Selection of eligible methods for pre-validation operations of endocrine disruptors characterization methods

WP3

“State of the art on the need for methods”

For

Pepper: Public-privatE Platform for the Pre-validation of Endocrine disRuptors characterization methods

By

Altertox – Benaki Phytopathological Institute – Université de Paris

(ABU)

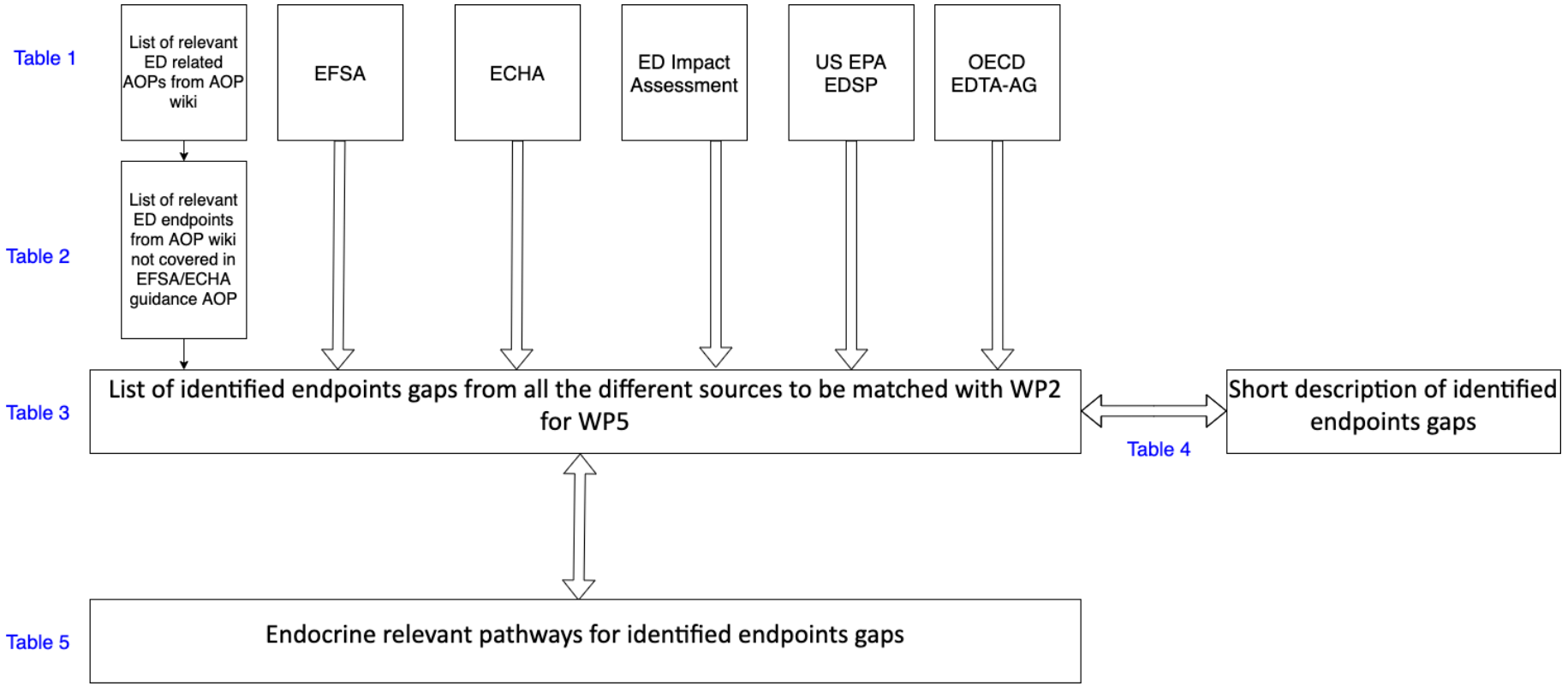
2020-15-06

Summary of the outcomes

Different sources of information, including the AOPwiki, the ECHA Endocrine Disruptor Expert Group, EFSA conclusions on the Peer Review of pesticide active substances, the Endocrine Disruptor Impact Assessment, the US EPA and the OECD, were used to identify ED-relevant endpoints.
Following the search in AOPwiki, 124 ED-relevant AOPs were identified (Table *1*), with the most frequent being under the Estrogen-Androgen-Thyroid-Steroidogenesis (EATS) pathways and then the Hypothalamic-Pituitary-Gonadal (HPG) axis, Epigenetics and other Non-EATS pathways. There is currently no or little information on AOPs for the somatotropic axis and the retinoid and vitamin D signalling pathways indicating knowledge gaps on endpoints involved. Overall, 90 endpoints were identified from the ED-relevant AOPs. When these endpoints were compared to endpoints measured using existing Test Guidelines (TGs) included in EFSA/ECHA guidance (2018), 32 endpoints gaps for mammals and 54 for non-mammals were identified as relevant to PEPPER (

Table *2*). The identified endpoint gaps (111) from all the different sources of information extracted from ECHA, EFSA, and ED IA, US EPA and OECD are presented in Table *3*. The endpoint gaps will be assessed taking into account the non-validated methods identified in WP2 and recommendations from WP4. Table 4 describes shortly the endpoint gaps reported in Table *3* whereas Table 5 organises the endpoint gaps under the relevant endocrine pathways. Figure 1 recapitulates all the above-mentioned steps.

Figure 1: workflow overview WP3 and aggregation of all the sources



Strategy for identification of needs in test methods for EDs characterization

The purpose of the report is to describe the strategy followed in order to identify needs in test methods to allow characterization of endocrine disruptors (EDs). The following sources of information were used:

1. Sources of information

1.1. Screening study performed for the Impact Assessment (IA).

Benaki Phytopathological Institute (BPI) exploited the experience obtained from the screening study performed between 2015-2016 to support the work of the European Commission (EC) in establishing scientific criteria for the identification of EDs in the context of an Impact Assessment, as the contractor of the EC. In that study, more than 600 substances were knowledge-screened (348 plant protection products, 96 biocides and 186 miscellaneous chemicals falling under REACH, Water Frame Directive and Cosmetics regulation) using existing evidence and toxicological data with the aim to determine which substances would be identified as potential EDs. Meta-analysis of the plausible link that had been established between adversity and mechanistic data indicative of an endocrine MoA was performed in order to identify pathways through which the substances were identified as potential EDs. Apart from the EATS modalities, analysis indicated also other modalities for which validated test methods do not currently exist, such as the HPA, HPG, somatotropic axis, retinoic signalling, PPAR signalling, *etc*. The non-EATS pathways are shown in Table 3. .

1.2. ECHA Endocrine Disruptor Expert Group (ECHA ED EG)

BPI participates with nominated experts in discussions of the ECHA ED EG for the assessment of the endocrine disrupting properties of chemicals regulated under Regulation (EU) No. 528/2012 and the REACH (Regulation (EC) No. 1907/2006). Information on the endocrine properties of 49 chemicals discussed since the implementation of the ECHA/EFSA guidance (2018) was considered with the purpose to identify endpoints that were either examined with non-validated protocols or were not examined due to lack of available validated methodology.

1.3. EFSA/BPI as competent authority for assessment of plant protection product active (PPPs) substances

BPI is the regulatory national competent authority for the assessment of PPPs in the frames of Regulation (EC) No. 1107/2009. In this context, the available validated methods as listed in EFSA/ECHA guidance (2018) and used for the evaluation of PPPs under Reg. 1107/2009 were screened to identify the already assessed endpoints (Appendix II). The final purpose of this task was to identify endpoints that were either examined with non-validated protocols or were not examined due to lack of available validated methodology.

The identification of these endpoints was based on information presented in OECD GD 150 (2018), OECD 178 (2012) and general knowledge on the anatomy and physiology of endocrine system of different organisms (mammals and non-mammals).

Furthermore, regulatory documents considered in EFSA Peer review discussions since the implementation of the ECHA/EFSA guidance (2018), such as renewal assessment reports (RARs), commenting/discussion tables and EFSA conclusions were screened (72 active substances).

1.4. Endpoints in ED relevant AOPs

AOP wiki (https://aopwiki.org/) was screened for ED relevant Adverse Outcome Pathways (AOPs). Keywords derived from the dictionary of terms as compiled in the frames of WP2 were used. All AOPs retrieved were collected and assessed manually for relevance. A total of 124 ED relevant AOPs were selected considering broad selection criteria. Only AOPs that involved an ED related mechanism were included. These AOPs were compiled in an excel spreadsheet including information on the AOP ID, the specific url for each AOP from the AOP wiki database, the AOP title and their relevance to EATS and non-EATS pathways (Table 1) and were considered as the basis for key event selection.

Table 1. List of ED-relevant AOPs from AOP wiki

			AOPs									
			Non-EATS pathways									
AOP ID	**AOP Link**	**AOP Title**	**HPA axis**	**HPG axis**	**somatotropic axis**	**retinoid signalling**	**HPT axis**	**Vitamin D signalling**	**PPAR signalling**	**epigenetics**	**Other non-EATS pathway**	**EATS**
4	https://aopwiki.org/aops/4	Ecdysone receptor agonism leading to incomplete ecdysis associated mortality									x	
6	https://aopwiki.org/aops/6	Antagonist binding to PPARα leading to body-weight loss							x			
7	https://aopwiki.org/aops/7	Aromatase (Cyp19a1) reduction leading to impaired fertility in adult female										S
8	https://aopwiki.org/aops/8	Upregulation of Thyroid Hormone Catabolism via Activation of Hepatic Nuclear Receptors, and Subsequent Adverse Neurodevelopmental Outcomes in Mammals										T
18	https://aopwiki.org/aops/18	PPARα activation in utero leading to impaired fertility in males							x			S
19	https://aopwiki.org/aops/19	Androgen receptor antagonism leading to adverse effects in the male foetus (mammals)										A
21	https://aopwiki.org/aops/21	Aryl hydrocarbon receptor activation leading to early life stage mortality, via increased COX-2	x					x	x		x	
23	https://aopwiki.org/aops/23	Androgen receptor agonism leading to reproductive dysfunction (in repeat-spawning fish)										A or S
25	https://aopwiki.org/aops/25	Aromatase inhibition leading to reproductive dysfunction										S
28	https://aopwiki.org/aops/28	Cyclooxygenase inhibition leading reproductive failure									x	
29	https://aopwiki.org/aops/29	Estrogen receptor agonism leading to reproductive dysfunction										E
30	https://aopwiki.org/aops/30	Estrogen receptor antagonism leading to reproductive dysfunction										E

			AOPs									
			Non-EATS pathways									
AOP ID	**AOP Link**	**AOP Title**	**HPA axis**	**HPG axis**	**somatotropic axis**	**retinoid signalling**	**HPT axis**	**Vitamin D signalling**	**PPAR signalling**	**epigenetics**	**Other non-EATS pathway**	**EATS**
42	https://aopwiki.org/aops/42	Inhibition of Thyroperoxidase and Subsequent Adverse Neurodevelopmental Outcomes in Mammals										T
51	https://aopwiki.org/aops/51	PPARα activation leading to impaired fertility in adult male rodents							x			
52	https://aopwiki.org/aops/52	ER agonism leading to skewed sex ratios due to altered sexual differentiation in males										E
53	https://aopwiki.org/aops/53	ER agonism leading to reduced survival due to renal failure										E
54	https://aopwiki.org/aops/54	Inhibition of Na+/I- symporter (NIS) leads to learning and memory impairment										T
63	https://aopwiki.org/aops/63	Cyclooxygenase inhibition leading to reproductive dysfunction									x	
64	https://aopwiki.org/aops/64	Glucocorticoid Receptor (GR) Mediated Adult Leydig Cell Dysfunction Leading to Decreased Male Fertility	x									
65	https://aopwiki.org/aops/65	XX Inhibition of Sodium Iodide Symporter and Subsequent Adverse Neurodevelopmental Outcomes in Mammals										T
66	https://aopwiki.org/aops/66	Modulation of Adult Leydig Cell Function Subsequent Glucocorticoid Activation in the Fetal Testis	x									S
67	https://aopwiki.org/aops/67	Modulation of Adult Leydig Cell Function Subsequent to Estradiaol Activation in the Fetal Testis		x								S
68	https://aopwiki.org/aops/68	Modulation of Adult Leydig Cell Function Subsequent to Alterations in the Fetal Testis Protome										S
69	https://aopwiki.org/aops/69	Modulation of Adult Leydig Cell Function Subsequent to Decreased Cholesterol Synthesis or Transport in the Adult Leydig Cell										S

			AOPs									
			Non-EATS pathways									
AOP ID	AOP Link	AOP Title	HPA axis	HPG axis	somatotropic axis	retinoid signalling	HPT axis	Vitamin D signalling	PPAR signalling	epigenetics	Other non-EATS pathway	EATS
70	https://aopwiki.org/aops/70	Modulation of Adult Leydig Cell Function Subsequent to Proteomic Alterations in the Adult Leydig Cell	x									S
71	https://aopwiki.org/aops/71	Modulation of Adult Leydig Cell Function Subsequent to Glucocorticoid Activation	x									S
72	https://aopwiki.org/aops/72	Epigenetic modification of PPARG leading to adipogenesis							x	x		
73	https://aopwiki.org/aops/73	Xenobiotic Inhibition of Dopamine-beta-Hydroxylase and subsequent reduced fecundity		x								
74	https://aopwiki.org/aops/74	Modulation of Adult Leydig Cell Function Subsequent to Hypermethylation in the Fetal Testis								x		S
77	https://aopwiki.org/aops/77	Nicotinic acetylcholine receptor activation contributes to abnormal foraging and leads to colony death/failure 1									x	
79	https://aopwiki.org/aops/79	Nicotinic acetylcholine receptor activation contributes to impaired hive thermoregulation and leads to colony loss/failure									x	
80	https://aopwiki.org/aops/80	Nicotinic acetylcholine receptor activation contributes to accumulation of damaged mitochondrial DNA and leads to colony loss/failure									x	
81	https://aopwiki.org/aops/81	Increased metabolic stress contributes to abnormal foraging and leads to colony loss/failure									x	
82	https://aopwiki.org/aops/82	Abnormal role change in worker caste contributes to reduced brood care and leads to colony loss/failure									x	
91	https://aopwiki.org/aops/91	Sodium channel inhibition leading to reduced survival									x	
93	https://aopwiki.org/aops/93	sodium channel inhibition leading to increased predation									x	

