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HABILITAČNÍ PRÁCE

Vybrané přírodní a antropogenní faktory způsobující endokrinní disrupci u ryb

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Prohlášení

Prohlašuji, že jsem předkládanou habilitační práci s názvem „Vybrané přírodní a antropogenní faktory způsobující endokrinní disrupci u ryb“ vypracovala samostatně s použitím dostupných literárních zdrojů a výsledků vlastní vědecké práce. Výsledků vlastní vědecké práce uvedených v habilitační práci jsem dosáhla ve spolupráci s kolegy na FROV, či na pracovištích, kde jsem působila od doby získání doktorského titulu do sepsání této práce (období 2008 – 2019).

Ve Vodňanech, dne 31.1.2019 Ing. Hana Kocour Kroupová, Ph.D.

Obsah

1. Úvod

Vodní ekosystémy jsou silně ohroženy chemickými látkami, které kontaminují povrchové i podzemní vody. Zdrojem chemických látek je jednak "vědomé" a v podstatě kontinuální znečištění, které pochází z čistíren průmyslových ale i komunálních odpadních vod, roli hrají ale i náhodné úniky chemických látek (havárie, splachy z okolí, atmosférické depozice atd.). Může se zdát paradoxní, že právě vody vypouštěné z čistíren odpadních vod (ČOV) jsou zdrojem řady chemických látek a v mnoha případech patří k nejzávažnějším znečišťovatelům povrchové vody v dané lokalitě. Musíme si ale uvědomit, že každodenně používáme cca 100 000 různých chemických látek, které se následně dostávají do surové odpadní vody (Sumpter, 2005). Některé z těchto látek nebo jejich přímých degradačních produktů nejsou, a ani nemohou být technologickými postupy běžně užívanými na ČOV kompletně odstraněny a nepřetržitě tak vstupují s vyčištěnými odpadními vodami do vod povrchových. Tlak na vodní prostředí se pak zvyšuje s rostoucí populací a většími nároky lidí na spotřebu vody. To vede v mnoha hustě osídlených zemích k tomu, že i více než 50 % toku některých řek tvoří vyčištěné odpadní vody. V období sucha (s malou frekvencí srážek a tím i nízkého průtoku) tento podíl může vzrůst až na 90 či více procent (Sumpter, 2005). Zvýšené koncentrace cizorodých látek pak mohou ve vodním prostředí negativně ovlivňovat fyziologické funkce vodních organismů a v případě dlouhodobého znečištění mohou vést i k změně druhového osídlení dané lokality.

Na druhou stranu fyziologii vodních organismů ovlivňují nejen antropogenní ale i přírodní faktory (Kloas et al., 2009 – **Příloha 1**). Příkladem jsou některé přírodní látky přirozeně se vyskytující ve vodním prostředí (Lutz et al., 2005; Jarosova et al., 2015) či změny fyzikálně-chemických parametrů kvality vody jako je pH (Ikuta a Kitamura, 1995; Zelennikov et al., 1999), teplota (Pankhurst et al., 1996; Watts et al., 2004) nebo koncentrace rozpuštěného kyslíku (Thomas et al., 2007; Wu et al., 2003). Dalším faktorem, který bývá v ekotoxikologických studiích obvykle ignorován, jsou parazitární infekce. Jak bylo prokázáno, přítomnost některých parazitů modifikuje fyziologické procesy svých hostitelů, což může vést například ke změnám citlivosti infikovaných jedinců vůči znečištění životního prostředí (Sures, 2008) nebo ke změnám hladin biomarkerů, které se analyzují v rámci biomonitoringu vodního prostředí. Znečištění může navíc ovlivnit strukturu a početnost parazitárních komunit (Blanar et al., 2009). Proto i tyto faktory je potřeba brát v

ekotoxikologických studiích v úvahu a věnovat velkou pozornost interpretaci výsledků, které byly v rámci monitorovací studie získány.

1.1. Endokrinní disrupce vyvolaná antropogenními faktory

Během několika posledních desetiletí byly shromážděny důkazy o tom, že některé sloučeniny vypouštěné do životního prostředí mohou negativně ovlivňovat endokrinní systém volně žijících živočichů i lidí. Tyto látky začaly být souhrnně označovány jako endokrinní disruptory (ED) (Kloas et al., 2009 – **Příloha 1**).

K objevu tohoto fenoménu ve vodním prostředí přispělo v sedmdesátých letech minulého století v podstatě náhodné zjištění neobvykle častého výskytu intersexních jedinců u populací ryb žijících v řece pod zaústěním dvou ČOV ve Velké Británii (Sumpter a Johnson, 2008). Následný podrobný monitoring prováděný v této oblasti nejen potvrdil přítomnost intersexních ryb, ale bylo dále prokázáno, že výskyt intersexuality se zvyšuje s věkem. Tedy, čím starší byly ryby, tím vyšší byl stupeň abnormalit jejich reprodukčního systému. Několik let pak ještě trvalo, než se prokázalo, že příčinou těchto reprodukčních abnormalit je expozice látkám s estrogenním účinkem, které byly přítomny ve vyčištěných odpadních vodách z ČOV (Purdom et al., 1994; Sumpter a Johnson, 2008). S postupem let pak byly zaznamenávány další případy endokrinní disrupce u vodních živočichů (Howell et al., 1980; Bryan et al., 1986; Colborn a Clement, 1992; Hinck et al., 2009) a získávány další důkazy o negativním působení různých endokrinních disruptorů (Jobling et al., 2006; Kloas et al., 2009 – **Příloha 1**).

