elimination of iodine is increased because of a higher glomerular
404 filtration rate, which itself constitutes another susceptibility factor of thyroid function. In both human

405 and sheep, total serum T4 and T3 concentrations rise to levels higher than those of non-pregnant
406 individuals. BPA is a good illustration on how a contaminant that does not seem to have any effect on
407 the thyroid function of adult non-pregnant animals might affect this function in pregnant animals
408 and/or their offspring. There are indeed data in both rodents and sheep showing that BPA can alter the
409 thyroid function of pregnant animals and/or their offspring (Guignard et al., 2017; Silva et al., 2019;
410 Vigiúé et al., 2013; Xu et al., 2019; Zoeller et al., 2005).

411 The dependency of the fetus on TH maternal supply is particularly important at the early stages of
412 the development (fig. 4). The fetal/ neonates thyroid function develops earlier in human and sheep
413 than in rodents. Fetal thyroid function starts after the first third of the pregnancy in human as well as in
414 sheep (fig. 4) while it starts much later, in the last third of pregnancy in rodents (Fisher, 1991; Fisher
415 et al., 1976; Howdeshell, 2002; Polk et al., 1991). Consequently, the window of sensitivity to thyroid
416 disruption might differ between humans and rodents. The sheep might thus represent a better
417 alternative than rodents when investigating the window of sensitivity during which an effect of thyroid
418 disruption on CNS development will be established.

419 In addition, the mode of exposure to contaminants differs between pre and post-natal life. During
420 prenatal life, the exposure occurs from the mother through the placental barrier that in some instances
421 protects the fetus. During post-natal life, the exposure will occur via oral route through lactation. Thus,
422 it is almost impossible to reproduce in rodents the same scheme of exposure to contaminants at the
423 final stage of the thyroid maturation as in humans. In addition to the effects of contaminants on the
424 maternal thyroid function, there is evidence that domestic and/or environmental contaminant can
425 disrupt the development of fetal thyroid. Maternal smoking and high maternal BMI are associated with
426 disturbed fetal thyroid gland development and endocrine function in a sex-specific manner during the
427 second trimester of pregnancy in humans (Filis et al., 2018). Similarly, peri-conceptional maternal
428 exposure to sewage sludge chemicals impedes fetal thyroid gland development in the sheep
429 (Hombach-Klonisch et al., 2013). BPA has also been shown to be a potential disruptor of maternal
430 and/or fetal thyroid function following chronic high (5 mg/kg/day) or low (5 µg/kg/day) dose
431 administration from the first to the last month of gestation in sheep (Guignard et al., 2017; Vigiúé et
432 al., 2013). Interestingly, some, although not all, epidemiological data from large human cohorts also

433 show a correlation between gestational BPA exposure and some degrees of thyroid disruption in
434 newborns (Aung et al., 2017; Chevrier et al., 2013; Romano et al., 2015).

435 The role of TH in brain development and homeostasis is well documented in animal experimental
436 models (Howdeshell, 2002) including for subtle thyroid disruption. In the human, although the effect
437 of moderate thyroid disruption on behaviour and cognitive function development is still debated, some
438 epidemiological studies suggest a potential link between maternal hypothyroxinemia (decreased free-
439 T4 concentration with or without modifications of TSH) during pregnancy and increased risk of
440 autism, impaired psychomotor development, Attention Deficit Hyperactivity Disorder, delay in
441 language development and lower mental scores (for review: (Henrichs et al., 2013). The overall costs
442 of such alterations in relation to ED exposures have been estimated at approximately 150 billions €
443 /year in the EU (Trasande et al., 2015). In the mouse, a 36% decrease in maternal T4 concentrations
444 without any modification of circulating T3 from gestational day (GD) 10 to GD13 is associated with a
445 cellular and molecular environment in the cortex that is unfavourable to neuronal migration (Cuevas et
446 al., 2005). In the rat, different models of chemically-induced moderate thyroid disruption during
447 pregnancy (e.g. iodine deficiency, perchlorate exposure, propylthiouracil) consistently show that these
448 manipulations are associated with functional modifications within the hippocampus with an alteration
449 of synaptic functions that can last until the adult stage even after a full recovery of the thyroid function
450 (Gilbert et al., 2017, 2012b; Gilbert and Sui, 2006). Subcortical band heterotopia (SBH) represent a
451 neuronal migration error in humans. In rats, even minor (<15%) reductions in maternal serum
452 thyroxine is associated with increased volume and presence of SBH in the corpus callosum of
453 offspring that persists in adulthood (Gilbert et al., 2014). In rodent models, as in humans, this
454 abnormality in brain development is associated with serious adverse neurological and/or behavioural
455 effects. The whole process of SBH development starts in the periventricular area *i.e* in close vicinity to
456 major sources of TH supply to the brain, the cerebrospinal fluid and vasculature.

457 Taken together, there is considerable rodent model evidence for the adverse effects of ED-induced
458 mild thyroid disruption in CNS development and potential mechanisms of action ((Gilbert and Lasley,
459 2013; Mughal et al., 2018; Remaud et al., 2014; Zoeller et al., 2002). However, the interspecies
460 temporal differences in the development of thyroid function, and of the CNS itself, emphasizes the

461 need for other animal models more relevant to human. This is especially important for the
462 determination of the critical windows of action of EDs in the brain, neurocognitive and behavioural
463 functions. From this standpoint, the sheep is an excellent model (Back and Hohimer, 2016), meeting
464 several critical criteria: i) being a reference model for physiology of gestation and development
465 (Morrison et al., 2018), ii) having similar regulation of thyroid function to that of human , iii)
466 exhibiting time-lines for the synchronised development of the thyroid and CNS that are similar to
467 human (fig. 4), iv) possessing considerable homology with human brain anatomy (gyrencephalic
468 species (Vink, 2018) and maturation (a marked difference with lissencephalic species like the mouse).
469 This makes the ovine model very valuable for the study of neurodevelopmental diseases and/or
470 alteration in the pattern of brain development.