			AOPs									
			Non-EATS pathways									
AOP ID	**AOP Link**	**AOP Title**	**HPA axis**	**HPG axis**	**somatotropic axis**	**retinoid signalling**	**HPT axis**	**Vitamin D signalling**	**PPAR signalling**	**epigenetics**	**Other non-EATS pathway**	**EATS**
95	https://aopwiki.org/aops/95	Ether-a-go-go (ERG) voltage-gated potassium channel inhibition leading to reduced survival									x	
96	https://aopwiki.org/aops/96	Axonal sodium channel modulation leading to acute mortality									x	
97	https://aopwiki.org/aops/97	5-hydroxytryptamine transporter (5-HTT; SERT) inhibition leading to population decline									x	
98	https://aopwiki.org/aops/98	5-hydroxytryptamine transporter (5-HTT; SERT) inhibition leading to decreased shelter seeking and increased predation									x	
99	https://aopwiki.org/aops/99	Histamine (H2) receptor antagonism leading to reduced survival									x	
100	https://aopwiki.org/aops/100	Cyclooxygenase inhibition leading to reproductive dysfunction via inhibition of female spawning behavior									x	
101	https://aopwiki.org/aops/101	Cyclooxygenase inhibition leading to reproductive dysfunction via inhibition of pheromone release									x	
102	https://aopwiki.org/aops/102	Cyclooxygenase inhibition leading to reproductive dysfunction via interference with meiotic prophase I /metaphase I transition									x	
103	https://aopwiki.org/aops/103	Cyclooxygenase inhibition leading to reproductive dysfunction via interference with spindle assembly checkpoint									x	
110	https://aopwiki.org/aops/110	Inhibition of iodide pump activity leading to follicular cell adenomas and carcinomas (in rat and mouse)										T
111	https://aopwiki.org/aops/111	Decrease in androgen receptor activity leading to Leydig cell tumors (in rat)										A
112	https://aopwiki.org/aops/112	Increased dopaminergic activity leading to endometrial adenocarcinomas (in Wistar rat)										E

			AOPs									
			Non-EATS pathways									
AOP ID	**AOP Link**	**AOP Title**	**HPA axis**	**HPG axis**	**somatotropic axis**	**retinoid signalling**	**HPT axis**	**Vitamin D signalling**	**PPAR signalling**	**epigenetics**	**Other non-EATS pathway**	**EATS**
119	https://aopwiki.org/aops/119	Inhibition of thyroid peroxidase leading to follicular cell adenomas and carcinomas (in rat and mouse)										T
120	https://aopwiki.org/aops/120	Inhibition of 5α-reductase leading to Leydig cell tumors (in rat)		x								
122	https://aopwiki.org/aops/122	Prolyl hydroxylase inhibition leading to reproductive dysfunction via increased HIF1 heterodimer formation										S
123	https://aopwiki.org/aops/123	Unknown MIE leading to reproductive dysfunction via increased HIF-1alpha transcription										S
124	https://aopwiki.org/aops/124	HMG-CoA reductase inhibition leading to decreased fertility		x								
126	https://aopwiki.org/aops/126	Alpha-noradrenergic antagonism leads to reduced fecundity via delayed ovulation	x									
128	https://aopwiki.org/aops/128	Kidney dysfunction by decreased thyroid hormone										T
134	https://aopwiki.org/aops/134	Sodium Iodide Symporter (NIS) Inhibition and Subsequent Adverse Neurodevelopmental Outcomes in Mammals										T
146	https://aopwiki.org/aops/146	Estrogen Receptor Activation and Female Precocious Puberty										E
152	https://aopwiki.org/aops/152	Interference with thyroid serum binding protein transthyretin and subsequent adverse human neurodevelopmental toxicity										T
153	https://aopwiki.org/aops/153	Aromatase Inhibition leading to Ovulation Inhibition and Decreased Fertility in Female Rats		x								S
155	https://aopwiki.org/aops/155	Deiodinase 2 inhibition leading to reduced young of year survival via posterior swim bladder inflation										T
156	https://aopwiki.org/aops/156	Deiodinase 2 inhibition leading to reduced young of year survival via anterior swim bladder inflation										T

			AOPs									
			Non-EATS pathways									
AOP ID	**AOP Link**	**AOP Title**	**HPA axis**	**HPG axis**	**somatotropic axis**	**retinoid signalling**	**HPT axis**	**Vitamin D signalling**	**PPAR signalling**	**epigenetics**	**Other non-EATS pathway**	**EATS**
157	https://aopwiki.org/aops/157	Deiodinase 1 inhibition leading to reduced young of year survival via posterior swim bladder inflation										T
158	https://aopwiki.org/aops/158	Deiodinase 1 inhibition leading to reduced young of year survival via anterior swim bladder inflation										T
159	https://aopwiki.org/aops/159	Thyroperoxidase inhibition leading to reduced young of year survival via anterior swim bladder inflation										T
162	https://aopwiki.org/aops/162	Enhanced hepatic clearance of thyroid hormones leading to thyroid follicular cell adenomas and carcinomas in the rat and mouse										T
165	https://aopwiki.org/aops/165	Antiestrogen activity leading to ovarian adenomas and granular cell tumors in the mouse										E
167	https://aopwiki.org/aops/167	Early-life estrogen receptor activity leading to endometrial carcinoma in the mouse.										E
168	https://aopwiki.org/aops/168	GnRH pulse disruption leading to mammary adenomas and carcinomas in the SD rat.		x								
169	https://aopwiki.org/aops/169	GnRH pulse disruption leading to pituitary adenomas and carcinomas in the SD rat.		x								
170	https://aopwiki.org/aops/170	Anti-dopaminergic activity leading to mammary adenomas and carcinomas in the SD rat									x	
175	https://aopwiki.org/aops/175	Thyroperoxidase inhibition leading to altered amphibian metamorphosis										T
176	https://aopwiki.org/aops/176	Sodium Iodide Symporter (NIS) Inhibition leading to altered amphibian metamorphosis										T
177	https://aopwiki.org/aops/177	Cyclooxygenase 1 (COX1) inhibition leading to renal failure and mortality									x	

			AOPs									
			Non-EATS pathways									
AOP ID	AOP Link	AOP Title	HPA axis	HPG axis	somatotropic axis	retinoid signalling	HPT axis	Vitamin D signalling	PPAR signalling	epigenetics	Other non-EATS pathway	EATS
188	https://aopwiki.org/aops/188	Iodotyrosine deiodinase (IYD) inhibition leading to altered amphibian metamorphosis										T
189	https://aopwiki.org/aops/189	Type I iodothyronine deiodinase (DIO1) inhibition leading to altered amphibian metamorphosis										T
190	https://aopwiki.org/aops/190	Type II iodothyronine deiodinase (DIO2) inhibition leading to altered amphibian metamorphosis										T
191	https://aopwiki.org/aops/191	Type III iodotyrosine deiodinase (DIO3) inhibition leading to altered amphibian metamorphosis										T
192	https://aopwiki.org/aops/192	Pendrin inhibition leading to altered amphibian metamorphosis										T
193	https://aopwiki.org/aops/193	Dual oxidase (DUOX) inhibition leading to altered amphibian metamorphosis										T
194	https://aopwiki.org/aops/194	Hepatic nuclear receptor activation leading to altered amphibian metamorphosis					x					T
195	https://aopwiki.org/aops/195	5-hydroxytryptamine transporter (5-HTT) inhibition leading to population increase									x	
199	https://aopwiki.org/aops/199	ER mediated breast cancer AOP										E
200	https://aopwiki.org/aops/200	Estrogen receptor activation leading to breast cancer										E
201	https://aopwiki.org/aops/201	Juvenile hormone receptor agonism leading to male offspring induction associated population decline									x	
203	https://aopwiki.org/aops/203	5-hydroxytryptamine transporter inhibition leading to decreased reproductive success and population decline									x	
204	https://aopwiki.org/aops/204	5-hydroxytryptamine transporter inhibition leading to increased reproductive success and population increase									x	

			AOPs									
			Non-EATS pathways									
AOP ID	**AOP Link**	**AOP Title**	**HPA axis**	**HPG axis**	**somatotropic axis**	**retinoid signalling**	**HPT axis**	**Vitamin D signalling**	**PPAR signalling**	**epigenetics**	**Other non-EATS pathway**	**EATS**
207	https://aopwiki.org/aops/207	NADPH oxidase and P38 MAPK activation leading to reproductive failure in Caenorhabditis elegans										
208	https://aopwiki.org/aops/208	Janus kinase (JAK)/Signal transducer and activator of transcription (STAT) and Transforming growth factor (TGF)-beta pathways activation leading to reproductive failure									x	
210	https://aopwiki.org/aops/210	Activation of c-Jun N-terminal kinase (JNK) and Forkhead box O (FOXO) and reduction of WNT pathways leading to reproductive failure: Integrated multi-OMICS approach for AOP building									x	
212	https://aopwiki.org/aops/212	Histone deacetylase inhibition leading to testicular atrophy								x		
218	https://aopwiki.org/aops/218	Inhibition of CYP7B activity leads to decreased reproductive success via decreased locomotor activity		x								
219	https://aopwiki.org/aops/219	Inhibition of CYP7B activity leads to decreased reproductive success via decreased sexual behavior		x								
263	https://aopwiki.org/aops/263	ATP synthase inhibition leading to reduced fecundity (#1)									x	
264	https://aopwiki.org/aops/264	ATP synthase inhibition leading to reduced fecundity (#2)									x	
271	https://aopwiki.org/aops/271	Inhibition of thyroid peroxidase leading to impaired fertility in fish										T, S
288	https://aopwiki.org/aops/288	Inhibition of 17α-hydrolase/C 10,20-lyase (Cyp17A1) activity leads to birth reproductive defects (cryptorchidism) in male (mammals)										A or S
289	https://aopwiki.org/aops/289	Inhibition of 5α-reductase leading to impaired fertility in female fish										S
290	https://aopwiki.org/aops/290	DNA methyltransferase inhibition leading to reduced fecundity associated population decline								x		

			AOPs									
			Non-EATS pathways									
AOP ID	**AOP Link**	**AOP Title**	**HPA axis**	**HPG axis**	**somatotropic axis**	**retinoid signalling**	**HPT axis**	**Vitamin D signalling**	**PPAR signalling**	**epigenetics**	**Other non-EATS pathway**	**EATS**
291	https://aopwiki.org/aops/291	DNA methyltransferase inhibition leading to transgenerational DNA methylation associated population decline								x		
295	https://aopwiki.org/aops/295	Early-life stromal estrogen receptor activation by endocrine disrupting chemicals in the mammary gland leading to enhanced cancer risk										E or S
297	https://aopwiki.org/aops/297	Inhibition of retinaldehyde dehydrogenase leads to population decline				x						
299	https://aopwiki.org/aops/299	S-adenosylmethionine depletion leading to reduced fecundity								x		
300	https://aopwiki.org/aops/300	Thyroid Receptor Antagonism and Subsequent Adverse Neurodevelopmental Outcomes in Mammals										T
305	https://aopwiki.org/aops/305	5α-reductase inhibition leading to short anogenital distance (AGD) in male (mammalian) offspring										A
306	https://aopwiki.org/aops/306	Androgen receptor (AR) antagonism leading to short anogenital distance (AGD) in male (mammalian) offspring										A
307	https://aopwiki.org/aops/307	Decreased testosterone synthesis leading to short anogenital distance (AGD) in male (mammalian) offspring										A or S
309	https://aopwiki.org/aops/309	Luteinizing hormone receptor antagonism leading to reproductive dysfunction		x								
310	https://aopwiki.org/aops/310	Embryonic Activation of the AHR leading to Reproductive failure, via epigenetic down-regulation of GnRHR		x						x		S
312	https://aopwiki.org/aops/312	Acetylcholinesterase Inhibition leading to Acute Mortality via Impaired Coordination & Movement									x	
314	https://aopwiki.org/aops/314	Activation of estrogen receptor in immune cells leading to exacerbation of systemic lupus erythematosus										E

			AOPs									
			Non-EATS pathways									
AOP ID	**AOP Link**	**AOP Title**	**HPA axis**	**HPG axis**	**somatotropic axis**	**retinoid signalling**	**HPT axis**	**Vitamin D signalling**	**PPAR signalling**	**epigenetics**	**Other non-EATS pathway**	**EATS**
321	https://aopwiki.org/aops/321	Reduced environmental pH leading to thinner shells in Mytilus edulis									x	
322	https://aopwiki.org/aops/322	Alkylation of DNA leading to reduced sperm count									x	
323	https://aopwiki.org/aops/323	PPARalpha Agonism Impairs Fish Reproduction				x			x			
333	https://aopwiki.org/aops/333	Mitochondrial complex inhibition leading to reproduction decline									x	
334	https://aopwiki.org/aops/334	Glucocorticoid Receptor Agonism Leading to Impaired Fin Regeneration	x	x								
336	https://aopwiki.org/aops/336	DNA methyltransferase inhibition leading to population decline (#1)								x		
337	https://aopwiki.org/aops/337	DNA methyltransferase inhibition leading to population decline (#2)								x		
338	https://aopwiki.org/aops/338	DNA methyltransferase inhibition leading to population decline (#3)								x		
339	https://aopwiki.org/aops/339	DNA methyltransferase inhibition leading to population decline (#4)								x		
340	https://aopwiki.org/aops/340	DNA methyltransferase inhibition leading to transgenerational effects (#1)								x		
341	https://aopwiki.org/aops/341	DNA methyltransferase inhibition leading to transgenerational effects (#2)								x		
342	https://aopwiki.org/aops/342	Chitin synthase inhibition leading to mortality									x	
343	https://aopwiki.org/aops/343	Sulfonylurea receptor binding leading to mortality									x	
344	https://aopwiki.org/aops/344	Androgen receptor (AR) antagonism leading to nipple retention (NR) in male (mammalian) offspring										A

EATS: Estrogen, Androgen, Thyroid, Steroidogenesis; HPA: Hypothalamus-Pituitary-Adrenal axis; HPG: Hypothalamus-Pituitary-Gonadal axis; HPT: Hypothalamus-Pituitary-Thyroid axis; PPAR: Peroxisome Proliferator Activated Receptor

1.5. US-EPA

The US-EPA has published the screening of ToxCast phase 1 and 2 chemical libraries including a total of 1074 unique chemicals (Hornung et al. 2018; Olker et al. 2019; Paul Friedman et al. 2016; Wang et al. 2018). Five (5) *in vitro* non-validated protocols indicative of thyroid endpoints were retrieved for endpoint selection. However, these assays are not widely accepted by EU regulatory authorities since they are not validated and their reliability and uncertainties are not well described. In addition, the US EPA EDSP[1] database was searched to include all the thyroid relevant assays. A complete overview of US-EPA test guidelines was performed under WP2.