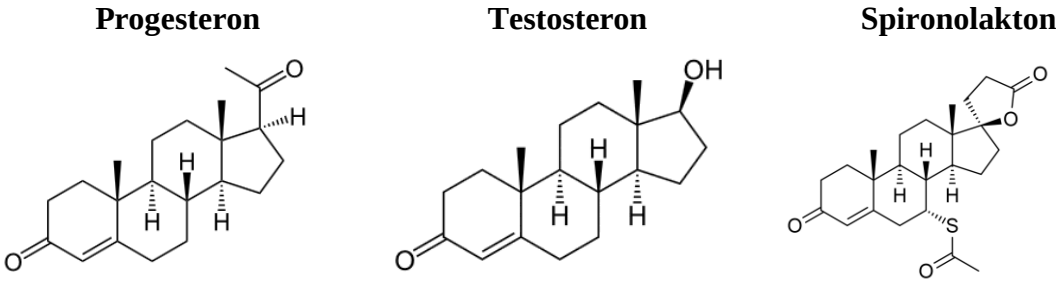
Mezi látky, které dosud vyvolaly největší rozruch, patří 17α-ethinylestradiol (EE2), který je součástí většiny perorálních antikoncepčních přípravků. U této látky bylo mimo jiné prokázáno, že hrála významnou roli při indukci intersexu u volně žijících sladkovodních ryb v řekách ve Velké Británii (Jobling et al., 2006) a dokonce způsobila kolaps populace divokých ryb v sedmiletém experimentu, který byl prováděn v jednom jezeře v Kanadě (Kidd et al., 2007).

Mnohem méně informací však je dosud známo o výskytu a ekotoxikologických účincích jiných syntetických steroidů, progestinů, i když jejich spotřeba je vyšší než u výše zmíněného EE2 (Besse a Garric, 2009).

1.1.1 Progestiny

Progestiny jsou širokou skupinou přírodních a syntetických látek, které mají jednu společnou vlastnost, vážou se na progesteronový receptor (PR). Mezi přírodní progestiny patří progesteron (P4), který hraje významnou úlohu v endokrinním systému většiny obratlovců včetně lidí. Je to důležitý hormon nezbytný pro regulaci menstruačního cyklu, udržení těhotenství a úspěšné dokončení embryogeneze u lidí (Graham a Clarke, 1997). U ryb progesteron tak významný není, zde se uplatňují více jiné progestiny, a to 17,20-β-dihydroxypregn-4-en-3-on či 17,20β,21-trihydroxypregn-4-en-3-on (Scott, 2010). Tyto progestiny mají důležitou roli při zahájení meiózy v průběhu spermatogeneze a oogeneze a podílejí se na konečném zrání oocytů a spermií (Scott, 2010).

Vedle přírodních progestinů existují ještě syntetické progestiny, které byly vyvinuty farmaceutickým průmyslem s cílem napodobovat přírodní hormon progesteron. Jedná se o syntetické deriváty progesteronu, testosteronu a spironolaktonu (Obr. 1). Seznam těch nejběžněji používaných syntetických progestinů včetně jejich struktury a fyzikálně chemických vlastností je uveden v **Příloze 2** (Kumar et al., 2015).



Obr. 1. Základní struktura progesteronu, testosteronu a spironolaktonu, které jsou prekurzory syntetických progestinů.

Syntetické progestiny jsou samostatně nebo spolu s malým množstvím výše zmíněného syntetického estrogenu (EE2) účinnými látkami v hormonální antikoncepci (Besse a Garric, 2009), která je každodenní součástí života milionů žen po celém světě. Vedle hormonální antikoncepce mají syntetické progestiny řadu dalších využití. Některé z nich se užívají například při léčbě neplodnosti, hormonální substituční terapii, léčbě endometriózy, jako prevence rakoviny endometria, léčbě dysfunkčního děložního krvácení, léčbě hormonálně podmíněných nádorů a při paliativní terapii pacientů nemocných rakovinou (pro stimulaci chutě k jídlu) (Kumar et al., 2015 – **Příloha 2**). V humánní medicíně má své uplatnění i přírodní progestin progesteron, který se používá k léčbě poruch menstruačního cyklu,

rakoviny prsu, dysfunkčního děložního krvácení a při asistované reprodukci (SÚKL, 2018). Díky tomuto poměrně širokému využití je spotřeba progestinů relativně vysoká. Například celková roční spotřeba progestinů (až 19 různých molekul) se odhaduje ve Francii a ve Spojeném království na 12 800 kg respektive 1 700 kg (obojí na cca 60 milionů obyvatel) a v ČR na 2 400 kg (na 10 milionů obyvatel) (Kumar et al., 2015 – **Příloha 2**). Progestiny jsou navíc používány i v chovech hospodářských zvířat pro regulaci reprodukce (Fent, 2015) nebo jako růstové promotory (Orlando a Ellestad, 2014, Schiffer et al., 2001). V některých zemích však již byla tato praxe zakázána.

Vzhledem k tomuto poměrně širokému využití není překvapením, že se progestiny dostávají do odpadních vod. Na čistírnách odpadních vod se je však nedaří zcela odstranit a tyto látky tak vstupují do vod povrchových (Fent, 2015, Kumar et al., 2015 – **Příloha 2**, Orlando a Ellestad, 2014). Dalším zdrojem progestinů jsou splachy z výše zmíněných chovů hospodářských zvířat, případně odpadní vody z farmaceutického průmyslu.

Přestože se pozornost na progestiny zaměřuje relativně krátkou dobu, máme již v současnosti k dispozici řadu dat o jejich výskytu v odpadních i povrchových vodách. Jedná se však spíše o útržkovité informace, protože důkladnější sledování se s výjimkou České republiky (**Přílohy 3 – 5**) a Číny (Liu et al., 2011; 2014; 2015; Shen et al., 2018) zatím neprovádělo. Níže uvádím informace o pozitivních záchytech progestinů v povrchových vodách ve světě (Tab. 1), údaje pro Českou republiku jsou pak uvedeny v sekci Výsledky a diskuze a v **Přílohách 3 – 5**. Koncentrace progestinů v povrchových vodách se většinou pohybují v nanogramech na litr.

Tab. 1. Pozitivní záchyty progestinů v povrchových vodách ve světě (v tabulce jsou uvedeny průměrné koncentrace).