471 Overall, it is clear that regulatory studies of thyroid disruption are limited due to both the use of
472 reference species which are controversial in terms of their relevance to human and the use of terminal
473 endpoints and periods of observation that might only partially reflect the thyroid status of the animals
474 and/or developmental exposure. There are very few recommendations for using other models than
475 rodents and therefore very few data for a proper evaluation of interspecies differences, that could serve
476 as a base for a sound extrapolation of experimental animal data to human.

477

478 **4. A HERBIVORE AT THE INTERFACE BETWEEN ENVIRONMENTAL**

479 **POLLUTANT AND PHYSIOLOGICAL EFFECT: A MODEL FIT FOR REAL**

480 **LIFE SCENARIO EXPOSURE**

481 The banning of disposal of human sewage into the seas and oceans by the EU in 1997 hastened the
482 development of alternatives for the repurposing and processing of this waste material. Human sewage
483 has long been used as a fertiliser (called “night soil”) and this trend has continued with the
484 employment of processed, dried, human sewage as a fertiliser. Subsequently, agricultural usage laws
485 and guidelines have been imposed. Sewage sludge is, of course, an exquisite read-out of combined
486 man-made and environmentally distributed pollution, including a rich cocktail of EDs. The Aberdeen
487 sewage sludge model, using outbred sheep, was established by Dr Stewart M. Rhind (deceased) at the
488 James Hutton Institute (previously the Macaulay Institute) and has been previously reviewed (Evans et

489 al., 2014; Fowler et al., 2012; Rhind et al., 2010a) These reviews present considerable support for the
490 use of sheep as a sentinel species for environmental ED effects throughout the life-course, with the
491 added issue that, of course, sheep meat enters the human food chain. This issue has attracted
492 considerable attention from both farming organisations and those opposed to the use of sewage sludge
493 fertiliser.

494 Here we present an update that has resulted from some 20 years of research with extensive support
495 from a range of organisations including the European Commission via the REEF project. Three main
496 study designs have been employed in terms of the exposure paradigms via different fertilisers applied
497 to pastures and these are summarised in fig. 5. In all three approaches fetal and maternal tissues have
498 been harvested, typically at 80, 110 or 140 days of gestation and offspring tissues in early adulthood at
499 around 18 months. The simplest model has been the *Always* exposure profile where mother, gestation
500 and offspring remain exposed to a single condition. These are Control fertilised, using inorganic
501 fertiliser balanced for nitrogen content and Sewage Sludge fertilised, using processed, dried, human
502 sewage sludge according to agricultural guidelines at the time. The next model was the *Cross-over*
503 exposure profile. In this model the ewes were either moved to the opposing fertiliser regime (or not, to
504 provide additional controls) and they and their offspring then remained on that pasture type until tissue
505 harvesting at 110 days and 18 months post-natal. The model used in the REEF project was the
506 *Windows* exposure profile. In this model the main study controls were animals reflecting both
507 treatment conditions of the *Always* profile, matched with ewes exposed in early (0-80 days), mid (30-
508 110 days) and late (60-140 days) gestational windows and tissues harvested at 80 and 140 days. This
509 design aimed to determine whether there were specific developmental windows of fetal sensitivity to
510 EDs in sewage sludge.

511 The results from these three study designs are summarised in fig. 6 and demonstrated the wide
512 range of adverse consequences from pre- and post- natal exposure to low dose, complex, mixtures of
513 EDs in sheep.

514

515 **4.1. Environmental and sheep tissue consequences of sewage sludge exposure**

516 Unsurprisingly, sewage sludge pellets themselves contain considerably higher concentrations of 33
517 EDs (including DEHP, PCBs, PBDEs, PAHs) measured and reported across 3 studies (Lind et al.,
518 2009a; Rhind et al., 2010b). The review from Evans (Evans et al., 2014) extensively assessed tissue
519 and soil ED levels following sewage sludge application.

520 EDs that were detectable in soil after sewage sludge application included phthalates, alkyphenols,
521 PAHs, PCBs, PBDEs and DEHPs. However, accumulation of EDs in soil from pastures treated with
522 sewage sludge fertiliser was highly variable across multiple studies, temporally and geographically
523 changing with considerable differences in accumulation of specific EDs (Rhind et al., 2002; Zhang et
524 al., 2014) and frequently at levels that in animal studies would be considered as No Observed Adverse
525 Effect levels (NOAEL). For any single study, therefore, accurately predicting ED load taken up by
526 grazing animals after sewage sludge application or extrapolating more widely, is difficult. Multiple
527 applications of sewage sludge lead to increased concentrations of chemicals and the number of
528 congeners (PBDEs) measurable (Rhind et al., 2013) but the magnitude of the increase is lower than
529 expected from the total sewage sludge quantities used on the pastures. An obvious explanation is that
530 degradation or flushing of chemicals from sewage sludge-treated pasture will reduce animal exposure
531 levels. Furthermore, since these EDs are in the environment from other sources, control pastures also
532 contained detectable levels of many of them, although usually at lower levels than seen in the sewage
533 sludge fertilised pastures. Relevantly for BPA, while levels in soils were initially similar between
534 control and sewage sludge pastures, the accumulation of this compound over 13 years of application
535 was considerable, rising from 3 to 63 ng BPA/g of soil (Zhang et al., 2015). Furthermore, there was
536 significant uptake by crops with up to 68 ng BPA/g of leafy vegetables.