1.6. OECD EDTA-AG

The OECD Draft Detailed Review Paper (DRP) on Retinoid Signalling Pathway (EDTA, April 2020), OECD GD 150 (2018) and OECD DRP 178 (2012) were considered to retrieve relevant endpoints. Also the OECD STA 207 (2014) for thyroid hormone signalling was also advised (https://www.oecd-ilibrary.org/docserver/9789264274716-en.pdf?expires=1592030156&id=id&accname=guest&checksum=F297753006E2E032BEAB2FED0C9AF909).

[1] https://comptox.epa.gov/dashboard

2. Results

2.1. Identified endpoints from different sources

The following endocrine-related endpoints relevant for mammals and non-mammalian species were identified from the different sources of information as described in Section 1 (See Box 1 for overview):

Box 1: Identified endpoints from different sources

Sources	ED IA	ECHA	EFSA	AOPwiki	US EPA	OECD
ED-relevant endpoints gaps for *in vitro*, *in vitro* complex, *in vivo* light	25	13	49	59	9	19

- From the ED IA, ECHA and EFSA, twenty-five (25), thirteen (13) and forty-nine (49) relevant endpoints were retrieved, respectively.
- AOP wiki screening methodology resulted in the identification of ninety (90) relevant key events IDs (KE IDs). Only the endpoints (MIE/KE/AO) presented in three (3) or more AOPs were considered. Exceptionally, certain endpoints occurred in less than 3 AOPs were selected as well. These endpoints are relevant for organisms with small number of available AOPs and considered to be important due to very limited endpoints assessed for these organisms in already validated assays. When more than one description and KE IDs were provided for the same endpoint this information was grouped resulting in a total of sixty-one (61) KEs. This list of KEs was subjected to further assessment in order to exclude the endpoints already addressed in validated Test Guidelines (TGs) included in the ECHA/EFSA ED guidance (2018) for mammals and/or non-mammals. From the selected endpoints, only those that can be assessed using *in vitro*, complex *in vitro* and "light" *in vivo* assays (see Box 2) were considered relevant in the frames of PEPPER (last column in Table 2). A total of 56 relevant endpoints were concluded (
-
-

- Table *2*).

Box 2: Terminology

- *In vitro* e.g. receptor transactivation assay
- *in vitro* complex: organoids, 3D models, battery of test
- *in vivo* light: amphibian, amphibian larva, birds embryos, fish, fish embryos, invertebrates (aquatic invertebrates, soil organisms, insects)

Table 2. List of relevant endpoints from selected AOPs vs. Endpoint addressed in validated TG relevant to mammals and non-mammals included in ECHA/EFSA ED guidance, 2018

Event ID	Endpoint description	Number of endpoint (MIE/KE/AO) occurrences in ED-relevant AOPs	Endpoint addressed in validated TG relevant to mammals included in ECHA/EFSA ED guidance, 2018	Endpoint addressed in validated TG relevant to non-mammals included in ECHA/EFSA ED guidance, 2018	Identified gap in validated assays for endpoints relevant to PEPPER
360, 442, 361, 679	Decrease, Population trajectory Decreased, Population trajectory Decline, Population Decline, Population trajectory	35	NR	No	No
426, 281	Thyroxine (T4) in serum, Decreased Decreased, Thyroxine (T4) in serum	15	Yes (OECD TG 407, 408, 414, 421, 422, 441, 443, OECD GD 115, OPPTS 890.1500, 890.1450)	Yes- birds (US EPA OCSPP 890.2100) No-amphibians, fish NR- invertebrates	Yes (non-mammals)
277	Thyroid hormone synthesis, Decreased	12	No	No	Yes (mammals and non-mammals)
675, 1141, 253, 1277	Reduced, Reproductive Success Decreased, Reproductive Success N/A, Reproductive failure Reproductive failure	12	Yes (OECD TG 421, 422)	Yes- fish (OECD TG 229, 240, JMASA)	No
328, 527	Decrease, Fecundity Decreased, Decreased fecundity	11	NR	Yes- fish (OECD TG 229, 240, JMASA)	Yes (non-mammals)
505, 520, 330, 406	Decreased sperm quantity / quality in the adult, Decreased fertility Decreased sperm quantity or quality in the adult, Decreased fertility Decrease, Fertility impaired, Fertility	11	Yes, in part (sperm morphology, motility, numbers) (OECD TG 408, 416, 443)	Yes- fish (fertility- OECD TG 229, 240, JMASA) Yes- birds (OECD TG 206, US EPA OCSPP 890.2100) NR- for other non-mammals	Yes (mammals and non-mammals- e.g sperm biomarkers)
78	Reduction, Cumulative fecundity and spawning	9	NR	Yes- fish (OECD TG 229, 240, JMASA) NR- other non-mammals	No
1101	Altered, Amphibian metamorphosis	9	NR	Yes- amphibians (OECD TG 231) NR- other non-mammals	No
219	Reduction, Plasma 17beta-estradiol concentrations	8	Yes (OECD TG 408, 441, GD 115)	Yes- birds (US EPA OCSPP 890.2100) No- other non-mammals	Yes (non-mammals)

Event ID	Endpoint description	Number of endpoint (MIE/KE/AO) occurrences in ED-relevant AOPs	Endpoint addressed in validated TG relevant to mammals included in ECHA/EFSA ED guidance, 2018	Endpoint addressed in validated TG relevant to non-mammals included in ECHA/EFSA ED guidance, 2018	Identified gap in validated assays for endpoints relevant to PEPPER
221	Reduction, Plasma vitellogenin concentrations	8	NR	Yes- fish and amphibians (Fish OECD TG 229, 230, 234, 240, JMASA Amphibians OECD TG 241) No- other non-mammals	No Knowledge gap for invertebrates
1116	Decreased, Triiodothyronine (T3) in tissues	8	No	Yes- birds (OCSPP 890.2100) No- fish and amphibians NR- invertebrates	Yes (non-mammals)
1619	Increase, DNA methyltransferase inhibition	8	No	No	Yes (mammals and non-mammals)
588, 623	Increased, predation Increase, predation	6	NR	No	No
3	Reduction, 17beta-estradiol synthesis by ovarian granulosa cells	6	No	No	Yes (mammals and non-mammals)
79	Inhibition, Cyclooxygenase activity	6	No	No	Yes (mammals and non-mammals)
280	Thyroxine (T4) in neuronal tissue, Decreased	6	No	No	No
285	Reduction, Vitellogenin synthesis in liver	6	NR	Yes- fish (OECD TG 229, 230, 234, 240, JMASA) No- amphibians, birds NR- invertebrates	No
309	Reduction, Vitellogenin accumulation into oocytes and oocyte growth/development	6	NR	No	No Knowledge gap for invertebrates
1003	Decreased, Triiodothyronine (T3) in serum	6	Yes (OECD TG 407, 408, 414, 421, 422, 443, OPPTS 890.1500, 890.1450)	Yes- birds (US EPA OCSPP 890.2100) No-amphibians and fish NR- invertebrates	Yes (non-mammals)
1005	Reduced, Swimming performance	6	NR	Yes- fish (OECD TG 229, 230, 234, 240, OPPTS 850.1500, JMASA) NR- for amphibians, birds and invertebrates	No
1093	Decreased, Thyroxine (T4) in tissues	6	No	Yes- birds (OCSPP 890.2100) No- fish and amphibians NR- invertebrates	Yes (non-mammals)
1366	Decrease, Oogenesis	6	Yes, in part (Number of corpora lutea) (OECD TG 414, 416, 421, 422, 443)	Yes- birds (egg production OECD TG 206, US EPA OCSPP 890.2100) NR- other non-mammals	Yes (mammals)
350, 351	Increase, Mortality Increased Mortality	6	Yes	Yes	No
286, 1687	Decreased, Transcription of genes by AR	5	No	No	Yes (mammals and non-mammals)

Event ID	Endpoint description	Number of endpoint (MIE/KE/AO) occurrences in ED-relevant AOPs	Endpoint addressed in validated TG relevant to mammals included in ECHA/EFSA ED guidance, 2018	Endpoint addressed in validated TG relevant to non-mammals included in ECHA/EFSA ED guidance, 2018	Identified gap in validated assays for endpoints relevant to PEPPER
	decrease, transcription of genes by AR				
279	Thyroperoxidase, Inhibition	5	No	No	Yes (mammals and non-mammals)
402	Cognitive Function, Decreased	5	No	NR	Yes (mammals)
563	Death/Failure, Colony	5	NR	No-invertebrates NR- other non-mammals	No
619	Inhibition, 5-hydroxytryptamine transporter (5-HTT; SERT)	5	No	No	No
626	Increased, serotonin (5-HT)	5	No	No	No
756	Hippocampal gene expression, Altered	5	No	No	No
757	Hippocampal anatomy, Altered	5	Yes (OECD TG 426, 443)	No	No
758	Hippocampal Physiology, Altered	5	No	No	No
1006	Reduced, Young of year survival	5	NR	No	No
1614	Decrease, androgen receptors (AR) activation	5	Yes (OECD 458)	Yes (OECD 458)	No
1007, 1044	Anterior/ Posterior swim bladder inflation	5	NR	No-fish NR-birds, amphibians, invertebrates	Yes# (non-mammals)
446, 1690	Reduction, testosterone level reduction, testosterone levels	4	Yes (OECD TG 408, OPPTS 890.1500)	No- amphibians and reptiles NR- other non-mammals	Yes (non-mammals)
177	N/A, Mitochondrial dysfunction 1	4	No	No	No
413	Reduction, Testosterone synthesis in Leydig cells	4	No	No	Yes (mammals and non-mammals)
424	Inhibition, Na+/I-symporter (NIS)	4	No	No NR- invertebrates	Yes (mammals and non-mammls)
425	Decrease of Thyroidal iodide	4	No	No NR- invertebrates	Yes (mammals and non-mammals)
655	Decreased testosterone by the fetal Leydig cells, Increased COUP-TFII in fetal Leydig cells	4	No	No	Yes (mammals and non-mammals)
1107	Weakened, Colony	4	NR	No- invertebrates NR- other non-mammals	No
1142	Increased, valve movement	4	NR	No- invertebrates NR- other non-mammals	Yes (non-mammals)
1613	Decrease, dihydrotestosterone (DHT) level	4	No	No- amphibians, birds NR- fish, invertebrates	Yes (mammals and non-mammals)
1773	Decrease, Global DNA methylation	4	No	No	Yes (mammals and non-mammals)

Event ID	Endpoint description	Number of endpoint (MIE/KE/AO) occurrences in ED-relevant AOPs	Endpoint addressed in validated TG relevant to mammals included in ECHA/EFSA ED guidance, 2018	Endpoint addressed in validated TG relevant to non-mammals included in ECHA/EFSA ED guidance, 2018	Identified gap in validated assays for endpoints relevant to PEPPER
1774	Increase, Caspase transcription	4	No	No	No
111, 112	Agonism, Estrogen receptor	4	Yes (OECD TG 455, OPPTS 890.1300)	Yes (OECD TG 455, OPPTS 890.1300)	No
227	Activation, PPARα	3	No	No	Yes (mammals and non-mammals)
341	Impairment, Learning and memory	3	No	No	Yes
559	Activation, Nicotinic acetylcholine receptor	3	No	No	No
585	Decreased, Sodium conductance 1	3	No	No	No
586	Reduced, swimming speed	3	NR	No- fish NR-other non-mammals	No
621	Increase, cilia movement	3	NR	No- invertebrates NR- other non-mammals	Yes (non-mammals)
656	Decreased number and function of adult Leydig cells, Decreased COUP-TFII stem Leydig cells	3	No	No	Yes (mammals and non-mammals)
1002	Inhibition, Deiodinase 2	3	No	No	Yes (mammals and non-mammals)
1009	Inhibition, Deiodinase 1	3	No	No	Yes (mammals and non-mammals)
1620	Increase, DNA hypomethylation	3	No	No	Yes (mammals and non-mammals)
1621	Increase, Gene expression	3	No	No	Yes* (mammals and non-mammals)
1688	decrease, male anogenital distance	3	Yes (OECD TG 414, 421, 422, 426, 416, 443)	NR	No
1775	Increase, Oocyte apoptosis	3	No	No	No
1104, 671	Prostaglandin F2alpha levels in plasma	3	No	No	Yes (non-mammals)
1387	7α-hydroxypregnenolone synthesis in the brain, decreased	2	No	No	Yes (mammals and non-mammals)
1777, 1784	Increase, Ovarian follicle breakdown	4	No	No	No
944	Dimerization, AHR/ARNT	2	No	No	Yes (mammals and non-mammals)
687	Reduced, Prostaglandin E2 concentration, hypothalamus	2	No	No	Yes (non-mammals)
689	Reduced, Gonadotropin releasing hormone, hypothalamus	2	No	No	Yes (non-mammals)
799	Increased, HIF-1 heterodimer	2	No	No	No
488, 1695	Decrease, Ovulation, Impaired ovulation	2	No	No	No

Event ID	Endpoint description	Number of endpoint (MIE/KE/AO) occurrences in ED-relevant AOPs	Endpoint addressed in validated TG relevant to mammals included in ECHA/EFSA ED guidance, 2018	Endpoint addressed in validated TG relevant to non-mammals included in ECHA/EFSA ED guidance, 2018	Identified gap in validated assays for endpoints relevant to PEPPER
1280	Activation, HIF-1	1	No	No	No
103	Increase, Ecdysone receptor agonism	1	NR	No	Yes (non-mammals)
1264	Increase, Nuclear receptor E75b gene expression	1	NR	No	Yes (non-mammals)
1265	Increase, Fushi tarazu factor-1 gene expression	1	NR	No	Yes (non-mammals)
988	Decrease, circulating ecdysis triggering hormone	1	NR	No	Yes (non-mammals)
990	Increase, Incomplete ecdysis	1	NR	No	Yes (non-mammals)
1205	Activation, Juvenile hormone receptor	1	NR	No	Yes (non-mammals)
1778	Decrease, Heritable DNA methylation (F3)	1	No	No	Yes (mammals and non-mammals)
129	Reduction, Gonadotropins, circulating concentrations	1	No	No	Yes (non-mammals)
619	Inhibition, 5-hydroxytryptamine transporter (5-HTT; SERT)	1	No	No	No
685	Reduced, Prostaglandins, ovary	1	No	No	Yes (non-mammals)
672	Reduced, Prostaglandin F2alpha synthesis, ovary	1	No	No	Yes (mammals and non-mammals)
802	Increased, HIF-1 alpha transcription	1	No	No	No
1637	Retinaldehyde dehydrogenase inhibition	1	No	No	Yes (mammals and non-mammals)
1691	Antagonism,,LH receptor	1	No	No	Yes (mammals and non-mammals)
1692	Reduction, Progesterone synthesis	1	No	No	Yes (mammals and non-mammals)
1693	Reduction, Plasma progesterone concentration	1	No	No	Yes (non-mammals)
1698	Increase, hypermethylation of the promotor region of gonadotropin releasing hormone receptor	1	No	No	Yes (mammals and non-mammals)
1756	Decreased, 11-ketotestosterone	1	NR	No-fish NR-birds, amphibians, invertebrates	Yes (non-mammals)
1759	Increase, Cripto-1 expression	1	No	No	Yes (mammals and non-mammals)
1760	Inhibition, Activin signalling	1	No	No	Yes (mammals and non-mammals)
1761	Inhibition, Fin regeneration	1	NR	No-fish NR-birds, amphibians, invertebrates	Yes (non-mammals)

*Refers to DNA methylation-induced gene deregulation
Depending on whether an appropriate "light *in vivo*" assay will be detected
NR: not relevant

- US-EPA data were considered to identify eight (8) endpoints relevant for the assessment of the **thyroid activity**. In addition, the endpoint of **glucocorticoid** receptor activation already retrieved from the ED IA and EFSA sources was added in the US-EPA EDSP outcome since that was the original reference.
- Eleven (11) endpoints related to the **retinoid signalling pathway** were recorded considering the respective OECD DRP.