Progestin	**Země**	**Konc.** (ng/l)	**Citace**
progesteron	Belgie	0,9	Pauwels et al., 2008
	Brazílie	9,4	Kuster et al., 2009
		9,5	Torres et al., 2015
	Čína	1,5	Shen et al., 2018
		2,5	Liu et al., 2011; Liu et al., 2014
	Francie	1,6	Vulliet a Cren-Olivé, 2011
		2,6	Vulliet et al., 2008
	Japonsko	0,07	Chang et al., 2008
	Kanada	3,0	Viglino et al., 2008
	Maďarsko	3,1	Avar et al., 2016
	Španělsko	0,88	Kuster et al., 2008
	Švýcarsko	6,3	Macikova et al., 2014
	USA	0,72-6,5[a]	Standley et al., 2008
		9,4	Velicu a Suri, 2009
		110[b]	Kolpin et al., 2002
levonorgestrel	Čína	4,77[c]	Liu et al., 2015
		7,5	Qiao et al., 2009
		22	Liu et al., 2011Liu
	Francie	3,6	Vulliet a Cren-Olivé, 2011
		6,2	Vulliet et al., 2008
	Maďarsko	1,9	Avar et al., 2016
	Malajsie	38	Al-Odaini et al., 2010

Tab. 1. *Pokračování.*

Progestin	Země	Konc. (ng/l)	Citace
gestoden	Čína	1,6	Shen et al., 2018
	Srbsko	3,6	Neale et al., 2015
norethisteron	Čína	0,48	Shen et al., 2018
	Francie	2	Vulliet a Cren-Olivé, 2011
		2,8	Vulliet et al., 2008
	USA	48[c]	Kolpin et al., 2002
medroxyprogesteron	Švýcarsko	2,7	Macikova et al., 2014
	USA	1,0	Kolodziej et al., 2004
megestrol acetát	Čína	0,15	Shen et al., 2018
cyproterone acetát	Čína	0,36	Shen et al., 2018
	Německo	0,82	Weizel et al., 2018
melengestrol acetát	Čína	0,60	Liu et al., 2014
dydrogesteron	Čína	3,8	Liu et al., 2015
		4,4	Shen et al., 2018
		9,6	Liu et al., 2014
dienogest	Německo	0,64	Weizel et al., 2018
drospirenon	Maďarsko	1,9	Avar et al., 2016

Pozn. [a] – uveden rozsah koncentrací, protože kompletní data nebyla k dispozici, [b] – medián, [c] – norgestrel (racemická směs 2 optických izomerů: levonorgestrelu a dextronorgestrelu)

Přestože byly syntetické progestiny navrženy tak, aby interagovaly s lidským progesteronovým receptorem a napodobovaly tak působení progesteronu, mají navíc schopnost vázat se u savců na řadu dalších jaderných receptorů, jako je androgenní receptor (AR), glukokortikoidní receptor (GR) a mineralokortikoidní receptor (MR) (Kumar et al., 2015 – **Příloha 2**). Navíc metabolity některých progestinů, například levonorgestrelu a norethisteronu, mohou vykazovat estrogenní aktivitu, která u jejich prekurzorů není patrná. Tyto nechtěné interakce s dalšími steroidními hormony mohou vést k nežádoucím vedlejším účinkům.

Ve vědecké literatuře se postupně začínají objevovat informace o negativním vlivu progestinů, zejména těch syntetických, na vodní organismy. Podrobné informace nejen o této problematice jsou uvedeny v naší rešerši v **Příloze 2** (Kumar et al., 2015).

Většina publikovaných studií se zatím zaměřovala především na účinky progestinů na reprodukci ryb (Fent, 2015; Kumar et al., 2015 – **Příloha 2**; Orlando a Ellestad, 2014) a obojživelníků (Kvarnryd et al., 2011; Safholm et al., 2012). Dosud získaná data jsou poměrně alarmující, protože negativní vliv syntetických progestinů, zejména derivátů testosteronu, na plodnost ryb byl zaznamenána již při velmi nízkých koncentracích, dosahujících pouze jednotek ng/l (DeQuattro et al., 2012; Paulos et al., 2010, Runnalls et al., 2013, Zeilinger et al., 2009). Ze všech testovaných progestinů byla nejnižší hodnota LOEC[1] stanovena pro levonorgestrel (0,8 ng/l) (Zeilinger et al., 2009; Kumar et al., 2015 – **Příloha 2**). Zároveň bylo zjištěno, že syntetické progestiny ovlivňují poměr pohlaví (Hou et al., 2018a, Shi et al., 2018) a indukují intersexní fenotyp u exponovaných ryb (Fent, 2015, Hou et al., 2018a). Objevilo se rovněž několik studií popisujících negativní vliv syntetických progestinů na reprodukční chování ryb (Frankel et al., 2016a,b; Hou et al., 2018b).

Progestiny mají také potenciál negativně ovlivňovat i jiné fyziologické funkce vodních organismů (Kumar et al., 2015 – **Příloha 2**), a to například cirkadiální rytmus (Shi et al., 2018; Zhao et al., 2015a; 2015b; 2018), funkce štítné žlázy (Lorenz et al., 2011; 2018; Liang et al., 2015), imunitní systém (Pietsch et al., 2009; 2011), případně feromonální komunikaci (Besse a Garric, 2009; Frankel et al., 2016a; Scott et al., 2010). Některé z výše zmíněných výsledků však byly buď zjištěny v *in vitro* testech, nebo v krátkodobých *in vivo* testech a změny byly zaznamenány pouze na molekulární úrovni. Není tedy zcela jisté, zda by se později promítly i do fyziologie exponovaných organismů.

V oblasti ekotoxikologie progestinů je ještě poměrně mnoho „bílých míst“. Podrobnější informace o jejich účincích na vodní organismy máme pouze pro úzkou skupinu těchto látek, a to zejména pro syntetické progestiny odvozené od testosteronu. Dalším látkám z této široké skupiny byla věnována mnohem menší či dokonce žádná pozornost. Neznámé jsou rovněž jejich účinky ve směsích, které se v přírodě vyskytují s větší pravděpodobností.

[1] LOEC = nejnižší koncentrace s pozorovaným účinkem

1.2. Endokrinní disrupce vyvolaná přírodními faktory

Poruchy fungování endokrinního systému, které byly pozorovány v posledních minimálně 40ti letech u vodních živočichů, a to zejména u ryb, bývají obvykle spojovány s lidskou činností, konkrétně s vypouštěním různých syntetických i přírodních látek do vodních ekosystémů. Postupně však vychází najevo, že i některé přírodní faktory, které nemají žádnou přímou souvislost s lidskou činností, mohou být příčinnou endokrinní disrupce. Níže uvádím stručný popis některých z nich.