537 Tissue concentrations of EDs also displayed considerable variability. These ED concentrations can
538 be influenced by the metabolic status of the animal and environmental contribution to routes of
539 exposures, including ingestion and inhalation. The release of many lipophilic EDs from adipose stores
540 when-energy demands increase during gestation, means ED exposures are likely to be quite different
541 between a dam and the fetus. Of the EDs of interest in the sewage sludge studies, tissue levels of
542 PCBs, PBDEs, PAHs, alkyl phenols and phthalates are variously measurable in tissue and/or milk
543 samples, collected from mothers, fetuses and lambs maintained on both the control and biosolids

544 treated pastures (Rhind et al., 2005, 2007, 2009, 2010b, 2011a) with up to 30% variation in
545 background tissue ED accumulation across Scotland (Rhind et al., 2011b) Overall, maternal and fetal
546 tissue levels in kidney fat, muscle and liver were elevated, even if relatively modestly and variably
547 following sewage sludge exposure in these studies. Furthermore, while many EDs are lipophilic, even
548 during lactation, lamb ED levels were not markedly elevated compared to the dams (Rhind et al.,
549 2007). Different EDs also showed considerable variability between mothers and offspring and
550 between studies. Tissue concentrations in 6 month old lambs on either treated or control pastures,
551 showed a broadly similar pattern of tissue EDC concentrations to those seen in the adult ewes.

552 Overall, fetal EDC concentrations and thus EDC exposure may be greater in early gestation as a
553 result of lower detoxification capacity of the fetal liver (Rhind et al., 2010b). In studies where
554 exposures of the ewes at conception or during gestation were altered (*Cross-over* and *Windows*
555 studies) different outcomes were observed. In the former, there were almost no differences between
556 control and cross-over animal tissue ED burdens (Rhind et al., 2010b). In the *Windows* study (Lea et
557 al., 2016), soil PAH, DEHP and PCB levels were increased by sewage sludge exposure. In dams 10 of
558 the 15 EDs that showed significant association with sewage sludge exposure were elevated, especially
559 in the mid and late exposure windows although changes in fetal ED burdens were highly variable.
560 Finally, we recently showed a sex difference in 18 month old sewage sludge exposed offspring, with
561 higher liver PAHs and lower PCBs in males than females. Furthermore, PAHs were elevated and
562 PCBs lowered in sewage sludge-exposed females compared to controls (Filis et al., 2019).

563

564 **4.2. Gonad and endocrine development**

565 While most of our reproductive studies focused more on the female, *Always* exposed 110 days of
566 gestation male fetuses exhibited a wide range of significant developmental defects, including reduced
567 circulating inhibin A, testosterone (nearly halved), Leydig and Sertoli cell numbers, body and testis
568 weights (Paul et al., 2005). A programme of analysis of the fetal ovary spanned *Always*, *Cross-over*
569 and *Windows* studies. There was some variability between the studies, for instance in the *Always*
570 (Fowler et al., 2008) and *Windows* (Lea et al., 2016) studies fetal ovarian weight was not altered while
571 sewage sludge exposed fetal ovaries were heavier in the *Cross-over* study (Bellingham et al., 2013) in

572 fetuses whose mothers remained on sewage sludge-fertilised pastures but not in those whose mothers
573 switched pasture type at conception. Fetal circulating prolactin and estradiol levels were reduced and
574 FSH unaffected in *Always* study exposed fetuses while inhibin A and FSH was increased and estradiol
575 decreased in animals constantly exposed to sewage sludge in the *Cross-over* study. In the *Windows*
576 study, clear signs of some virilisation were seen, with reduced uterine weight and increased anogenital
577 distance in the late window of fetal sewage sludge exposure in particular, even though fetal
578 testosterone was unaltered in this group.

579 In terms of ovarian morphology, histological analysis showed that oocyte density was reduced in
580 fetuses exposed to sewage sludge pasture (*Always* study, Fowler et al., 2008) with a shifted balance
581 between the pro- (BCL2) and anti- (BAX) apoptotic ovarian markers. In the *Cross-over* study
582 (Bellingham et al., 2013) in contrast, the only observed effect on follicle numbers was a reduction in
583 the number of type 1a follicles in the fetuses whose mothers had been moved to sewage sludge
584 pastures at mating. This was despite alterations in ovarian expression of a number of proteins/genes
585 associated with follicle recruitment, activation, apoptosis and health. Subsequently, in the *Windows*
586 study (Lea et al., 2016) there were several changes in numbers of different follicle classes, most
587 notably a decrease in healthy and increase in atretic type 1a follicles in all exposed groups and
588 indications of delayed follicle development ovaries from fetuses in the late exposure window. These
589 changes were associated with a large number of proteome and transcriptome changes in the mid and
590 late sewage sludge-exposed ovaries in particular. From these data network analyses predicted
591 methylation associated changes were occurring in these 2 groups.