A list of the endpoints retrieved from the different sources of information is presented in Table 3. . The excel file is embedded in the Endocrine relevant pathways for identified endpoints

The identified ED-related endpoints could be involved in one or more EATS and/or non-EATS pathways as depicted in Table 5. From the excel file embedded in the APPENDIX, it is possible to also retrieve the endpoints involved in each pathway, noting that this allocation of endpoints in the different pathways is not exhaustive but reflective of the most frequently observed pathways.

Table 5. Endocrine relevant pathways for identified endpoint

Endpoint	HPA	HPG	HPT	Somatotropic	Retinoid	Vitamin D	PPAR	Epigenetics	Other non-EATS pathway	EATS
11-ketotestosterone levels		x								S
17alpha-hydroxyprogesterone (17α-OHP) synthesis	x	x								S
17beta-estradiol plasma levels		x								S
17beta-estradiol synthesis in ovarian granulosa cells		x								S
5a-reductase activity		x								S
7α-hydroxypregnenolone synthesis in the brain										S
Activin signalling pathway		x								
Adrenocorticotropic hormone (ACTH) levels in tissues	x									
Androgen receptor (AR) expression										A
Androgen receptor (AR)-induced gene expression										A
Androstenedione synthesis		x								S
Annetocin levels									x	
Arginine vasotocin (AVT) levels in tissues	x									

Endpoint	HPA	HPG	HPT	Somatotropic	Retinoid	Vitamin D	PPAR	Epigenetics	Other non-EATS pathway	EATS
Aryl hydrocarbon receptor (AhR) activation	x		x		x	x				
Aryl hydrocarbon receptor (AhR) expression	x		x		x	x				
Aryl hydrocarbon receptor (AHR/ARNT) dimerization					x					
Calcitonin levels (plasma or relevant tissues)						x				
Cilia movement									x	
Cognitive Function										S, T
Constitutive androstane receptor (CAR) activation and CAR-related genes (Alas1, Slco1b2, and NADPH-Cyp-reductase, Cyp2b10)			x		x				x	
Corticotropin-releasing hormone (CRH) (or CRH-like) levels in tissues	x									
Cripto-1 expression levels		x							x	
Cyclooxygenase activity (Cox1, Cox2)									x	
Cyp26 activity					x					
Deiodinase 1 (DIO1) activity			x							T
Deiodinase 2 (DIO2) activity			x							T
Deiodinase 3 (DIO3) activity			x							T
Dihydrotestosterone circulating (DHT) levels		x								S
DNA hypomethylation								x		
DNA methylation, global								x		
DNA methylation, heritable								x		
DNA methylation-induced gene deregulation								x		
DNA methyltransferase activity								x		
Ecdysis triggering hormone (ETH) levels					x				x	
Ecdysone receptor (EcR) activation					x				x	
Ecdysteroids levels					x				x	
Eggshell formation/ calcification (mechanistic studies)						x			x	
Estrogen receptor (ER) expression										E
Fecundity		x							x	E, A, S, T
Fertility (female)		x			x	x		x		E, S
Fertility (male)	x	x			x	x				A, S
Fin development/ regeneration	x	x			x					

Endpoint	HPA	HPG	HPT	Somatotropic	Retinoid	Vitamin D	PPAR	Epigenetics	Other non-EATS pathway	EATS
Follicle-stimulating hormone-like (FSH-like) levels in tissues		x								
Fushi tarazu factor-1 gene expression									x	
Gene/protein expression related to the transfer of thyroid hormone (TH) in embryonic circulation.			X							T
Glucocorticoid receptor (GR) receptor activation	x									
Gonadotropin circulating levels		x								
Gonadotropin levels in hypothalamus		x								
Gonadotropin releasing hormone receptor (GnRHR) promoter region hypermethylation		x						x		
Gonadotropin-releasing hormone receptor (GnRHR) activation		x								
Growth hormone (GH) levels in tissues				x						
Growth hormone receptor (GHR) activation				x						
Insulin-like growth factor (IGF1, IGF2) levels				x						
Interrenal (adrenal) gland histopathology	x									
Juvenile hormone (JH) levels									x	
Juvenile hormone (JH) receptor activation									x	
Learning and memory	x								x	T, S
Luteinizing hormone (LH) receptor activation		x								
Luteinizing hormone-like (LH-like) hormone levels in tissues		x								
Melanocyte-stimulating hormone (MSH) levels									x	
Mineralocorticoid receptor (MR) activation	x									
Molting/ ecdysis incomplete					x				x	
Na+/I- symporter (NIS) activity			x							T
Nuclear receptor E75b gene expression									x	
Number and function of adult Leydig cells, levels of COUP-TFII in stem Leydig cells		x								S
Obesity					x		x			
Oogenesis					x			x	x	
Ovarian steroidogenesis		x			x					S
Parathyroid hormone (PTH) levels in plasma or in relevant tissues						x				
Peroxisome proliferator-activated receptor alpha (PPARα) activation			x		x		x			S

Endpoint	HPA	HPG	HPT	Somatotropic	Retinoid	Vitamin D	PPAR	Epigenetics	Other non-EATS pathway	EATS
Peroxisome proliferator-activated receptor gamma (PPARγ) activation			x		x		x			
Pigmentation (retinal and body)									x	T
Pregnane X Receptor (PXR) activation					x				x	
Progesterone levels in plasma	x	x								
Progesterone Receptor (PR) activation	x	x								
Progesterone synthesis	x	x								S
Prolactin (PRL) levels in tissues		x	x							
Prostaglandin D2 (PGD2) synthesis									x	
Prostaglandin E2 (PGE2) levels in hypothalamus									x	
Prostaglandin F2alpha levels in plasma									x	
Prostaglandin F2alpha synthesis in ovary									x	E
Prostaglandin levels in ovary									x	E
Reproductive pheromones		x								
Retinaldehyde dehydrogenase (RALDH) activity					x					
Retinoid acid receptor (RAR), Retinoid X receptor (RXR), RAR/RXR transactivation					x					
Spontaneous acrosome reaction (SAR), progesterone-induced acrosome reaction (PIAR), capacitation		x								E, A
Steroid hormone levels in tissues										S
Steroidogenesis- related gene and protein expression (Star, 17b-HSD3, Cyp11a1, 3b-HSD, Cyp17a1, Cyp19a1)										S
Stra8 induction					x					
Swim bladder inflation (anterior/posterior)			x							T
Testosterone serum levels		x								S

Endpoint	HPA	HPG	HPT	Somatotropic	Retinoid	Vitamin D	PPAR	Epigenetics	Other non-EATS pathway	EATS
Testosterone synthesis in fetal Leydig cells, COUP-TFII levels in fetal Leydig cells		x								S
Testosterone synthesis in Leydig cells		x								S
Thyroid hormone clearance			x							T
Thyroid hormone receptor (THR) activation			x							T
Thyroid hormone receptors (THRs) expression levels			x							T
Thyroid hormone synthesis			x							T
Thyroid- stimulating hormone receptor (TSHR) activation			x							T
Thyroidal iodide uptake in thyroid follicular cells			x							T
Thyroperoxidase (TPO) activity			x							T
Thyrotropin releasing hormone receptor (TRHR) binding activity			x							T
Thyroxine (T4) in serum			x							T
Thyroxine (T4) in tissues			x							T
Transthyretin (TTR) binding to T4			x							T
Triiodothyronine (T3) levels in serum			x							T
Triiodothyronine (T3) levels in tissues			x							T
TSH- stimulated iodide efflux block in thyroid cells			x							T
Valve movement									x	

This allocation of endpoints in the different pathways is not exhaustive but reflective of the most frequently observed pathways.

. Information in Table 3. is organized in alphabetical order for endpoints related to “mammals”, “non-mammals” and “both”. All endpoints are marked with an “x” under at least one of the different information sources. In the “AOPs” column, the number of occurrences of the specific endpoint in the ED-relevant AOPs examined is also depicted in brackets.

A short description of the identified endpoints presented in Table 3. is included in Table 4. The description provides information on the role of each endpoint in the several endocrine pathways and the organism(s) in which it was more frequently observed in the sources of information considered (see Section 1). This does not preclude the relevance of these endpoints in other organisms.

Based on the info gathered in Table 3, a prioritisation of WP2 non-validated test methods will be performed for WP5. The methods will be ranked “high” or “low “in case they assess the endpoint identified in this work package.

A supplementary excel file named “Table 3_AO_WP3” is summarising all the adverse outcomes for the missing regulatory endpoints.

Table 3. List of identified endpoints gaps from all the different sources.

Mammals/Non-mammals	Identified endpoint gaps	ED IA	ECHA	National competent authority and EFSA	AOPs	US-EPA EDSP	OECD EDTA-AG
Non-mammals	11-ketotestosterone levels				x (1)		
Both	17alpha-hydroxyprogesterone (17α-OHP) synthesis	x					
Both	17beta-estradiol plasma levels	x		x	x (8)		
Both	17beta-estradiol synthesis in ovarian granulosa cells				x (6)		
Both	5a-reductase activity			x	x (4)		
Both	7α-hydroxypregnenolone synthesis in the brain				x (2)		
Both	Activin signalling pathway				x (1)		
Both	Adrenocorticotropic hormone (ACTH) levels in tissues			x			
Both	Androgen receptor (AR) expression	x					
Both	Androgen receptor (AR)- induced gene expression				x (5)		
Both	Androstenedione synthesis	x					
Non-mammals	Annetocin levels			x			
Both	Arginine vasotocin (AVT) levels in tissues			x			
Both	Aryl hydrocarbon receptor (AhR) activation	x		x			
Both	Aryl hydrocarbon receptor (AhR) expression	x					
Both	Aryl hydrocarbon receptor (AHR/ARNT) dimerization			x			
Non-mammals	Calcitonin levels in plasma or in relevant tissues			x			
Non-mammals	Cilia movement				x (3)		
Both	Cognitive Function				x (5)		
Both	Constitutive androstane receptor (CAR) activation and CAR-related genes (Alas1, Slco1b2, and NADPH-Cyp-reductase, Cyp2b10)			x			
Both	Corticotropin-releasing hormone (CRH) (or CRH-like) levels in tissues			x			
Non-mammals	Cripto-1 expression levels				x (1)		
Both	Cyclooxygenase activity (Cox1, Cox2)			x	x (6)		

Mammals/Non-mammals	Identified endpoint gaps	ED IA	ECHA	National competent authority and EFSA	AOPs	US-EPA EDSP	OECD EDTA-AG
Both	Cyp26 activity			x			x (OECD DRP on retinoid signalling pathway EDTA April 2020)
Both	Deiodinase 1 (DIO1) activity				x (3)	x	
Both	Deiodinase 2 (DIO2) activity				x (3)	x	
Both	Deiodinase 3 (DIO3) activity					x	
Both	Dihydrotestosterone (DHT) circulating levels				x (4)		
Both	DNA hypomethylation				x (3)		
Both	DNA methylation, global				x (4)		
Both	DNA methylation, heritable				x (2)		
Both	DNA methylation-induced gene deregulation				x (3)		
Both	DNA methyltransferase activity				x (8)		
Non-mammals	Ecdysis triggering hormone (ETH) levels				x (1)		x (OECD GD 150)
Non-mammals	Ecdysone receptor (EcR) activation				x (1)		x (OECD GD 150)
Non-mammals	Ecdysteroid levels			x	x (1)		x (OECD GD 150)
Non-mammals	Eggshell formation / calcification (mechanistic studies)			x			
Both	Estrogen receptor (ER) expression	x					
Non-mammals	Fecundity				x (11)		
Both	Fertility (female)				x (3)		x
Both	Fertility (male)				x (9)		x
Non-mammals	Fin development		x				
Non-mammals	Follicle-stimulating hormone-like (FSH-like) levels in tissues			x			

Mammals/Non-mammals	Identified endpoint gaps	ED IA	ECHA	National competent authority and EFSA	AOPs	US-EPA EDSP	OECD EDTA-AG
Non-mammals	Fushi tarazu factor-1 gene expression				x (1)		
Both	Gene/protein expression related to the transfer of thyroid hormone (TH) in embryonic circulation.			x			
Both	Glucocorticoid receptor (GR) activation	x		x		x	x
Both	Gonadotropin circulating levels				x (1)		
Both	Gonadotropin levels in hypothalamus			x	x (2)		
Both	Gonadotropin releasing hormone receptor (GnRHR) promoter region hypermethylation				x (1)		
Both	Gonadotropin-releasing hormone receptor (GnRHR) activation		x				
Both	Growth hormone (GH) levels in tissues			x			
Both	Growth hormone receptor (GHR) activation	x		x			
Both	Insulin-like growth factors (IGF1, IGF2) levels			x			
Non-mammals	Interrenal (adrenal) gland histopathology			x			
Non-mammals	Juvenile hormone (JH) levels		x				x (OECD GD 150)
Non-mammals	Juvenile hormone (JH) receptor activation		x		x (1)		x (OECD GD 150)
Both	Learning and memory				x (3)		
Non-mammals	Juvenile hormone (JH) levels		x				x (OECD GD 150)
Non-mammals	Juvenile hormone (JH) receptor activation		x		x (1)		x (OECD GD 150)
Both	Learning and memory				x (3)		
Both	Luteinizing hormone (LH) receptor activation			x	x (1)		
Non-mammals	Luteinizing hormone-like (LH-like) hormone levels in tissues			x	x		
Both	Melanocyte-stimulating hormone (MSH) levels		x	x			
Both	Mineralocorticoid receptor (MR) activation						x
Non-mammals	Molting / ecdysis incomplete			x	x (1)		