1.2.1. Přírodní látky

Poměrně dobře popsaná je skupina látek označovaná jako fytoestrogeny. Fytoestrogeny, jak jejich název napovídá, vykazují estrogenní účinky u exponovaných organismů (Jarošová et al., 2015). Jedná se o vícesytné fenoly, které jsou svou strukturou podobné steroidní hormonům. Jejich přirozeným zdrojem ve vodním prostředí mohou být některá makrofyta, řasy, sinice, případně rostliny rostoucí poblíž vodních toků (Jarosova et al., 2015). Estrogenní aktivita byla rovněž zaznamenána u huminových látek (Lutz et al., 2005; Chen et al., 2012), což je široká skupina přírodních organických látek vznikajících rozkladem převážně rostlinných zbytků. Huminové látky patří mezi vysokomolekulární převážně cyklické sloučeniny aromatického charakteru a obvykle tvoří více než 60 % rozpuštěného organického uhlíku v povrchových vodách (Pitter, 2015). Kromě estrogenní aktivity byla u některých z těchto látek zaznamenána i relativně silná anti-androgenní aktivita (Bittner et al., 2012). Endokrinně aktivní látky, které ovšem nebyly dosud identifikovány, jsou obsaženy i v listech některých rostlin, přičemž spadané listí tvoří až 30 % vstupů organických látek do vodního prostředí (Meyer et al., 1998). Například vodné extrakty z listů rákosu, buku a dubu vykazují anti-estrogenní účinky a extrakt dubu navíc relativně silnou anti-androgenní aktivitu (Hermelink et al., 2010).

1.2.2. Paraziti

Kromě látek přírodního původu může být endokrinní disrupce u vodních živočichů vyvolána také parazitární infekcí. Někteří paraziti totiž výrazně ovlivňují reprodukční fyziologii svých hostitelů (Baudoin, 1975; Werren et al., 2008; Lafferty a Kuris, 2009), což obvykle činí dvěma různými způsoby: buď mění u svých hostitelů poměr pohlaví, nebo způsobují pokles jejich plodnosti. Změny v poměru pohlavní jsou běžné u bezobratlých

hostitelů, zejména členovců, infikovaných vertikálně přenášenými (od rodičů až po potomky) intracelulárními parazity (Bandi et al., 2001). Protože vertikální přenos probíhá většinou prostřednictvím samičího hostitele, dochází k posunu poměru pohlaví směrem k hostitelům samičího pohlaví, a to různými mechanismy, včetně narušení endokrinního systému (Rodgers-Gray et al., 2004). Kompletní nebo částečná feminizace samců (intersex) bývá často zaznamenávána ve vodním prostředí u členovců infikovaných mikroskopickými houbami mikrosporidiemi (Rodgers-Gray et al., 2004; Terry et al., 2004). U ryb byl výskyt intersexů dáván do souvislosti s infekcí mikrosporidiemi *Pleistophora mirrandellae* u plotice obecné (*Rutilus rutilus*; Wiklund et al., 1996) a myxosporidiemi *Sphaerospora testicularis* u morčáka evropského (*Dicentrarchus labrax*; Sitja-Bobadilla, 2009). Spojitost mezi výskytem intersexu u ryb a infekcí parazity však nebyla zatím zcela spolehlivě prokázána. Wiklund et al. (1996) například pouze uvádějí, že u plotic obecných, u kterých byl pozorován intersex, byla větší pravděpodobnost, že budou zároveň infikovány mikrosporidiemi (*Pleistophora mirrandellae*). Další studie, prováděná autory Bjerregaard et al. (2006), však žádný vztah mezi výskytem intersexu a přítomností tohoto parazita neodhalila.

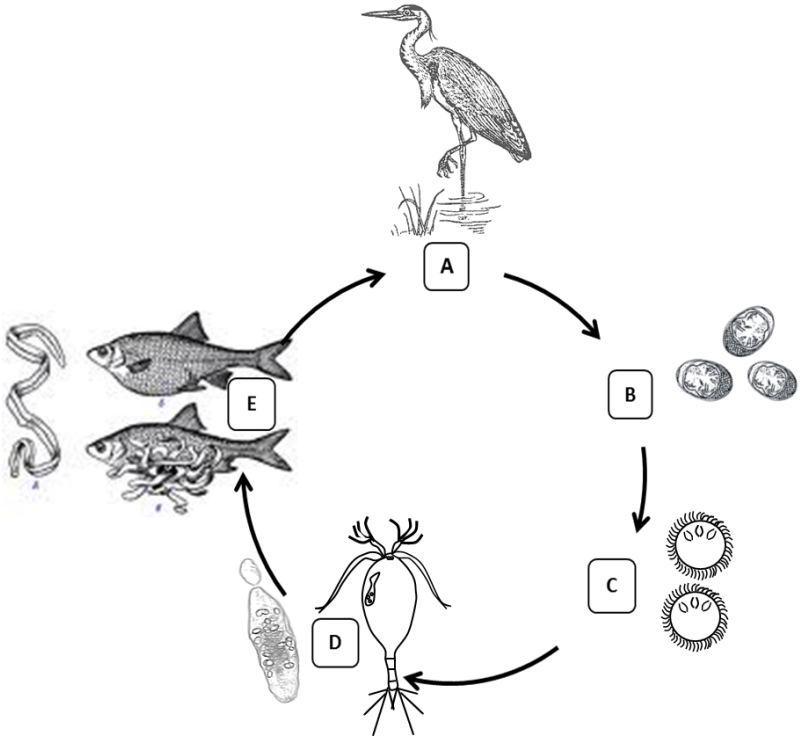
Kromě feminizace je dalším velmi častým důsledkem infekce parazity snižování plodnosti hostitele. Pokles plodnosti může být důsledkem neadaptivních vedlejších účinků infekce (zvýšené energetické nároky na hostitele) nebo i obrannou adaptací hostitele s cílem zmírnit dopady parazitismu. Může se však také jednat o adaptivní manipulaci parazita, který se tak snaží podpořit svoje další šíření. Výše uvedené příčiny se zároveň nemusí navzájem vylučovat (Hurd, 2001; 2009; Lafferty a Kuris, 2009). Paraziti způsobující první typ účinků bývají zjednodušeně označováni jako konzumenti a paraziti zodpovědní za druhý typ účinků jako kastrátoři (Hall et al., 2007). Baudoin (1975) definoval pojem parazitická kastrace jako: „poškození nebo změny v tkáni gonád, reprodukčním chování, hormonální rovnováze apod., které vedou ke snížení reprodukčního úspěchu hostitele nad rámec toho, co vyplývá z neselektivního využití hostitelských energetických rezerv parazitem“. Fenomén snižování plodnosti bývá nejčastěji zaznamenáván u měkkýšů infikovaných motolicemi nebo u korýšů infikovaných parazity z řádu stejnonožců nebo infratřídy svijonožců (Hurd, 2001, Lafferty a Kuris, 2009). U ryb způsobují podobné efekty buď 1) někteří drobní parazitičtí korýši jako například *Riggia paranensis* napadající sladkovodní ryby druhu *Cyphocharax gilbert* (Azevedo et al., 2006; Lima et al., 2007), *Ichthyoxenus japonensis* u karase stříbřitého (*Carassius auratus*) (Hua et al., 2017) a *Anilocra apogonae* u mořské tropické ryby druhu *Cheilodipterus quinquelineatus* (Fogelman et al., 2009) nebo 2) larvy (plerocerkoidy)