592

593 **4.3. Brain, pituitary and thyroid development and function**

594 Expression of GnRH and its receptor (Bellingham et al., 2010) and afferent systems to the GnRH
595 neuronal network including Kisspeptin (Kiss-1)/GPR54, estradiol receptor alpha (ER α) (Bellingham
596 et al., 2009), galanin and its receptors (Bellingham et al., 2010, 2009) within the hypothalamus and
597 LH β , Kisspeptin and ER α in the pituitary gland were affected in the sewage sludge-exposed fetuses,
598 unlike their mothers (*Always* exposure model). GnRH and GnRH receptor mRNA concentrations were

599 reduced in the hypothalamus but unaffected in the pituitary glands of fetuses exposed to sewage
600 sludge (Bellingham et al., 2010). Important steroid sensitive afferents to the GnRH neurosecretory
601 system, galanin and kisspeptin were disrupted in fetuses exposed to sewage sludge: expression of
602 *KISS1* transcript and the three galanin receptor subtypes (1, 2 and 3) in both the hypothalamus and
603 pituitary glands were reduced following maternal sewage sludge exposure. Estradiol receptor
604 transcript expression within the hypothalamus and pituitary gland were not affected by sewage sludge
605 exposure in either the mothers or their 110 day old fetuses. However, fetal pituitary ER α protein
606 expression was reduced in sewage sludge-exposed fetuses. A similar range of endpoints were assessed
607 in further study using the *Cross-over* study model (Bellingham et al., 2016). Fetal plasma FSH,
608 estradiol and inhibin A were affected by sewage sludge exposure while LH and estradiol, but not
609 testosterone, were affected in males. The direction of disruption varied according to exposure type:
610 constant or cross-over, with sex-specific consequences. Overall, fetuses from mothers whose exposure
611 type changed at mating showed more extensively disrupted hypothalamic and pituitary expression
612 patterns of neuroendocrine regulators, such as the GnRH and Kisspeptin systems, than fetuses
613 constantly exposed to sewage sludge. Male fetuses were more affected than females and exhibited
614 more female-like patterns of neuroendocrine gene expression. Finally, 5 month old lambs were
615 assessed using behavioural tests indicative of emotional reactivity and exploratory behaviour. Sewage
616 sludge exposure *in utero* and post-natally was associated with a decrease in emotional reactivity and
617 decreased activity levels. Furthermore, the exploratory behaviour of females was unaltered but that of
618 males increased to match female levels (Erhard and Rhind, 2004). This shows that early exposure to
619 low levels of a cocktail of pollutants, alters brain development and dimorphism that could have long-
620 term detrimental effects on reproductive behaviour.

621 The fetal thyroid gland was also investigated in the *Cross-over* study (Hombach-Klonisch et al.,
622 2013). Fetal thyroid gland weight was only affected in the group moved from sewage sludge to control
623 pastures at mating group. Similarly, fetal blood thyroid hormone levels were not affected by
624 treatment. In terms of fetal thyroid morphology, changing either from or to sewage sludge-treated
625 pasture at mating was associated with a reduction in the number of thyroid follicles. Variable effects
626 were observed in terms of blood vessel number, together with areas of immature thyroid follicle

627 development, reduced NIS (sodium-iodide-symporter) expression and focal lack of mature angio-
628 follicular differentiation in continuously exposed group fetal females and sewage sludge moved to
629 control group fetal males being most affected. In contrast, in the Windows study (Lea et al., 2016),
630 fetal thyroid weight was reduced in the late exposure window, together with reduced free T3 and
631 increased free T4 in the same group.

632

633 **4.4. Adverse consequences of sewage sludge exposure in adulthood (18 months)**

634 Fetal and post-natal sewage sludge exposure affected male lamb bones more than the females (12/23
635 bone parameters significantly different in males vs 2/23 parameters affected in females). In general,
636 the bones of sewage sludge-exposed animals were more fragile compared to the controls (Lind et al.,
637 2009b). In contrast, fetal bones were markedly less affected by the EDs present in sewage sludge
638 pastures, unlike their mothers who showed reduced mineralisation and cross-sectional areas of some
639 bones (Lind et al., 2010).

640 Testes from *in utero* and post-natally sewage sludge-exposed rams were analysed at age 18 months
641 old (Bellingham et al., 2012) and although there was no effect on mean testis weights, a subset of
642 exposed rams had markedly lighter testes. While there were no differences in total mean number of
643 germ cells, 42% of the exposed rams had total number of germ cells were lower than the minimum
644 numbers in the control group. When this subgroup was analysed separately the number of germ cells
645 per testes, germ cells per Sertoli cell, germ cell absolute volume, number of round spermatids, number
646 of spermatocytes were all reduced by sewage sludge exposure. They also exhibited an increase in
647 tubule number, number of tubules with dilated lumens and an increase in Sertoli cell only tubules
648 which lacked germ cells in the testes collected from biosolids exposed animals. The sewage sludge-
649 exposed rams with small testes also had lowered FSH. While the *in utero* + post-natally exposed adult
650 animals (Bellingham et al., 2012) showed different consequences of sewage sludge-exposure than the
651 *in utero* exposed fetuses (Paul et al., 2005), changes observed in both studies, especially the reduction
652 in the number of fetal Sertoli cells and adult Sertoli cell-only tubules and the reduction in germ cells in
653 a subset of the adults, would disrupt sperm producing capacity. Analysis of ovaries from our study of
654 *in utero* + post-natally sewage exposed 18 month old ewes has not currently been published. However,

655 (Fowler, personal communication), the consequences of the sewage sludge exposure include adverse
656 morphological and molecular markers of ovarian function and health. One striking aspect is the
657 retention of some fetal ovarian characteristics in the sewage sludge-exposed group.