Mammals/Non-mammals	Identified endpoint gaps	ED IA	ECHA	National competent authority and EFSA	AOPs	US-EPA EDSP	OECD EDTA-AG
Both	Na+/I- symporter (NIS) activity		x		x (4)	x	
Non-mammals	Nuclear receptor E75b gene expression				x (1)		
Both	Number and function of adult Leydig cells, levels of COUP-TFII in stem Leydig cells				x (3)		
Mammals	Obesity						X (OECD DRP 178)
Both	Oogenesis				x (6)		x
Both	Ovarian steroidogenesis	x		x			
Both	Parathyroid hormone (PTH) levels in plasma or in relevant tissues			x			
Both	Peroxisome proliferator-activated receptor α (PPARα) activation	x		x	x (3)		
Both	Peroxisome proliferator-activated receptor γ (PPARγ) activation	x		x			
Both	Pigmentation (retinal and body)		x				
Both	Pregnane X receptor (PXR) activation	x		x			
Both	Progesterone levels in plasma				X (1)		
Both	Progesterone Receptor (PR) activation	x	x				x
Both	Progesterone synthesis			x	x (1)		
Both	Prolactin (PRL) levels in tissues			x			
Both	Prostaglandin D2 (PGD2) synthesis	x		x			x
Non-mammals	Prostaglandin E2 (PGE2) levels in hypothalamus			x			
Non-mammals	Prostaglandin F2alpha levels in plasma				x (3)		
Non-mammals	Prostaglandin F2alpha synthesis in ovary			x	x (2)		
Non-mammals	Prostaglandin levels in ovary			x	x		
Non-mammals	Reproductive pheromones			x			
Both	Retinaldehyde dehydrogenase (RALDH) activity				x (1)		x (OECD DRP on retinoid signalling

Mammals/Non-mammals	Identified endpoint gaps	ED IA	ECHA	National competent authority and EFSA	AOPs	US-EPA EDSP	OECD EDTA-AG
							pathway EDTA April 2020)
Both	Retinoid acid receptor (RAR), Retinoid X receptor (RXR), RAR/RXR transactivation	x					x
Mammals	Spontaneous acrosome reaction (SAR), progesterone-induced acrosome reaction (PIAR), capacitation	x					
Both	Steroid hormone levels in tissues			x			
Both	Steroidogenesis- related gene and protein expression (Star, 17b- HSD3, Cyp11a1, 3b-HSD, Cyp17a1, Cyp19a1)	x					
Mammals	Stra8 induction						x (OECD DRP on retinoid signalling pathway EDTA April 2020)
Non-mammals	Swim bladder inflation (anterior / posterior)				x (3)		
Both	Testosterone serum levels	x		x	x (4)		
Both	Testosterone synthesis in fetal Leydig cells, COUP-TFII levels in fetal Leydig cells				x (4)		
Both	Testosterone synthesis in Leydig cells				x (4)		
Mammals	Thyroid hormone clearance	x	x	x			
Both	Thyroid hormone receptor (THR) activation	x	x	x		x	
Both	Thyroid hormone receptors (THRs) expression levels			x			
Both	Thyroid hormone synthesis			x	x (12)		
Both	Thyroid-stimulating hormone receptor (TSHR) activation			x		x	
Both	Thyroidal iodide uptake in thyroid follicular cells				x (4)		
Both	Thyroperoxidase (TPO) activity	x	x	x	x (5)	x	

Mammals/Non-mammals	Identified endpoint gaps	ED IA	ECHA	National competent authority and EFSA	AOPs	US-EPA EDSP	OECD EDTA-AG
Both	Thyrotropin releasing hormone receptor (TRHR) binding activity	x		x		x	
Both	Thyroxine (T4) levels in serum			x	x (15)		
Non-mammals	Thyroxine (T4) levels in tissues				x (6)		
Both	Transthyretin (TTR) binding to T4	x		x			
Both	Triiodothyronine (T3) levels in serum				x (6)		
Both	Triiodothyronine (T3) levels in tissues				x (8)		
Both	TSH- stimulated iodide efflux block in thyroid cells	x					
Non-mammals	Valve movement				x (4)		

Table 4. Short description of identified endpoints gaps

Endpoint	Short description of endpoint
11-ketotestosterone levels	A decrease in cholesterol causes a decrease in reproductive hormones, notably 11-ketotestosterone. Endpoint observed in fish.
17alpha-hydroxyprogesterone (17α-OHP) synthesis	17α-OHP is derived from progesterone via 17α-hydroxylase (encoded by Cyp17a1). It is a chemical intermediate in the biosynthesis of androgens, estrogens, glucocorticoids, mineralocorticoids and neurosteroids, primarily produced in the adrenal glands and to some degree in in the gonads, specifically the corpus luteum. Endpoint observed in mammals and amphibians.
17beta-estradiol plasma levels	Estradiol synthesized by the gonads is transported to other tissues via blood circulation. Endpoint observed in fish.
17beta-estradiol synthesis in ovarian granulosa cells	Estradiol synthesis in ovary is mediated by a number of enzyme- catalyzed reactions. Aromatase enzyme, mainly responsible for estradiol synthesis, is primarily localized in the granulosa cells within the ovary. Endpoint observed in mammals and fish.
5a-reductase activity	5α-reductase catalyzes the conversion of testosterone to 5α-dihydrotestosterone (DHT). Endpoint observed in mammals and fish.
7α-hydroxypregnenolone synthesis in the brain	7α-hydroxypregnenolone is an active neurosteroid synthesized in the brain from pregnenolone via a reaction catalyzed by CYP7B (R08943). Pregnenolone can also be synthesized in most vertebrate brain by CYP11A from cholesterol. Endpoint observed in mammals, birds, fish and amphibians.
Activin signalling pathway	In mammals, activin stimulates FSH secretion. It participates in androgen synthesis enhancing LH action in the ovary and testis. In the male, activin enhances spermatogenesis. In fish, the activin signalling pathway is involved with the promotion of blastemal cell proliferation in the fin regeneration process. Endpoint observed in mammals and fish.
Adrenocorticotropic hormone (ACTH) levels in tissues	In mammals, adrenocorticotropin hormone (ACTH) is released from the pituitary gland and regulates levels of cortisol, released from the adrenal gland. In birds and amphibians, ACTH stimulates interrenal gland cells to produce steroids and thus regulates sodium balance, water balance, and metabolism. Endpoint observed in mammals, birds and amphibians.
Androgen receptor (AR) expression	Gene/ protein expression of AR. Endpoint observed in mammals and fish.
Androgen receptor (AR)-induced gene expression	Expression levels of specific genes, induced by AR activation.
Androstenedione synthesis	Androstenedione is defined as an obligatory intermediate in sex steroid biosynthesis being the precursor of steroid hormones such as testosterone and estradiol. Endpoint observed in mammals.
Annetocin levels	Annetocin is expressed in annelid worms within the neurons of the central nervous system. It has been shown that annetocin is involved in the induction of egg-laying behavior. Endpoint observed in invertebrates (worms).
Arginine vasotocin (AVT) levels in tissues	Arginine vasotocin (AVT) released by pituitary in all non-mammalian vertebrates and fetal mammals. Vasotocin is a hybrid of oxytocin and vasopressin, and it appears to have the biologic properties of both oxytocin (which stimulates contraction of muscles of the reproductive tract, thus playing a role in egg-laying or birth) and vasopressin (with either diuretic or antidiuretic properties). Endpoint observed in amphibians and birds.
Aryl hydrocarbon receptor (AhR) activation	Aryl hydrocarbon receptor (AhR) is implicated in the HPA, HPT axis, the retinoid and Vitamin D signalling pathways. Endpoint observed in mammals and fish.
Aryl hydrocarbon receptor (AhR) expression	Aryl hydrocarbon receptor (AhR) is implicated in the HPA, HPT axis, the retinoid and Vitamin D signalling pathways. Endpoint observed in mammals and fish.

Endpoint	Short description of endpoint
Aryl hydrocarbon receptor (AHR/ARNT) dimerization	One of the key steps of the aryl hydrocarbon signalling pathway. Endpoint observed in mammals and fish.
Calcitonin levels (plasma or relevant tissues)	Calcitonin secretion is regulated primarily by rising plasma Ca^{+2} levels, which lead to increased secretion from the C cells. Calbindin and osteopontin (OPN) are known to be involved in calcium metabolism of the eggshell gland. Calcium for eggshell formation is derived in part directly from the gut and in part from the mobilization of medullary bone. Deposition of medullary bone is under estrogenic control. Endpoint observed in birds.
Cilia movement	Cilia in the gills and pedal of mollusks are under serotonergic control. Endpoint observed in invertebrates (mollusks).
Cognitive Function	Cognitive abilities are brain-based skills needed to carry out various tasks. Endpoint observed in mammals.
Constitutive androstane receptor (CAR) activation and CAR-related genes (Alas1, Slco1b2, and NADPH-Cyp-reductase, Cyp2b10)	Constitutive androstane receptor (CAR) is implicated in HPT axis and retinoid signalling pathway. Endpoint observed in mammals.
Corticotropin-releasing hormone (CRH) (or CRH-like) levels in tissues	Corticotropin-releasing hormone (CHR) is secreted by the hypothalamus and in non-mammal vertebrates regulates both the adrenal and thyroid axes, especially in development. In mammals it is mainly expressed in placenta and brain. Endpoint observed in mammals, amphibians, birds.
Cripto-1 expression levels	Cripto-1 is responsible for growth factor activity, as well as activin binding on the cell membrane. It is involved in the fin regeneration process. Endpoint observed in fish.
Cyclooxygenase activity (Cox1, Cox2)	Cyclooxygenase site (COX) of prostaglandin- endoperoxide synthase (PTGS) catalyzes conversion of arachidonic acid into endoperoxide prostaglandin G2 (PGG2). The inhibition of COX can lead to reduced efficiency of converting arachidonic acid to PGG2. Inhibition of COX can decrease the rate of prostaglandin production. Endpoint observed in mammals, birds and fish.
Cyp26 activity	The CYP26 enzymes metabolize retinoid acid (RA) and they are inducible by RA in selected systems. Endpoint observed in mammals.
Deiodinase 1 (DIO1) activity	Deiodinases are integral membrane proteins of the thioredoxin superfamily. Type I deiodinase is capable of both outer ring deiodination and inner ring deiodination, including the conversion of T4 into T3, as well as the conversion of rT3 to the inactive thyroid hormone 3,3' T2. Endpoint observed in mammals, fish, amphibians and birds.
Deiodinase 2 (DIO2) activity	All deiodinases are integral membrane proteins of the thioredoxin superfamily. Type II deiodinase (DIO2) is only capable of outer ring deiodination activity with T4 as a preferred substrate (i.e., activation of T4 to T3). Endpoint observed in mammals, fish, amphibians and birds.
Deiodinase 3 (DIO3) activity	Deiodinase 3 (DIO3) is a selenoenzyme that inactivates TH by catalyzing its conversion to biologically inactive metabolites. DIO3 mRNA expression and DIO3 activity are regulated by a number of hormones and growth factors as well as by epigenetic mechanisms. Endpoint observed in mammals, fish, amphibians and birds.
Dihydrotestosterone circulating (DHT) levels	Reduction in DHT synthesis leads to a reduction in DHT circulating levels. DHT is inactivated in the liver and excreted in the urine. DHT is not measured in OECD TG 456 assay. Endpoint observed in mammals.
DNA hypomethylation	DNA hypomethylation refers to the loss of the methyl group in the 5-methylcytosine nucleotide. It can also refer to the unmethylated state of CpG sites in a sequence (e.g LINE-1 and Alu genes in neuroendocrine tumors) or as a more general phenomenon affecting the bulk of the genome. Endpoint observed in mammals and invertebrates (arthropods).
DNA methylation, global	DNA methylation influences gene expression by regulating the activity of DNA segments without changing the sequence (epigenetic control) (e.g *in vitro*

Endpoint	Short description of endpoint
	epigenetic experiments with N2A cells). Global DNA methylation refers to the methylation of the genome total. Endpoint observed in mammals and invertebrates (arthropods).
DNA methylation, heritable	DNA methylation influences gene expression by regulating the activity of DNA segments without changing the sequence (epigenetic control). DNA methylation can be heritable (e.g. transgenerational epigenetic deregulation of microRNAs in germ cells) Endpoint observed in mammals and invertebrates (arthropods).
DNA methylation-induced gene deregulation	DNA methylation influences gene expression by regulating the activity of DNA segments without changing the sequence (epigenetic control) (e.g. transgenerational epigenetic deregulation of microRNAs in germ cells). ED-related effects on the epigenome relate to fertility, neurodevelopment and metabolism. Examples of endocrine genes regulated by DNA methylation include Cyp11a1, Cyp17a1, Hsd3b1/2, AR, ESR, PGR, GR, MR, RAR, FSHR, TSHR (Miriam N. Jacobs et al. 2017) Endpoint observed in mammals.
DNA methyltransferase activity	DNA methyltransferases catalyze the addition of the methyl group in the 5-methylcytosine nucleotide. EDCs can modulate the expression of such enzymes (e.g O^6- MGMT in *Kryptolebias sp*.) Endpoint observed in mammals, invertebrates (arthropods) and fish.
Ecdysis triggering hormone (ETH) levels	Molting is controlled by complex multi-hormone systems, with 20-hydroxyecdysone (20E) being the key effective hormone to mediate different biological processes that are necessary for molting. The hormonal actions of 20E are exerted through binding and modulation of the ecdysone receptors (EcRs), nuclear transcriptional factors that regulate a wide range of physiological and behavioral changes. Endpoint observed in invertebrates (arthropods).
Ecdysone receptor (EcR) activation	The hormonal actions of 20E are exerted through binding and modulation of the ecdysone receptors (EcRs), which are nuclear transcriptional factors that regulate a wide range of physiological and behavioral changes. Endpoint observed in invertebrates (arthropods).
Ecdysteroid levels	Ecdysteroids (e.g. ecdysone, ecdysterone, turkesterone and 2-deoxyecdysone) are arthropod steroid hormones that are mainly responsible for molting, development and reproduction. Endpoint observed in invertebrates (arthropods).
Eggshell formation / calcification (mechanistic studies)	The shell gland (tubular and pouch portions) is the place where the calcification of the eggs occurs. Calcification within the shell gland is associated with stimuli initiated by ovulation or by neuroendocrine factors that control and coordinate both, ovulation and calcium secretion. Endpoint observed in birds.
Estrogen receptor (ER) expression	Estrogen receptor (ER) as hormone receptors for sex steroids (steroid hormone receptors) are important for sexual maturation and gestation. Endpoint observed in mammals and fish.
Fecundity	The reproductive capacity of a single person or a population. Endpoint observed in invertebrates (mollusks) and fish.
Fertility (female)	Fertility is the capacity to conceive or induce conception. Impairment of fertility represents disorders of male or female reproductive functions or capacity. Endpoint observed in mammals.
Fertility (male)	Fertility is the capacity to conceive or induce conception. Impairment of fertility represents disorders of male or female reproductive functions or capacity. Endpoint observed in mammals.
Fin development/regeneration	Fins serve different purposes such as moving forward, turning, keeping an upright position or stopping. Developmental abnormalities affect these skills and thus the survivability of the fish. Endpoint observed in fish.
Follicle-stimulating hormone-like (FSH-like) levels in tissues	Follicle-stimulating hormone-like (FSH-like) hormones released by pituitary and promote development of eggs and sperm. Endpoint observed in amphibians, birds and invertebrates.
Fushi tarazu factor-1 gene expression	Molting is controlled by complex multi-hormone systems. Fushi tarazu factor-1 is a transcriptional factor expressed a few hours prior to ecdysis.