tasemnic jako například *Schistocephalus solidus* u koljušky tříostné (*Gasterosteus aculeatus*; Heins a Baker, 2008), *Schistocephalus pungitii* u koljušky devítiostné (*Pungitius pungitius*; Heins, 2017) a řemenatka ptačí (*Ligula intestinalis*) zejména u kaprovitých ryb (Arme, 1997; Hoole et al., 2010).

Řemenatka ptačí (*Ligula intestinalis*)

Z výše jmenovaných parazitů byla dosud asi největší pozornost věnována řemenatce ptačí. Studiem vlivu tohoto parazita na endokrinní systém plotice obecné jsem se v rámci post-doktorandského projektu financovaného nadací Alexandra von Humboldta při svém pobytu na IGB v Berlíně v Německu zabývala i já.

Tasemnice řemenatka ptačí (*Ligula intestinalis*), která je široce rozšířena po celé Evropě a Asii, má tří hostitelský životní cyklus (Obr. 2; Dubinina, 1980). Dospělé tasemnice cizopasí u rybožravých ptáků, jako jsou například volavky či racci. Vajíčka tasemnice se s trusem těchto ptáků dostávají do vody, kde se z nich uvolní koracidia. Koracidium se pak stává potravou prvního mezihostitele, kterým je buchanka. V těle buchanky se vyvine larva označovaná jako procerkoid. Druhým mezihostitelem jsou pak ryby, převážně kaprovité, v jejichž tělní dutině dozrává infekční larva (plerocerkoid). U ryb mohou larvy tasemnic přežívat i několik let než se dostanou do svého definitivního hostitele.



Obr. 2. Životní cyklus řemenatky ptačí (*Ligula intestinalis*). A – definitivní hostitel (rybožravý pták), B – vajíčka, C – koracidia, D – první mezihostitel (buchanka) a procerkoid, E – druhý mezihostitel (ryba) s plerocerkoidem.

Jak už bylo zmíněno výše, nejpozoruhodnějším důsledkem infekce ryb řemenatkou ptačí je inhibice reprodukce jejího hostitele (Arme a Owen, 1968). U obou pohlaví infikovaných ryb zůstávají gonády v nezralém stavu bez ohledu na sezónu a věk ryb (Arme a Owen, 1968; Arme, 1997).

1.3. Cíle práce

Prvním cílem této práce bylo i) získat informace o výskytu syntetických progestinů a progesteronu ve vodním prostředí, ii) zjistit, zda tyto látky přispívají k výskytu některý typů hormonálních aktivit ve vodním prostředí (zejména progestagenní a (ani-)androgenní aktivitě) a iii) popsat, jaké účinky mohou mít vybrané syntetické progestiny na endokrinní systém ryb a jejich chování v laboratorních podmínkách. Druhým cílem práce pak bylo zjistit, jaký vliv má infekce parazitem řemenatkou ptačí (modelový „přírodní endokrinní disruptor“) na endokrinní systém plotice obecné.

Výsledky této práce jsou stručně shrnuty v následujících kapitolách a podrobně popsány v publikovaných článcích, které jsou přílohou této habilitační práce.

2. Výsledky a diskuse

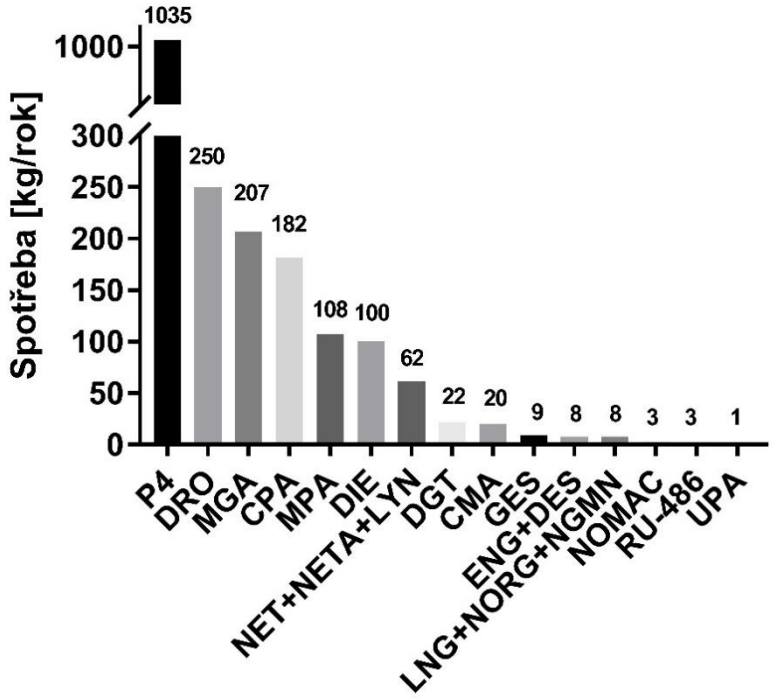
2.1. Progestiny

2.1.1. Vývoj metody pro analýzu progestinů ve vodě

Aby bylo možné zjistit, zda a v jakých koncentracích se progestiny vyskytují ve vodním prostředí České republiky, bylo nejdříve nutné vyvinout spolehlivou a hlavně citlivou analytickou metodu. Tato metoda byla vyvinuta ve spolupráci s pracovní skupinou doc. Romana Grabice z Laboratoře environmentální chemie a biochemie, FROV JU.