658 In Filis et al. (Filis et a., 2019), we investigated the livers from the adult animals discussed in the
659 preceding paragraph. These animals exhibited sexually dimorphic patterns of ED burdens as discussed
660 at the start of this section. The life-long sewage sludge-exposure of these animals manifested as altered
661 xenobiotic and detoxification responses. The liver proteomes of both sexes were disrupted by sewage
662 sludge exposure and proteins with altered expression profiles included major plasma-secreted and
663 blood proteins, and metabolic enzymes. Pathway analysis predicted dysregulation of cancer-related
664 pathways and altered lipid dynamics. Follow-up confirmed these predictions, with a reduction in total
665 lipids in female livers and up-regulation of cancer related transcript markers in male livers respectively
666 by sewage sludge exposure.

667 To draw together the sheep sewage sludge model section, this complex picture of highly variable
668 and sometimes inconsistent changes in ED levels in the animals are reminiscent of the data held in
669 human exposure cohorts, such as NHANES (National Health & Nutrition Survey:
670 <https://www.cdc.gov/nchs/nhanes/index.htm>). In addition, this variable ED exposure profile is highly
671 likely to contribute to the variable tissue- and sex- specific morphological, endocrine and molecular
672 marker alterations observed in the sewage sludge exposed fetuses and adult offspring. In many ways
673 this variability is very similar to data seen in human studies.

674

675 5. CONCLUSION

676 The complexity of the regulatory pathways triggered by EDs underlines the need for the identification
677 of systemic biomarkers that can be robustly linked to ED exposures with minimal possible uncertainty.
678 Choosing the right model is one of the best ways to reduce uncertainties. Rodents have proven to be
679 very useful in evidencing the potential of chemicals to induce endocrine disruption and to provide data
680 on their potential mechanisms of action and associated adverse effects. However, because the endocrine
681 system is very plastic, it is clear that it is necessary to integrate the notion of interspecies differences
682 into our way of thinking with respect to EDs. Sheep studies have clearly established that non-rodent

683 mammalian models contribute strongly to a better understanding of human health risk assessment than
684 necessarily achieved using classical rodent studies. It should be acknowledged however, that like every
685 other model, the large animal models, including sheep, also exhibit some limitations. Many of these
686 limitations are practical, reflecting the need for large scale animal facilities. Sheep have long gestation
687 lengths, making them more similar to human from the standpoint of duration of exposure and usually
688 the timelines of ontogenesis. The price to pay, however, is that experiments can take years to complete.
689 Sheep are, therefore, not suitable for rapid screening studies. Sheep placentation has some differences
690 from the human and may not accurately predict human fetal exposure. However, the rodent placenta is
691 not any better from this point of view and rodent models also do not allow kinetic investigations
692 required for modelling predictive approaches. Furthermore, investigators do not have access to as many
693 tools for sheep as for rodents, in particular molecular ones for mechanistic investigations. Finally, for
694 some endpoints of adverse effects, such as cognitive or behavioural alterations, there are almost no well
695 standardised tools or validated sheep models for human neurobehavioural abnormalities.

696 Because of its relevance to human physiology, we conclude that the sheep model and more
697 generally large animal models can take us one step further for a better understanding of EDs. But
698 because of their limitations in particular technical ones, those models have to be seen as
699 complementary to rodent models. Anyway, the best possible models should be used to address
700 scientific questions and it is our responsibility to question which model/models is/are the most precise
701 to give the right answers to each specific question for a better understanding and prediction of ED
702 adverse effects.

703

704 **FIG. LEGENDS**

705 **Fig. 1:** Transplacental exchange rates of BPA, BPS and their main glucuronidated metabolites. The %
706 represent the fraction of a given dose that crosses the placental barrier or the rate at which the parent
707 molecules are metabolised into glucuronidated conjugates, or, alternatively, the reactivation rate of the
708 conjugate into native molecules. These data were generated in an ovine model of catheterised fetus at a
709 late gestational stage.

710
711 **Fig. 2:** Possible interactions of endocrine disruptors with extra-thyroidal metabolism of thyroid
712 hormones. The thyroid gland synthesises and secretes both T4 and the more biologically active T3.
713 Thyroid secretion accounts for only 20% of circulating T3. Once in the blood more than 99% of the
714 TH are bound to proteins. Two of these proteins, thyroid binding globulin (TBG) and transthyretin
715 (TTR) are of high affinity and specificity. The TH-bound fraction is in equilibrium with the free
716 hormone fraction, the only accessible one for the liver and the target tissues. Once in the liver, TH can
717 be deiodinated by specific deiodinases (DOIs) or metabolised mainly through conjugation reactions by
718 UGTs or sulphotransferase enzymes. The expression of these enzymes can be induced following the
719 activation of xenosensor nuclear receptors (XNRs) thus leading to increased clearance of TH.

720
721 **Fig. 3:** Pharmacological properties of the two specific binding proteins transthyretin (TTR) and thyroxin
722 binding globulin (TBG) of thyroid hormones (TH) in both humans and sheep. K_d is the dissociation
723 constant that characterises the affinity of the protein for T4, the lower K_d , the higher affinity of the
724 binding proteins for TH.

725
726 **Fig. 4:** Timelines of fetal thyroid axis maturation and neural development in sheep (Barlow, 1969;
727 Ferreiro et al., 1987; Fisher, 1991; Fisher et al., 1976; McIntosh et al., 1979; Polk et al.,
728 1991; Thomas et al., 1993), humans (Howdeshell, 2002) and rats (Howdeshell, 2002) from
729 conception to birth or to PND22 for the rat. Only global starting points of the different processes are
730 indicated, there is no indication about the quantitative changes of the different parameters throughout
731 fetal development (except for the fetal thyroid function). All these processes keep evolving during

732 subsequent postnatal development. The human and rat elements are adapted from Howdeshell;
733 2002.