Endpoint	Short description of endpoint
	Endpoint observed in invertebrates (arthropods).
Gene/protein expression related to the transfer of thyroid hormone (TH) in embryonic circulation.	Both transthyretin (TTR) and albumin (ALB) are expressed in embryonic tissues and may contribute to TH transfer to the embryonic circulation. Yolk sac membrane also expresses certain TH transporters. Expression profiles are gene-specific and dynamic throughout embryonic development. Endpoint observed in mammals and birds.
Glucocorticoid receptor (GR) activation	The glucocorticoid receptor (GR) is implicated in the HPA axis and mediates the physiologic action of endogenous corticosteroids and the pharmacologic action of therapeutic corticosteroids on gene transcriptional regulation. Endpoint observed in mammals.
Gonadotropin circulating levels	Gonadotropin (luteinizing hormone- LH and follicle-stimulating hormone- FSH) secretion from the pituitary is a key regulator of gonadal steroid biosynthesis. Gonadotropin secretion by pituitary gonadotropes is regulated via gonadotropin releasing hormone (GnRH) signalling from the hypothalamus as well as by intrapituitary regulators of gonadotropin expression (e.g., activin, follistatin, inhibin). Endpoint observed in fish.
Gonadotropin levels in hypothalamus	Gonadotropin (luteinizing hormone- LH and follicle-stimulating hormone- FSH) secretion from the pituitary is a key regulator of gonadal steroid biosynthesis. Gonadotropin secretion by pituitary gonadotropes is regulated via gonadotropin releasing hormone (GnRH) signalling from the hypothalamus as well as by intrapituitary regulators of gonadotropin expression (e.g., activin, follistatin, inhibin). Endpoint observed in fish.
Gonadotropin releasing hormone receptor (GnRHR) promoter region hypermethylation	Embryonic activation of the AhR (Aryl hydrocarbon Receptor) leads to reproductive failure, via epigenetic down-regulation of GnRHR. Endpoint observed in mammals and fish.
Gonadotropin-releasing hormone receptor (GnRHR) activation	Gonadotropin (luteinizing hormone- LH and follicle-stimulating hormone- FSH) secretion from the pituitary is a key regulator of gonadal steroid biosynthesis. Gonadotropin secretion by pituitary gonadotropes is regulated via gonadotropin releasing hormone (GnRH) signalling from the hypothalamus as well as by intrapituitary regulators of gonadotropin expression (e.g. activin, follistatin, inhibin). Endpoint observed in mammals and fish.
Growth hormone (GH) levels in tissues	Growth hormone (GH) (released by pituitary) influences protein metabolism, growth and reproduction. Endpoint observed in amphibians, birds and fish.
Growth hormone receptor (GHR) activation	Growth hormone receptors (GHRs) are located on the cell surfaces of many tissues (liver, muscle, adipose, and kidney, and in early embryonic and fetal tissue) and are implicated in the somatotropic axis. Endpoint observed in mammals, fish, amphibians and birds.
Insulin-like growth factor (IGF1, IGF2) levels	Reproduction is regulated by the integration of endocrine signals with exogenous factors. Gonadal IGF1 and IGF2 are targets of endocrine-disrupting compounds (particularly estrogens). Endpoint observed in fish.
Interrenal (adrenal) gland histopathology	The interrenal (adrenal) gland in non- mammals consists of interrenal and chromaffin cells. In amphibians the interrenal and chromaffin cells are distributed diffusely along the surface of the kidneys. Endpoint observed in amphibians.
Juvenile hormone (JH) levels	Juvenile hormone regulates many aspects of arthropod physiology and development, mainly metamorphosis and molting. In adults it plays a key role in the control of reproduction. Endpoint observed in invertebrates (arthropods).
Juvenile hormone (JH) receptor activation	Juvenile hormone regulates many aspects of arthropod physiology and development, mainly metamorphosis and molting. In adults it plays a key role in the control of reproduction. Endpoint observed in invertebrates (arthropods).
Learning and memory	Learning can be defined as the process by which new information is acquired to establish knowledge by systematic study or by trial and error. The memory

Endpoint	Short description of endpoint
	formation requires acquisition, retention and retrieval of information in the brain, which is characterized by the non-conscious recall of information.
Luteinizing hormone (LH) receptor activation	Luteinizing hormone receptor antagonism can lead to reproductive dysfunction. Endpoint observed in mammals and fish.
Luteinizing hormone-like (LH-like) hormone levels in tissues	Luteinizing hormone- like (LH-like) hormones are released by pituitary. LH-like hormones cause ovulation and sperm release and stimulate secretion of steroid hormones (androgens, estrogens and in some cases progesterone) from gonads. Endpoint observed in amphibians, invertebrates and fish.
Melanocyte-stimulating hormone (MSH) levels	MSH circulates in blood and binds to melanocortin receptors of melanocytes (mammals) and chromatophores (lower vertebrates), regulating melanin pigment concentrations and distribution. Endpoint observed in fish, amphibians and reptiles.
Mineralocorticoid receptor (MR) activation	Mineralocorticoid receptors- MRs (HPA axis) bind both mineralocorticoids and glucocorticoids with high affinity and are found in both epithelial and nonepithelial tissues. Activation of the mineralocorticoid receptor, upon the binding of its ligand aldosterone, results in its translocation to the cell nucleus and binding to response elements in gene promoter regions. Endpoint observed in mammals.
Molting/ ecdysis incomplete	Molting (or ecdysis) is a natural biological process in arthropods. During a molt cycle, the animals generate new exoskeletons by the epidermis and shed the old ones in order to grow. Successful molting is key to survival, development and reproduction. Endpoint observed in invertebrates (arthropods).
Na+/I- symporter (NIS) activity	Decreased iodine intake can decrease TH production. It is induced by TSH. Endpoint observed in mammals.
Nuclear receptor E75b gene expression	Molting (or ecdysis) is a natural biological process in arthropods. The hormonal actions of 20E are exerted through binding and modulation of the ecdysone receptors (EcR) like E75b, which regulate a wide range of physiological and behavioral changes. Endpoint observed in invertebrates (arthropods).
Number and function of adult Leydig cells, levels of COUP-TFII in stem Leydig cells	Leydig cells are located in the seminiferous tubules of the testis and secrete testosterone. COUP-TFII plays a critical developmental role. The glucocorticoid receptor (GR) stimulates COUP-TFII-induced transactivation while COUP-TFII represses the GR transcriptional activity. Endpoint observed in mammals.
Obesity	The association of weight gain (e.g. assessed with adipocyte differentiation) with other disorders, such as type 2 diabetes and metabolic syndrome (which includes hyperlipidemia and cardiovascular disease), has provided added support for a mechanistic linkage between exposure to EDs and these conditions. Endpoint observed in mammals.
Oogenesis	The formation, development, and maturation of an ovum (e.g. Stra8 induction). Endpoint observed in mammals.
Ovarian steroidogenesis	Two types of somatic cells, follicular granulosa cells and surrounding theca cells, are responsible for ovarian steroidogenesis. Theca cells autonomously synthesize progesterone and androgen, whereas immature granulosa cells only convert theca cell-produced androgens into estrogens. Endpoint observed in mammals and fish.
Parathyroid hormone (PTH) levels in plasma or in relevant tissues	The parathyroid gland secretes parathyroid hormone (PTH). The primary targets of PTH in birds are bone and the kidneys, as in mammals. Plasma Ca^{+2} levels regulate PTH secretion. Endpoint observed in birds.
Peroxisome proliferator-activated receptor alpha (PPARα) activation	PPARs are activated by fatty acids and their derivatives; they are sensors of dietary lipids and are involved in lipid and carbohydrate metabolism, immune response and peroxisome proliferation. PPRα is also a target of hypothalamic hormone signalling and plays a role in embryonic development. Endpoint observed in mammals.
Peroxisome proliferator-activated receptor gamma (PPARγ) activation	PPARs are activated by fatty acids and their derivatives; they are sensors of dietary lipids and are involved in lipid and carbohydrate metabolism, immune response and peroxisome proliferation. Endpoint observed in mammals.

Endpoint	Short description of endpoint
Pigmentation (retinal and body)	The pigmentation of many animals is adapted to their environment and aids in their survival. However, pigments not only provide external coloration but also function in some important physiological processes (e.g eye pigment cells, synthesis of vitamins) Endpoint observed in fish, amphibians and reptiles.
Pregnane X Receptor (PXR) activation	Pregnane X Receptor (PXR) binding, (trans)activation. PXR is a transcription factor that controls the expression of genes involved in transcellular transport and xenobiotic metabolism, lipid metabolism, glucose homeostasis, and inflammation. Endpoint observed in mammals.
Progesterone levels in plasma	Progesterone is a steroid hormone involved in reproduction in many species. Endpoint observed in mammals and fish.
Progesterone receptor (PR) activation	Progesterone Receptor (PR) binding, (trans)activation. After progesterone binds to the receptor, restructuring occurs and the complex enters the nucleus and binds to DNA. Ligands for this receptor include endogenous and synthetic progestogens, selective progesterone receptor modulators and antiprogestogens. Endpoint observed in mammals and fish.
Progesterone synthesis	Luteinizing hormone receptor (LHR) inhibition leads to a reduction of the synthesis of progesterone, which is also indispensable for ovulation and fertility in fish. Endpoint observed in mammals and fish.
Prolactin (PRL) levels in tissues	Prolactin (PRL) (released by pituitary) is involved in the differentiation and function of many sex accessory structures and secondary sexual characteristics and in the reproduction. In addition, PRL is involved in other physiological functions in amphibians e.g. metamorphosis, regulation of water and salt balance, water-seeking behavior. Endpoint observed in amphibians and birds.
Prostaglandin D2 (PGD2) synthesis	Prostaglandin D synthases known as the lipocalin prostaglandin D synthase (L-PGDS) and the hematopoietic prostaglandin D-synthase (H-PGDS) are responsible for the formation of PGD2 from Prostaglandin H2. Endpoint observed in mammals.
Prostaglandin E2 (PGE2) levels in hypothalamus	Prostaglandins are well known to be central regulators of vertebrate ovulation. PGE2 is an important metabolic hormone in fish liver. Endpoint observed in fish.
Prostaglandin F2alpha levels in plasma	Cyclooxygenase 1 (COX1), officially known as prostaglandin-endoperoxide synthase (PTGS), is an enzyme that is responsible for formation of prostanoids, including thromboxane and prostaglandins such as prostacyclin, from arachidonic acid. Inhibition of COX can decrease the rate of prostaglandin production. Endpoint observed in birds and fish.
Prostaglandin F2alpha synthesis in ovary	Cyclooxygenase inhibition leads to reproductive dysfunction via inhibition of female spawning behaviour by decreasing the rate of prostaglandin production. Endpoint observed in fish.
Prostaglandin levels in ovary	Cyclooxygenase inhibition leads to reproductive dysfunction by decreasing the rate of prostaglandin production. Endpoint observed in fish.
Reproductive pheromones	Pheromones induce developmental and endocrinological changes. They are present in endocrine bioassays that affect reproduction. Endpoint observed in fish and invertebrates (arthropods).
Retinaldehyde dehydrogenase (RALDH) activity	In the postnatal male, RALDH inhibition and the subsequent decrease in RA levels would interfere with spermatogenesis (OECD draft retinoid DRP, 2020) The role of RALDH in retinol metabolism is to catalyse the chemical reaction converting retinal to retinoic acid (RA). RALDH inhibition leads to decreased optical elements of the eye leading to population decline. Endpoint observed in mammals and fish.
Retinoid acid receptor (RAR), Retinoid X receptor (RXR), RAR/RXR transactivation	The retinoic acid receptors are transcriptional activators and contain two autonomous transactivation functions, AF-1 and AF-2. Endpoint observed in mammals.