Prvním krokem bylo vypracování seznamu progestinů, které by mohly být přítomny ve vodách v České republice, přičemž hlavním kritériem při výběru analytů byla spotřeba těchto látek. Data o spotřebě (distribuci) léčiv s progestiny jako účinnými látkami jsou volně přístupná na webových stránkách Státního ústavu pro kontrolu léčiv (SÚKL; http://www.sukl.cz). Výsledkem analýzy těchto dat byl seznam 16ti progestinů (Obr. 3), na jejichž analýzu v odpadních a povrchových vodách jsme se následně zaměřili. Do seznamu byl dále přidán medroxyprogesteron, který sice není účinnou látkou žádného předepisovaného léku, ale jedná se o jeden z degradačních produktů jiného, často předepisovaného progestinu, jímž je medroxyprogesteron acetát. Medroxyprogesteron byl navíc několikrát detekován ve vodním prostředí různých zemí včetně České republiky (Macikova et al., 2014).

Detailní informace o vývoji a parametrech analytické metody pro stanovení progestinů ve vodních matricích jsou uvedeny v publikaci Golovko et al. (2018) (**Příloha 3**). Pro zakoncentrování vzorků vody byla použita automatická extrakce na pevné fázi (SPE). Při analýzách byla použita kapalinová chromatografie s kombinovanou ionizací (chemickou ionizací a fotoionizací za atmosférického tlaku) s hmotnostně spektrometrickou detekcí (hybridní kvadrupólový analyzátor/orbitální past) provozovanou v režimu produktového skenu ve vysokém rozlišení (LC-APCI/APPI-HRPS).



Obr. 3. Spotřeba progesteronu a syntetických progestinů v České republice v roce 2014. P4 – progesteron, DRO – drospirenon, MGA – megestrol acetát, CPA – cyproteron acetát, MPA – medroxyprogesteron acetát, DIE – dienogest, NET – norethisteron, NETA – norethisteron acetát, LYN – lynestrenol, DGT – dydrogesteron, CMA – chlormadinon acetát, GES – gestoden, ENG – etonogestrel, DES – desogestrel, LNG – levonorgestrel, NORG – norgestimát, NGMN – norelgestromin, NOMAC – nomegestrol acetát, RU-486 – mifepriston, UPA – ulipristal acetát

Metoda je unikátní hned v několika ohledech: 1) Zahrnuje všechny progestiny předepisované v jedné zemi, přičemž dosud byla vždy prováděna analýza pouze několika vybraných progestinů bez jasného vztahu k jejich spotřebě v dané zemi; 2) Seznam analytů poprvé zahrnuje altrenogest, etonogestrel, dienogest, nomegestrol acetát a ulipristal acetát spolu s dalšími 12 progestiny, které dosud nebyly analyzovány v jednom analytickém cyklu; 3) Je relativně citlivá, hodnoty limitu kvantifikace (LOQ) se pohybují v rozmezí od 0,02 do 0,87 ng/l.

Vyvinutá metoda byla následně použita pro stanovení progestinů v reálných vzorcích odpadních vod z několika čistíren odpadních vod (ČOV) a v povrchových vodách pod a nad zaústěním těchto ČOV. Výsledky analýz jsou shrnuty v následující kapitole.

2.1.2. Výskyt progestinů ve vodním prostředí v České republice

Data o výskytu progestinů ve vodním prostředí České republiky (v odpadních a povrchových vodách) jsou zahrnuta ve třech publikacích, konkrétně v Golovko et al. (2018) (**Příloha 3**), Šauer et al. (2018a) (**Příloha 4**) a Šauer et al. (2018b) (**Příloha 5**). Tato data jsem se pokusila shrnout v Tabulce 2, kde jsou uvedeny informace o maximálních koncentracích, mediánech koncentrací a počtu záchytů jednotlivých látek v odpadních vodách na přítoku na ČOV, na odtoku z ČOV a v recipientu pod zaústěním odpadních vod z ČOV.

Většina progestinů, na které jsme se při našich analýzách zaměřili, byla nalezena v odpadních vodách na přítoku na ČOV a jejich koncentrace se pohybovaly v rozmezí od 0,19 do 110 ng/l. Progestin s nejvyšší spotřebou, přírodní hormon progesteron, byl zároveň nejčastěji detekovaným progestinem ve všech studovaných matricích. Výběr progestinů (jako analytů) na základě údajů o jejich spotřebě se ukázal jako správná cesta, kterou lze doporučit při zahájení sledování výskytu těchto látek i v jiných zemích.

Na odtoku z ČOV se koncentrace jednotlivých progestinů nacházely v rozmezí 0,11 až 3,2 ng/l. Progesteron, megestrol acetát a dienogest patřily mezi nejčastěji nacházené progestiny v průběhu všech našich odběrových kampaní.

V povrchových vodách byly identifikovány (detekovány) pouze dva progestiny, progesteron a medroxyprogesteron, a jejich koncentrace navíc nepřesahovaly 1,3 ng/l a 0,12 ng/l v daném pořadí. To znamená, že koncentrace progestinů v povrchových vodách ČR zatím naštěstí nedosahují tak vysokých hodnot, jaké byly zaznamenány v jiných evropských a asijských státech či v USA (Kolpin et al., 2002; Vulliet et al., 2008; Al-Odaini et al., 2010, Tab. 1).