734
735 **Fig. 5:** Schematic summary of the experimental scheduling used in the Aberdeen sewage sludge studies.
736 Days of gestation along the top between “sex determination” and “birth”. Animals were harvested at 80,
737 110, 140 days of gestation and at 18 months. While the *Windows* and *Cross-over* studies were single
738 large studies, the *Always* study where animals were not changed from pre-gestation exposure conditions
739 (sewage sludge vs control [inorganic] fertiliser) was run several times.

740
741 **Fig. 6:** Global summary of the findings of the Aberdeen sewage sludge studies. Processed human
742 sewage sludge pellets were used according to agricultural guidelines. There was accumulation of EDs in
743 the soil but only modest changes in maternal and fetal tissue levels of EDs. Nevertheless, extensive
744 morphological, endocrine, molecular and behavioural consequences for the fetus, some of which have
745 been shown to persist into adulthood. *personal communication P.A. Fowler, University of Aberdeen,
746 manuscript in preparation. For references see relevant text.

747

748 **Competing interest statement**

749 **None of the authors has competing interests.**

750

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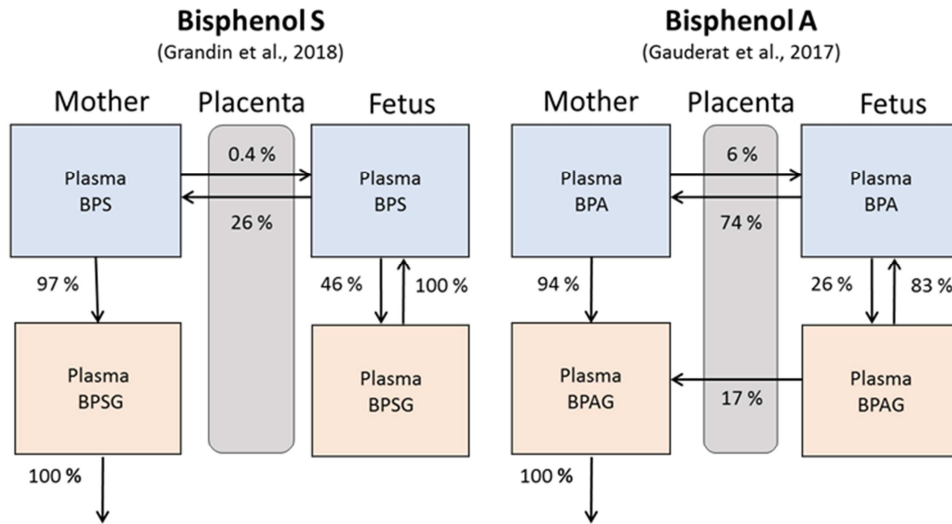


Figure 1

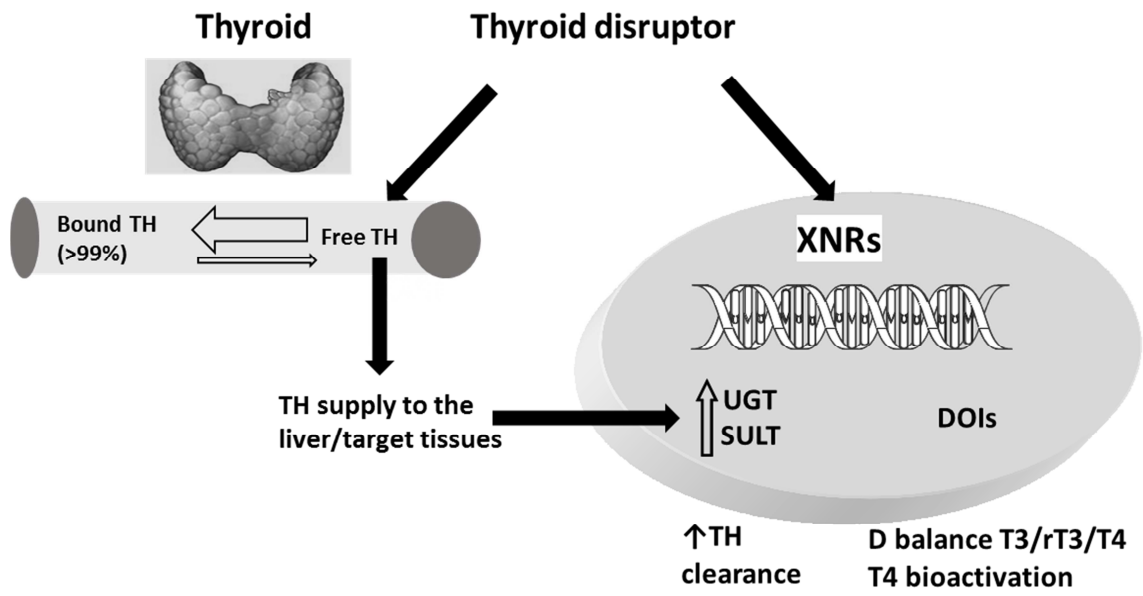


Figure 2



TBG	Concentration	270 nM	160 nM
	Kd	0.11 nM	0.11 nM
TTR	Concentration	2952 nM	4621 nM
	Kd	7 nM	6 nM