Endpoint	Short description of endpoint
Spontaneous acrosome reaction (SAR), progesterone-induced acrosome reaction (PIAR), capacitation	Spermatozoa can initiate the acrosomal reaction well in advance of reaching the zona pellucida, as well as *in vitro* in an appropriate culture medium (spontaneous acrosome reaction- SAR). Mammalian sperm must undergo a series of biochemical and physiological modifications, collectively called capacitation, in the female reproductive tract prior to the acrosome reaction (AR). Endpoint observed in mammals.
Steroid hormone levels in tissues	Progesterone is essential for normal gestation in many fish, amphibians, and reptiles, while androgens and estrogens influence male and female characteristics and behavior. Endpoint observed in fish, amphibians and reptiles.
Steroidogenesis- related gene and protein expression (Star, 17b- HSD3, Cyp11a1, 3b-HSD, Cyp17a1, Cyp19a1)	Gene expression of steroidogenesis-related enzymes. Endpoint observed in mammals.
Stra8 induction	Stra8 is regulated by retinoic acid (RA) and is expressed only in germ cells (Mark et al. 2008; Oulad-Abdelghani et al. 1996). *Stra8* is required for germ cells to enter meiosis (Baltus et al. 2006). Therefore, an *in vitro* screening assay for Stra8 expression could be used to identify substances that, through disturbed RA signalling, affect meiosis initiation (OECD draft Retinoid DRP, 2020) Endpoint observed in mammals.
Swim bladder inflation (anterior/posterior)	Inflation of the anterior/posterior swim bladder chamber is part of the larval-to-juvenile transition in fish. Endpoint observed in fish.
Testosterone serum levels	Testosterone is synthesized by the gonads and other steroidogenic tissues (e.g., brain, adipose), acts locally and/or is transported to other tissues via blood circulation. Leydig cells are the testosterone-producing cells of the testis. Endpoint observed in fish.
Testosterone synthesis in fetal Leydig cells, COUP-TFII levels in fetal Leydig cells	COUP transcription factor 2 transactivation. Leydig cells are located in the seminiferous tubules of the testis and secrete testosterone. COUP-TFII plays a critical developmental role. The glucocorticoid receptor (GR) stimulates COUP-TFII-induced transactivation while COUP-TFII represses the GR transcriptional activity. Endpoint observed in mammals.
Testosterone synthesis in Leydig cells	In humans and other mammals, testosterone is secreted primarily by the testicles of males and, to a lesser extent, the ovaries of females and other steroidogenic tissues (e.g., brain, adipose). Testosterone synthesis takes place within the mitochondria of Leydig cells, the testosterone-producing cells of the testis upon stimulation by Luteinizing hormone (LH). Endpoint observed in mammals.
Thyroid hormone clearance	Rate of clearance of thyroid hormones in liver (comparative physiology). Comparative assay between rodent and human primary hepatocytes.
Thyroid hormone receptor (THR) activation	The thyroid hormone receptors (THRs) are nuclear receptors that exhibit a dual role as activators or repressors of gene transcription in response to thyroid hormone (T3). Endpoint observed in mammals.
Thyroid hormone receptors (THRs) expression levels	The majority of Thyroid Hormone (TH) signalling occurs via binding of T3 to nuclear thyroid hormone receptors (THRs), so their presence is essential for TH action. Endpoint observed in mammals and birds
Thyroid hormone synthesis	The thyroid hormones (TH), triiodothyronine (T3) and thyroxine (T4) are thyrosine based hormones. Synthesis of TH is regulated by thyroid-stimulating hormone (TSH) binding to its receptor and thyroidal availability of iodine via the sodium iodide symporter (NIS). Other proteins contributing to TH production in the thyroid gland, including thyroperoxidase (TPO), dual oxidase enzymes (DUOX), and pendrin are also necessary for iodothyronine production. Decreased T4 and T3 serum concentrations activate the hypothalamus-pituitary-thyroid (HPT) axis which upregulates thyroid-stimulating hormone (TSH) that acts to increase production of additional THs.TRH and the TSH primarily regulate the production of T4 and T3. Endpoint observed in mammals, amphibians, invertebrates and fish.

Endpoint	Short description of endpoint
Thyroid- stimulating hormone receptor (TSHR) activation	The thyroid-stimulating hormone (TSH) receptor (TSHR) is a class A G protein-coupled receptor (GPCR). TSHR activation induces adenylyl cyclase activity via the Gsa-protein. At higher TSH concentrations an activation of the phospholipase C cascade by Gq/G11 has also been shown. Endpoint observed in mammals.
Thyroidal iodide uptake in thyroid follicular cells	A sodium-iodide (Na/I) symporter pumps iodide (IO) actively into the cell. This iodide enters the follicular lumen from the cytoplasm by the transporter pendrin, in a purportedly passive manner. In the colloid, iodide (I−) is oxidized to iodine (I0) by an enzyme called thyroid peroxidase (TPO). IO is very reactive and iodinates the thyroglobulin at tyrosyl residues in its protein chain. In conjugation, adjacent tyrosyl residues are paired together. Thyroglobulin binds the megalin receptor for endocytosis back into the follicular cell. Proteolysis by various proteases liberates thyroxine (T4) and triiodothyronine molecules (T3), which enter the bloodstream where they are bound to thyroid hormone binding proteins, mainly thyroxin binding globulin (TBG). Endpoint observed in mammals and amphibians.
Thyroperoxidase (TPO) activity	The outcome of TPO inhibition is decreased synthesis of thyroxine (T4) and triiodothyronine (T3), a decrease in release of these hormones from the gland into circulation, and unless compensated, a consequent decrease in systemic concentrations of T4, and possibly T3. Endpoint observed in mammals.
Thyrotropin releasing hormone receptor (TRHR) binding activity	TRHR is a G protein-coupled receptor which binds thyrotropin-releasing hormone. TRHR is found in the anterior pituitary. TRHR binds TRH and thus activates phospholipase C, which causes the formation of inositol triphosphate (IP3) and diacylglycerol (DAG). This leads to an increase in cytoplasmic calcium ion concentrations which induces the release of thyroid-stimulating hormone (TSH) into the blood. Endpoint observed in mammals.
Thyroxine (T4) in serum	There are major species differences in the predominant binding proteins and their affinities for THs. However, there is broad agreement that changes in serum concentrations of THs is diagnostic of thyroid disease or chemical-induced disruption of thyroid homeostasis. Endpoint observed in fish and amphibians.
Thyroxine (T4) in tissues	Thyroxine (T4) uptake from serum into tissues plays a substantial role in thyroid hormone action, as it can then be available for enzymatic conversion to the active hormone, triiodothyronine (T3). Uptake of T4 into cells/tissues is mediated by active transport proteins that exhibit unique expression profiles depending on tissue type and timing of development. Decreases in tissue T4 could potentially occur in several ways individually or in combination, (1) circulating levels of T4 decrease to critical levels that even compensatory increases in active transport cannot overcome, (2) TH-specific transporters are non-functional either due to mutation, inhibited by a xenobiotic or their transcriptional expression is repressed, (3) enhanced T4 catabolism by type III deiodinase in peripheral tissues or by phase II metabolic enzymes in the liver. Endpoint observed in fish and amphibians.
Transthyretin (TTR) binding to T4	Serum binding proteins are responsible for the maintenance of a large extrathyroidal pool of thyroid hormone of which only the minute fraction of free hormone (<0.5%) is immediately available to tissues. TTR is the major T4 binding protein in birds, amphibians, fish, and rodents, xenobiotics that interfere with TTR binding of T4 may have greater negative effects in wildlife species than in humans (OECD 207, 2014). The function of the serum protein TTR is to deliver T4 to target cells in the liver, tight junctions, etc. where it is facilitated across the membrane via specific receptors and converted to the active form T3, where it can activate nuclear receptors. TTR facilitates passage across key tight junctions, such as the blood-brain barrier, the CSF barrier and transplacentally, and the interruption of thyroid serum protein-assisted transport during certain developmental windows can lead to profound developmental neurotoxicity (i.e. cretinism). Endpoint observed in mammals, birds, amphibians and fish.
Triiodothyronine (T3) levels in serum	There are major species differences in the predominant binding proteins and their affinities for THs. However, there is broad agreement that changes in

Endpoint	Short description of endpoint
	serum concentrations of THs is diagnostic of thyroid disease or chemical-induced disruption of thyroid homeostasis. Endpoint observed in fish and amphibians.
Triiodothyronine (T3) levels in tissues	T3 can only be derived from T4, which can occur in any tissue that expresses either type I or II iodothyronine deiodinases (DIO1, DIO2), whereas T4 can only be synthesized in the thyroid gland. The local concentration of T3 in any given cell or tissue will be a function of, (1) local T4 availability, which is a function of plasma T4 concentration and active transport capacity across cell membranes, (2) local DIO1 and/or DIO2 activity, and (3) circulating levels of T3, as a result of remote activation of T4 by either DIO1 or DIO2 and release of T3 to the plasma. Endpoint observed in fish and amphibians.
TSH- stimulated iodide efflux block in thyroid cells	Iodide efflux into the thyroid by the Na+/I- symporter (NIS), is the first and rate-limiting step in thyroid hormone synthesis. Thyrotropin (TSH) increases NIS mRNA and protein levels, as well as iodide uptake activity. Blocking the efflux reduces/eliminates TH synthesis. Endpoint observed in mammals.
Valve movement	In bivalves, the muscle involved in valve movement is the anterior byssus retractor muscle (ABRM). This muscle and other muscles can undergo a catch state of contraction, which is characterized by a very slowly decaying force in the absence of stimulation. Endpoint observed in invertebrates (mollusks).

2.2. Endocrine relevant pathways for identified endpoints

The identified ED-related endpoints could be involved in one or more EATS and/or non-EATS pathways as depicted in Table 5. From the excel file embedded in the APPENDIX, it is possible to also retrieve the endpoints involved in each pathway, noting that this allocation of endpoints in the different pathways is not exhaustive but reflective of the most frequently observed pathways.

Table 5. Endocrine relevant pathways for identified endpoint

Endpoint	HPA	HPG	HPT	Somatotropic	Retinoid	Vitamin D	PPAR	Epigenetics	Other non-EATS pathway	EATS
11-ketotestosterone levels		x								S
17alpha-hydroxyprogesterone (17α-OHP) synthesis	x	x								S
17beta-estradiol plasma levels		x								S
17beta-estradiol synthesis in ovarian granulosa cells		x								S
5a-reductase activity		x								S
7α-hydroxypregnenolone synthesis in the brain										S
Activin signalling pathway		x								
Adrenocorticotropic hormone (ACTH) levels in tissues	x									
Androgen receptor (AR) expression										A

Endpoint	HPA	HPG	HPT	Somatotropic	Retinoid	Vitamin D	PPAR	Epigenetics	Other non-EATS pathway	EATS
Androgen receptor (AR)-induced gene expression										A
Androstenedione synthesis		x								S
Annetocin levels									x	
Arginine vasotocin (AVT) levels in tissues	x									
Aryl hydrocarbon receptor (AhR) activation	x		x		x	x				
Aryl hydrocarbon receptor (AhR) expression	x		x		x	x				
Aryl hydrocarbon receptor (AHR/ARNT) dimerization					x					
Calcitonin levels (plasma or relevant tissues)						x				
Cilia movement									x	
Cognitive Function										S, T
Constitutive androstane receptor (CAR) activation and CAR-related genes (Alas1, Slco1b2, and NADPH-Cyp-reductase, Cyp2b10)			x		x				x	
Corticotropin-releasing hormone (CRH) (or CRH-like) levels in tissues	x									
Cripto-1 expression levels		x							x	
Cyclooxygenase activity (Cox1, Cox2)									x	
Cyp26 activity					x					
Deiodinase 1 (DIO1) activity			x							T
Deiodinase 2 (DIO2) activity			x							T
Deiodinase 3 (DIO3) activity			x							T
Dihydrotestosterone circulating (DHT) levels		x								S
DNA hypomethylation								x		
DNA methylation, global								x		
DNA methylation, heritable								x		
DNA methylation-induced gene deregulation								x		
DNA methyltransferase activity								x		
Ecdysis triggering hormone (ETH) levels					x				x	
Ecdysone receptor (EcR) activation					x				x	
Ecdysteroids levels					x				x	
Eggshell formation/ calcification (mechanistic studies)						x			x	
Estrogen receptor (ER) expression										E

Endpoint	HPA	HPG	HPT	Somatotropic	Retinoid	Vitamin D	PPAR	Epigenetics	Other non-EATS pathway	EATS
Fecundity		x							x	E, A, S, T
Fertility (female)		x			x	x		x		E, S
Fertility (male)	x	x			x	x				A, S
Fin development/ regeneration	x	x			x					
Follicle-stimulating hormone-like (FSH-like) levels in tissues		x								
Fushi tarazu factor-1 gene expression									x	
Gene/protein expression related to the transfer of thyroid hormone (TH) in embryonic circulation.			X							T
Glucocorticoid receptor (GR) receptor activation	x									
Gonadotropin circulating levels		x								
Gonadotropin levels in hypothalamus		x								
Gonadotropin releasing hormone receptor (GnRHR) promoter region hypermethylation		x						x		
Gonadotropin-releasing hormone receptor (GnRHR) activation		x								
Growth hormone (GH) levels in tissues				x						
Growth hormone receptor (GHR) activation				x						
Insulin-like growth factor (IGF1, IGF2) levels				x						
Interrenal (adrenal) gland histopathology	x									
Juvenile hormone (JH) levels									x	
Juvenile hormone (JH) receptor activation									x	
Learning and memory	x								x	T, S
Luteinizing hormone (LH) receptor activation		x								
Luteinizing hormone-like (LH-like) hormone levels in tissues		x								
Melanocyte-stimulating hormone (MSH) levels									x	
Mineralocorticoid receptor (MR) activation	x									
Molting/ ecdysis incomplete					x				x	
Na+/I- symporter (NIS) activity			x							T
Nuclear receptor E75b gene expression									x	
Number and function of adult Leydig cells, levels of COUP-TFII in stem Leydig cells		x								S
Obesity					x		x			

Endpoint	HPA	HPG	HPT	Somatotropic	Retinoid	Vitamin D	PPAR	Epigenetics	Other non-EATS pathway	EATS
Oogenesis					x			x	x	
Ovarian steroidogenesis		x			x					S
Parathyroid hormone (PTH) levels in plasma or in relevant tissues						x				
Peroxisome proliferator-activated receptor alpha (PPARα) activation			x		x		x			S
Peroxisome proliferator-activated receptor gamma (PPARγ) activation			x		x		x			
Pigmentation (retinal and body)									x	T
Pregnane X Receptor (PXR) activation					x				x	
Progesterone levels in plasma	x	x								
Progesterone Receptor (PR) activation	x	x								
Progesterone synthesis	x	x								S
Prolactin (PRL) levels in tissues		x	x							
Prostaglandin D2 (PGD2) synthesis									x	
Prostaglandin E2 (PGE2) levels in hypothalamus									x	
Prostaglandin F2alpha levels in plasma									x	
Prostaglandin F2alpha synthesis in ovary									x	E
Prostaglandin levels in ovary									x	E
Reproductive pheromones		x								
Retinaldehyde dehydrogenase (RALDH) activity					x					
Retinoid acid receptor (RAR), Retinoid X receptor (RXR), RAR/RXR transactivation					x					
Spontaneous acrosome reaction (SAR), progesterone-induced acrosome reaction (PIAR), capacitation		x								E, A
Steroid hormone levels in tissues										S
Steroidogenesis- related gene and protein expression (Star, 17b-										S

Endpoint	HPA	HPG	HPT	Somatotropic	Retinoid	Vitamin D	PPAR	Epigenetics	Other non-EATS pathway	EATS
HSD3, Cyp11a1, 3b-HSD, Cyp17a1, Cyp19a1)										
Stra8 induction					x					
Swim bladder inflation (anterior/posterior)			x							T
Testosterone serum levels		x								S
Testosterone synthesis in fetal Leydig cells, COUP-TFII levels in fetal Leydig cells		x								S
Testosterone synthesis in Leydig cells		x								S
Thyroid hormone clearance			x							T
Thyroid hormone receptor (THR) activation			x							T
Thyroid hormone receptors (THRs) expression levels			x							T
Thyroid hormone synthesis			x							T
Thyroid- stimulating hormone receptor (TSHR) activation			x							T
Thyroidal iodide uptake in thyroid follicular cells			x							T
Thyroperoxidase (TPO) activity			x							T
Thyrotropin releasing hormone receptor (TRHR) binding activity			x							T
Thyroxine (T4) in serum			x							T
Thyroxine (T4) in tissues			x							T
Transthyretin (TTR) binding to T4			x							T
Triiodothyronine (T3) levels in serum			x							T
Triiodothyronine (T3) levels in tissues			x							T
TSH- stimulated iodide efflux block in thyroid cells			x							T
Valve movement									x	

This allocation of endpoints in the different pathways is not exhaustive but reflective of the most frequently observed pathways.