Tab. 2. Koncentrace progestinů v odpadních a povrchových vodách České republiky.

Progestin	Přítok na ČOV		Odtok z ČOV		Povrchová voda[a]	
	Konc. (Max/Me) ng/l	**Počet** (n/N)	**Konc.** (Max/Me) ng/l	**Počet** (n/N)	**Konc.** (Max/Me) ng/l	**Počet** (n/N)
P4	**110/34**	13/13	**2,7/0,63**	11/13	**1,3/0,58**	8/12
DIE	**15/7,0**	13/13	**1,0/0,51**	5/13	< LOQ	0/12
NET	< LOQ	0/13	**0,85/0,85**	1/13	< LOQ	0/12
GES	**75/7,7**	9/13	**0,71/0,71**	1/13	< LOQ	0/12
DRO	**6,7/2,5**	6/13	**0,29/0,20**	2/13	< LOQ	0/12
LNG	< LOQ	0/13	< LOQ	0/13	< LOQ	0/12
ENG	< LOQ	0/13	< LOQ	0/13	< LOQ	0/12
NOMAC	**10/5,3**	3/13	**0,26/0,26**	1/13	< LOQ	0/12
DGT	**0,28/0,28**	1/13	**0,51/0,51**	1/13	< LOQ	0/12
MP	**0,19/0,19**	1/13	**0,95/0,59**	2/13	**0,12/0,12**	1/12
MPA	**8,1/3,3**	5/13	**0,58/0,30**	4/13	< LOQ	0/12
RU-486	**3,0/1,1**	3/13	**0,50/0,50**	1/13	< LOQ	0/12
UPA	< LOQ	0/13	< LOQ	0/13	< LOQ	0/12
CMA	**1,5/1,5**	1/13	< LOQ	0/13	< LOQ	0/12
CPA	**12/2,1**	6/13	**2,8/1,7**	2/13	< LOQ	0/12
ALT	**0,35/0,35**	1/13	**0,15/0,15**	1/13	< LOQ	0/12
MGA	**13/6,3**	9/13	**1,0/0,23**	7/13	< LOQ	0/12

Pozn.: [a] – recipient pod zaústěním vyčištěných odpadních vod, Max – maximální koncentrace, Me – medián koncentrace, n – počet záchytů, N – počet odebraných vzorků, P4 – progesteron, DIE – dienogest, NET – norethisteron, GES – gestoden, DRO – drospirenon, LNG – levonorgestrel, ENG – etonogestrel, NOMAC – nomegestrol acetát, DGT – dydrogesteron, MP – medroxyprogesteron, MPA – medroxyprogesteron acetát, RU-486 – mifepriston, UPA – ulipristal acetát, CMA – chlormadinon acetát, CPA – cyproteron acetát, ALT – altrenogest, MGA – megestrol acetát.

2.1.3. Hormonální aktivity progestinů

Progestagenní aktivita

Již delší dobu je známo, že odpadní i povrchové vody jsou často kontaminovány látkami antropogenního i přírodního původu, které mají estrogenní účinky. Estrogenní aktivita ve vodním prostředí bývá proto poměrně často monitorována. Kromě estrogenní aktivity je pozornost věnována i anti-estrogenním či (anti-)androgenním aktivitám. Látky přispívající k těmto aktivitám však zůstávají často neidentifikovány. V relativně nedávné době se začaly ve vědecké literatuře objevovat informace o dalším typu hormonálních aktivit vyskytujících se ve vodním prostředí, a to progestagenních aktivitách (van der Linden et al., 2008; Leusch et al., 2014).

Syntetické progestiny byly navrženy tak, aby fungovaly jako agonisté progesteronového receptoru (PR) a tím napodobovaly funkci přírodního hormonu progesteronu (Stanczyk et al., 2013). Většina těchto látek má dokonce silnější progestagenní aktivitu než samotný progesteron (Besse a Garric, 2009). Přestože je tato jejich vlastnost poměrně dobře známa, existuje překvapivě velmi málo informací o příspěvku progestinů k progestagenní aktivitě v komunálních odpadních vodách a povrchových vodách. Cílem naší práce bylo tudíž zjistit, zda a do jaké míry progestiny ve vodách přispívají k progestagenním aktivitám (Šauer et al., 2018a – **Příloha 4**).

Vzorky odpadní vody (na přítoku a odtoku z ČOV) a vody z recipientu (nad a pod zaústěním vyčištěných OV) ze šesti komunálních ČOV byly nejdříve analyzovány na přítomnost progestinů pomocí instrumentální chemické analýzy. Dále byla ve stejných vzorcích vody zjišťována i *in vitro* progestagenní aktivita. Progestagenní aktivita byla měřena pomocí PR-CALUX biotestu. Jedná se o *in vitro* test využívající buňky U2-OS, které jsou transfektovány plazmidy pro reportérový protein luciferázu a progesteronový receptor. Tyto geneticky modifikované buňky specificky reagují na sloučeniny, které mohou aktivovat nebo blokovat vložený receptor (Sonneveld et al., 2005). Abychom mohli zjistit, do jaké míry progestiny přítomné ve vzorcích vody přispívají k progestagenní aktivitě, museli jsme nejdříve otestovat *in vitro* aktivitu jednotlivých látek. Zjišťovali jsme jejich relativní účinek (potenciál), tzn. schopnost aktivovat progesteronový receptor ve srovnání se standardem. Jako standard byl využit silný syntetický progestin ORG 2058. Celková progestagenní aktivita zjištěná ve vzorku vody (vyjádřená v ng/l ekvivalentů ORG 2058) byla pak porovnána se součtem účinků jednotlivých sloučenin zjištěných chemickou analýzou.