Figure 3

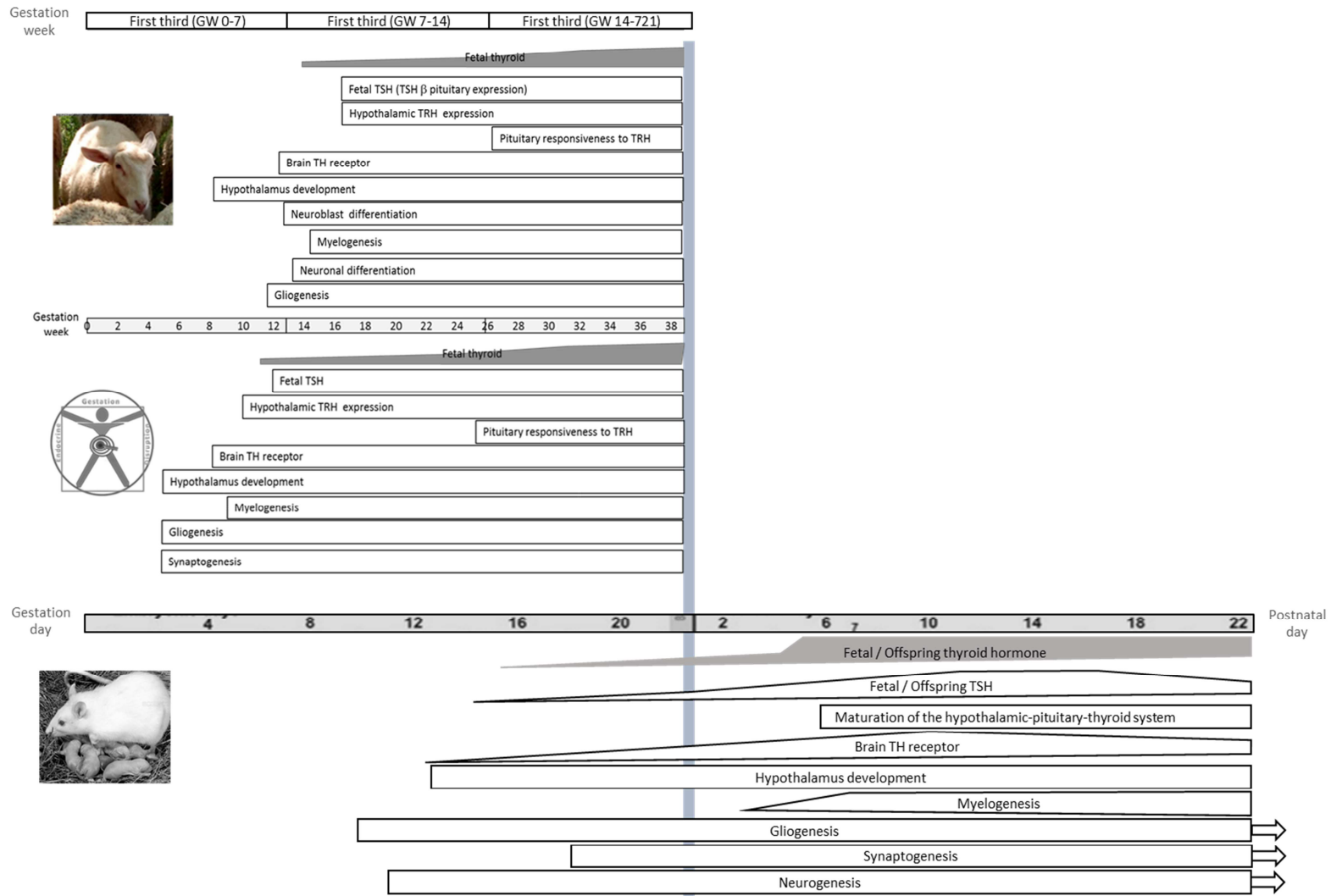


Figure 4

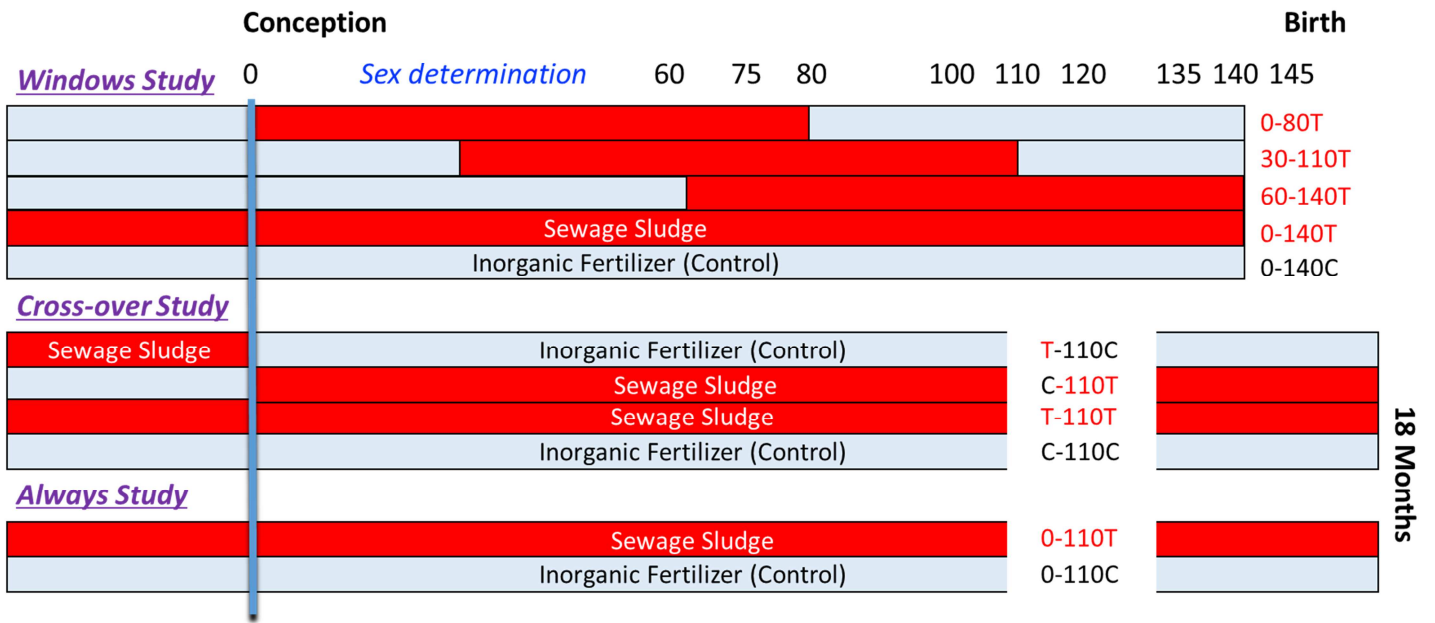


Figure 5

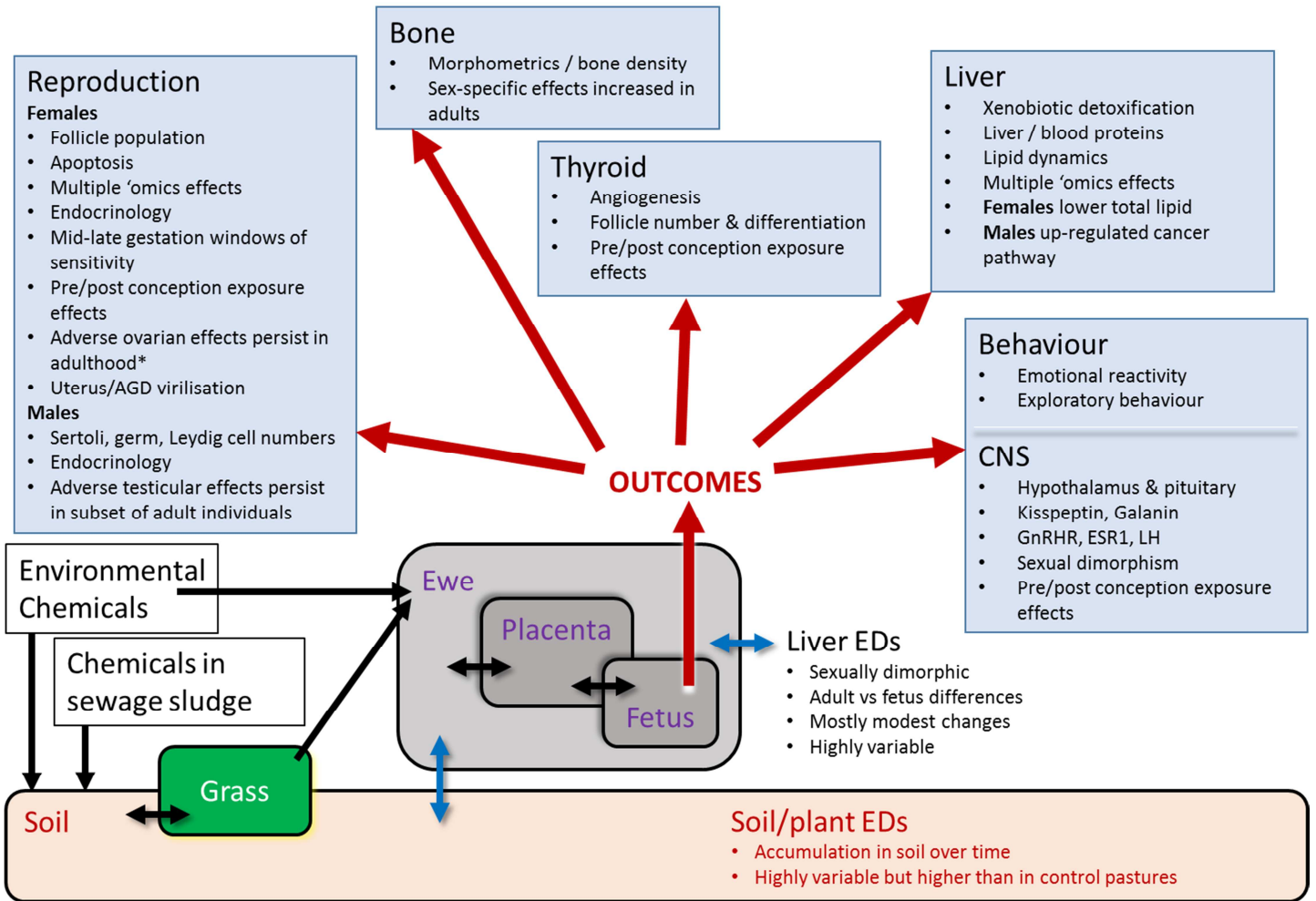


Figure 6

Viguié et al., Highlights

- Endocrine disruption is a very complex and critical public health issue
- There is a need for human-relevant models to assess endocrine physiology and the physiology of period at risks
- The sheep is a good alternative to classical rodent models to understand fetal exposure, thyroid disruption and its consequences on brain development
- The sheep allows real-life exposure scenario to complex mixtures.

Journal Pre-proof

Author contributions

Catherine Viguié coordinated the manuscript preparation.

All author equally contributed to the redaction according to their own field of competences :

- C Viguié: introduction – thyroid and brain -conclusion
- Elodie Chaillou: relevance of the sheep for brain development
- Véronique Gayraud and Nicole Picard-Hagen: fetal exposure
- Paul Fowler: the sheep a model fitted for real life scenario exposure.

All authors have contributed to raise different fundings covering their investigations the results of which have been used for this review.