3. References

Baltus, A. E., D. B. Menke, Y. C. Hu, M. L. Goodheart, A. E. Carpenter, D. G. de Rooij, and D. C. Page. 2006. 'In germ cells of mouse embryonic ovaries, the decision to enter meiosis precedes premeiotic DNA replication', *Nat Genet*, 38: 1430-4.

Hornung, M. W., J. J. Korte, J. H. Olker, J. S. Denny, C. Knutsen, P. C. Hartig, M. C. Cardon, and S. J. Degitz. 2018. 'Screening the ToxCast Phase 1 Chemical Library for Inhibition of Deiodinase Type 1 Activity', *Toxicol Sci*, 162: 570-81.

Mark, M., H. Jacobs, M. Oulad-Abdelghani, C. Dennefeld, B. Feret, N. Vernet, C. A. Codreanu, P. Chambon, and N. B. Ghyselinck. 2008. 'STRA8-deficient spermatocytes initiate, but fail to complete, meiosis and undergo premature chromosome condensation', *J Cell Sci*, 121: 3233-42.

Miriam N. Jacobs, Emma L. Marczylo, Carlos Guerrero-Bosagna, and Joelle Ruegg. 2017. 'Marked for Life: Epigenetic Effects of Endocrine Disrupting Chemicals', *Annual Review of Environment and Resources*: 105-60.

Olker, J. H., J. J. Korte, J. S. Denny, P. C. Hartig, M. C. Cardon, C. N. Knutsen, P. M. Kent, J. P. Christensen, S. J. Degitz, and M. W. Hornung. 2019. 'Screening the ToxCast Phase 1, Phase 2, and e1k Chemical Libraries for Inhibitors of Iodothyronine Deiodinases', *Toxicol Sci*, 168: 430-42.

Oulad-Abdelghani, M., P. Bouillet, D. Decimo, A. Gansmuller, S. Heyberger, P. Dolle, S. Bronner, Y. Lutz, and P. Chambon. 1996. 'Characterization of a premeiotic germ cell-specific cytoplasmic protein encoded by Stra8, a novel retinoic acid-responsive gene', *J Cell Biol*, 135: 469-77.

Paul Friedman, K., E. D. Watt, M. W. Hornung, J. M. Hedge, R. S. Judson, K. M. Crofton, K. A. Houck, and S. O. Simmons. 2016. 'Tiered High-Throughput Screening Approach to Identify Thyroperoxidase Inhibitors Within the ToxCast Phase I and II Chemical Libraries', *Toxicol Sci*, 151: 160-80.

Wang, J., D. R. Hallinger, A. S. Murr, A. R. Buckalew, S. O. Simmons, S. C. Laws, and T. E. Stoker. 2018. 'High-Throughput Screening and Quantitative Chemical Ranking for Sodium-Iodide Symporter Inhibitors in ToxCast Phase I Chemical Library', *Environ Sci Technol*, 52: 5417-26.

APPENDIX

I. Identified ED-relevant AOPs and KEs involved

ED-relevant AOPs and KEs.xlsx

II. Endpoints assessed in validated assays.

Table A-1: Parameters in OECD CF level 2 '*in vitro* mechanistic', for which guidance is provided in OECD GD 150

Test guideline	**OECD TG**	**455**	**493**		**458**		**456**
	US EPA OPPTS		**890.1250**	**890.1150**		**890.1200**	
Species/ *in vitro* test system		ER TA (human) cells	Binding to rat (EPA) or human	Binding to rat androgen	AR TA (human AR-	Human recombinant microsomes	Human H295R cells
Indicative of: [(a)]		E	E	A	A	S	S
Androgen receptor binding/ transactivation				X	X		
Aromatase						X	
Estrogen receptor binding/ transactivation		X	X				
Steroidogenesis (oestradiol and/or testosterone synthesis)							X

Table A-2: Validated assays for birds and assessed endpoints

Organism	**Birds**	
Available validated assays	**Relevant to….**	**Endpoints**
OECD TG 206 – Avian Reproduction test (Apr.1984) Level 4	-	Mortality and signs of toxicity Body w eight (adults and young) Food consumption (adults and young) Gross pathological examination <u>Reproduction parameters:</u> Egg production – number of eggs laid per hen Percentage of cracked eggs Viability (per cent viable embryos of eggs set) Hatchability (per cent hatching of eggs set) Percentage of hatchlings that survive to 14 days Number of 14-day old survivors per hen Eggshell thickness (mm)

Avian Two-generation toxicity test in the Japanese quail (ATGT) US EPA TG OCSPP 890.2100/740-C-15-003 (July 2015) Level 5 (insufficiently validated according to OECD standards)	EAT	Mortality and clinical signs of toxicity Food consumption Body weight Observations of secondary sex characteristics and timing of sexual maturity or absence Egg endpoints: - Total egg production per hen (per day) - Number of eggs cracked / broken (% of eggs laid) - Number of eggs set (per pair) - Number of abnormal eggs (plus description of abnormalities) - Eggshell strength (per egg, Newtons) - Eggshell thickness (mean nm per egg) - Fertile eggs (ED8) (% of eggs set) - Viable embryos (ED15) (% of eggs set) - Hatchability (% Number of eggs that hatch) - Number of 14-day old survivors per hen Chick endpoints: - Day of hatch - Abnormal hatchling morphology/clinical signs of toxicity or disease - Hatching mortality - Chick body weight at hatching - Number of 14-day-old surviving chicks - Chick body weight at 14 days after hatching - Phenotypic sex (F1, F2) - Genetic sex (F1, F2) - Time of sexual maturity (F1, F2) Necropsy and gross examination Measurements of hormone levels (in blood, egg yolk or thyroid gland) - Glandular T4 in thyroid gland (embryos) - Thyroxine (T4) in plasma or/and thyroid - Triiodothyronine (T3) in plasma or/and thyroid - Steroid hormones measurements in plasma and/or egg yolks Hormone levels in thyroid tissue Histopathological examination (and tissue weight): - Kidneys - Liver - Adrenal glands - Thyroid - Reproductive organs and associated structures (Cloacal gland and bursa) - Pancreas - Spleen

Table A-3: Validated assays for amphibians and assessed endpoints

Organism	**Amphibian**	
Available validated assays	**Indicative of**	**Endpoints**
OECD 231_AMA test (Sep.2009) Level 3	T	Developmental stage Hind Limp Length Snout-Vent Length (SVL) / growth Body Weight Thyroid Gland Histopathology Malformations Behaviour Mortality
OECD 248_XETA test (June 2019) Level 3	T	The assay measures the ability of a chemical to activate or inhibit transcription of the genetic construct, whether directly through binding to the thyroid hormone receptor (TR) or modifying the binding of TH to the TR, or indirectly by modifying the amount of TH available to activate the

		TR and thereby transcription of the TH/bZIP-GFP construct (fluorescent measurements)
OECD 241_LAGDA test (July 2015) Level 4	E, A, T	Mortality Behaviour Liver-somatic index (LSI) (juvenile sample) Kidney histopathology (juvenile sample) Liver histopathology (juvenile sample) Body weight Thyroid histopathology (larval sample) Gonad and gonad duct histopathology (juvenile sample) Snout-Vent Length (SVL) / growth Genotypic/phenotypic sex ratios (juvenile sample) Plasma vitellogenin (VTG) (optional) (juvenile sample) Time to metamorphosis Malformations / abnormal development

Table A-4: Validated assays for fish and assessed endpoints

Organism	**Fish**	
Available validated assays	**Relevant to….**	**Endpoints**
OECD 229_FSTRA test Level 3	E, A, S	VTG (males, females) in plasma Spiggin secondary sex characteristics gonad histopathology behaviour morphological abnormalities reproduction survival
OECD 230_fish assay Level 3	E, A, S	VTG (males, females) in plasma secondary sex characteristics sex ratio (male, female) behaviour morphological abnormalities survival
OECD 234_FSDT test Level 4	E, A, S	VTG (males, females) in plasma or liver or head/tail homogenate secondary sex characteristics gonad histopathology sex ratio (male, female) behaviour length morphological abnormalities survival (larval and embryos) hatching success body weight
OECD 240_MEOGRT test Level 5	E, A, S	VTG (males, females) in liver sex ratio (male, female) behaviour length reproduction survival time to maturity hatching success body weight

Table A-5: Validated assays for arthropods (aquatic and terrestrial) assessed endpoints

Organism	**Arthropods**	
Available validated assays	**Relevant to**	**Endpoints**
OECD TG 218-219 Chironomid toxicity test (Nov 2004) Level 4	-	Chironomid emergence (sex and number) and development rate is measured observation of any abnormal behaviour the number of visible pupae that have failed to emerge and any egg masses deposition.
OECD TG 233 Sediment water chironomid life cycle toxicity test (July 2010) Level 5	-	Chironomid emergence, time to emergence, and sex ratio of the fully emerged and alive midges are assessed. The number of egg ropes produced and their fertility are assessed. From these egg ropes, first instar larvae of the 2nd generation are obtained. These larvae are placed into freshly prepared test beakers (spiking procedure as for the 1st generation) to determine the viability of the 2nd generation through an assessment of their emergence, time to emergence and the sex ratio of the fully emerged and alive midges.
OECD TG 232 Collembolan reproduction test in soil (July 2016) Level 4	-	Mortality of adults and reproduction are assessed
OECD TG 226 Predatory mite reproduction test in soil (July 2016) Level 4	-	Numbers of juveniles of *Hypoaspis aculeifer* (i.e. larvae, protonymphs and deutonymphs) and adults are counted separately
OECD TG 211 Daphnia magna Reproduction Test (Oct 2008) Level 4	-	Assess the effect of chemicals on the reproductive output of *Daphnia magna* total number of living offspring produced per parent animal alive at the end of the test is assessed
OECD GD 201 Harpacticoid Copepod Development and Reproduction Test with *Amphiascus* (Jul. 2014) Level 4	-	Larval/juvenile development rates, stage-specific mortality, and percent mating success of the F0 may all be analysed statistically for significant differences relative to controls. Reproductive endpoints may be analysed as single end-of-test measurements or relative to different time intervals during the exposure. The mean number of F1 offspring produced from fertilised females (F0) in each replicate is calculated. Generally, the reproduction data is not adjusted for mortality but are presented on a *per female or per mating-pair* basis. The mean number of offspring produced by each control and treatment matingpair is calculated through two broods or up to 36 days – whichever arrives first. If a female dies after producing young, the total number of offspring produced up to the time of death may be used. However, those mating pairs that die during the exposure (*i.e.*, before giving birth to any young) are not included in the fertility or fecundity endpoint calculations and subsequent statistical analyses The test is expected (by extension from experience with other copepods) to be responsive to juvenile hormone (JH) (ant)agonists and ecdysteroid (Ec) (ant)agonists which can interfere with such processes as metamorphosis, moulting, growth and reproduction.
Short-Term Juvenile Hormone Activity Screening Assay Using Daphnia magna (draft OECD TG) Level 3	Other non-EATS pathway	The SJHASA exposes 17-day-old (i.e. adult) female *D. magna* to dilutions of the test chemical for 5-7 days. Their first brood after exposure is discarded, but all individuals of the second brood are sexed by observation of their longer first antenna. Juvenile hormone (JH) and other JH agonists cause the production of males due to exposure during a short critical period (52-53 hours after ovulation). An adverse outcome

		pathway for this process is under development – significant male production in a population could potentially lead to its decline. However, due to the very short-term nature of SJHASA, the endpoint of male production should not be considered as an adverse apical endpoint without further investigation in longer term tests
Daphnia multigeneration test for assessment of endocrine disrupting chemicals (draft OECD TG) Level 5	-	Modality detected/endpoints: This long-term *in vivo* assay with *Daphnia magna* is responsive to juvenile hormone (JH) agonists which lead to the production of male offspring. It exposes the test organisms over two generations. The lack of internationally validated mechanistic assays for endocrine activity in crustaceans may prevent firm conclusions about whether test chemicals are endocrine disruptors (EDs) in this taxon, although *in vitro* assays for JH and ecdysteroid (Ec) activity are available in the literature.

Table A-6: Validated assays for annelida and assessed endpoints

Organism	**Annelida**	
Available validated assays	**Relevant to….**	**Endpoints**
OECD TG 222 Earthworm Reproduction Test (Eisenia fetida/Eisenia andrei) (July 16) Level 4		Mortality / Reproduction living adult worms are observed and counted and weighed number of juveniles hatched from the cocoons in the test soil and cocoon numbers are determined
OECD TG 220 Enchytraeid reproduction test (July 2016) Level 4		At the end the total number of juveniles produced by parent animal and the survival of parent animals are assessed The study report should include the adult morphological changes, the number of offspring The duration of the definitive test is six weeks.
OECD TG 225 Sediment-Water Lumbriculus Toxicity Test Using Spiked Sediment (Oct 2007) Level 4		Total number of living and dead individuals per replicate should be recorded and assessed Additionally, the living worms can be assigned to one of three groups: a) large complete worms (adults) without regenerated body regions b) complete worms with regenerated, lighter-coloured body regions (i.e., with new posterior part, with new anterior part, or with both new posterior and anterior parts) c) incomplete worms (i.e., recently fragmented worms with non-regenerated body regions) Worm dry weight is determined Asexual reproduction results in two fragments, which do not feed for a certain period until the head or tail part is regenerated

Table A-7: Validated assays for mollusks and assessed endpoints

Organism	**Mollusks**	
Available validated assays	**Relevant to….**	**Endpoints**
OECD TG 242 *Potamopyrgus antipodarum* reproduction test (July 2016) Level 4	-	P. antipodarum (mudsnail) survival over the 28 days exposure period and reproduction at the end of the test after 28 days are examined. Reproduction is evaluated by counting the number of embryos in the brood pouch (without distinction of developmental stages) at the end of 28 days exposure.
OECD TG 243 *Lymnaea stagnalis* reproduction test (July 2016) Level 4	-	Reproducing adults of *L. stagnalis* are exposed to a concentration range of the test chemical and monitored for 28 days for their survival and reproduction (number of egg clutches). As additional information, the number of eggs per clutch may also be determined. Adult shell length increase may also be measured.