Progestagenní aktivity byly detekovány ve všech odpadních vodách a pohybovaly se v rozmezí od 0,09 do 0,6 ng/l ekvivalentů ORG 2058 na přítoku a od 0,04 do 0,47 ng/l ekvivalentů ORG 2058 na odtoku z ČOV. Většina progestagenní aktivity ve vyčištěných odpadních vodách z ČOV byla způsobena přítomností syntetických progestinů a progesteronu. Tyto látky přispěly k 65 – 96 % této aktivity (ve vzorcích, kde nebyla zjištěna žádná antagonistická aktivita). Progestiny medroxyprogesteron acetát, megestrol acetát a progesteron přispívaly k progestagenní aktivitě nejvíce. V povrchových vodách dosahovaly progestagenní aktivity o jeden řád nižších hodnot než v odpadních vodách a pohybovaly se v rozmezí od 0,03 do 0,06 ng/l ekvivalentů ORG 2058 (což odpovídá přibližně 0,4 – 0,8 ng/l ekvivalentů progesteronu). Progestiny se podílely na celkové progestagenní aktivitě až z 83 % a nejvýznamnějším přispěvatelem byl přírodní hormon progesteron.

Na tomto místě je ovšem nutno uvést, že syntetické progestiny se u ryb na progesteronový receptor vůbec nevážou (např. levonorgstrel, etonogestrel, medroxyprogesteron acetát) nebo jsou jeho jen velmi slabými agonisty (drospirenon, gestoden) (Bain et al., 2015; Elllestad et al., 2014). Přírodní hormon progesteron je rovněž slabým agonistou rybího PR (Bain et al., 2015; Elllestad et al., 2014). Mechanismus účinku syntetických progestinů a progesteronu se u ryb pravděpodobně liší od jejich účinků u člověka (Kumar et al., 2015). Detekce progestagenní aktivity pomocí *in vitro* testu (PR-CALUXu), který je založen na lidském PR, nemusí být tedy pro posuzování případného rizika pro ryby relevantní. Určitým indikátorem rizika může být ale pro obojživelníky, protože se předpokládá, že PR u obojživelníků má podobnou sekvenci aminokyselin (v doméně vázající hormon) jako u lidí (Bayaa et al., 2000).

(Anti-)androgenní aktivita

Anti-androgenní aktivity bývají poměrně často detekovány ve vodním prostředí po celém světě, ale sloučeniny odpovědné za tyto aktivity bývá obvykle problém identifikovat. Progestiny patří mezi látky, které jsou schopné interferovat se signální drahou androgenního receptoru (AR) a vykazovat tak (anti-)androgenní aktivitu.

Cílem naší další práce tak bylo zjistit, zda 15 vybraných progestinů přispívá k (anti-) androgenní aktivitě v komunálních odpadních vodách a povrchových vodách (Šauer et al., 2018b – **Příloha 5**). Vzorky odpadní vody (na přítoku a odtoku z ČOV) a vody z recipientu (nad a pod zaústěním vyčištěných OV) ze čtyř komunálních ČOV byly nejdříve analyzovány na přítomnost progestinů pomocí instrumentální chemické analýzy. (Anti-)androgenní

aktivita v těchto vzorcích vody pak byla měřena pomocí (anti-)AR-CALUX biotestu (buňky U2-OS, které jsou transfektovány plazmidy pro reportérový protein luciferázu a androgenní receptor). U sledovaných progestinů jsme zároveň zjišťovali jejich relativní účinky (potenciál), tzn. schopnost aktivovat či blokovat androgenní receptor ve srovnání se standardem. Jako standard androgenní aktivity byl využit dihydrotestosteron (DHT) a pro anti-androgenní aktivitu flutamid (FLU). Celková androgenní a anti-androgenní aktivita zjištěná ve vzorku vody (vyjádřená v ng/L ekvivalentů DHT nebo μg/l ekvivalentů FLU) byla pak porovnána se součtem účinků jednotlivých sloučenin zjištěných chemickou analýzou. V odebraných vzorcích vody byla detekována androgenní aktivita v rozsahu 0,08 – 59 ng/l ekvivalentů DHT, anti-androgenní aktivita v rozsahu 2,4 – 26 μg/l ekvivalentů FLU a koncentrace progestinů se pohybovaly od 0,19 do 75 ng/l. Některé progestiny vykazovaly relativně silné androgenní (0,01 – 0,22 násobek DHT) a anti-androgenní (9 až 62 násobek FLU) účinky. Testovali jsme i aktivitu ekvimolárních směsí nalezených progestinů a výsledky naznačily, že tyto látky působí aditivně.

Progestiny přispívaly do určité míry k androgenní (0,3 – 29 %) a anti-androgenní (4,6 – 27 %) aktivitě v odpadních vodách na přítoku na ČOV, ale jejich příspěvek k (anti-) androgenním aktivitám v odpadních vodách na odtoku z ČOV a v povrchových vodách byl zanedbatelný (≤ 2,1 %). I když mají progestiny relativně silné (anti-)androgenní účinky, není pravděpodobné, že by hrály významnou roli jako přispěvatelé k (anti-)androgenní aktivitě ve vodním prostředí České republiky, protože se vyskytují jen v relativně nízkých koncentracích (desetiny až maximálně jednotky ng/l).

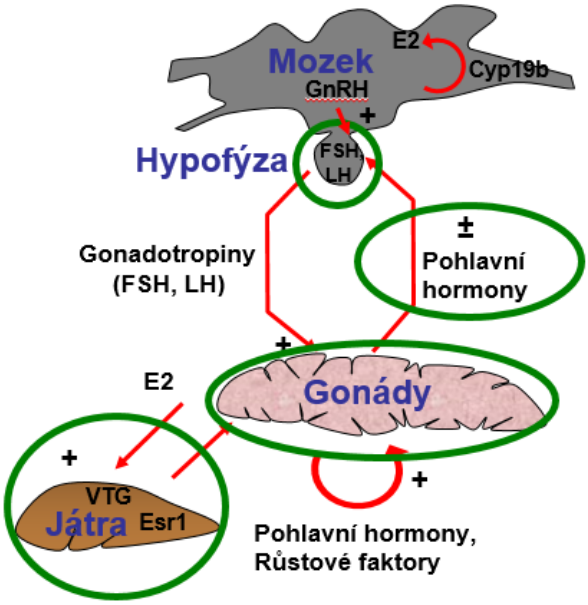
2.1.4. Vliv progestinů na ryby

Vliv syntetického progestinu levonorgestrelu na ryby

První článek popisující negativní vliv syntetických progestinů na reprodukci ryb vyšel teprve v roce 2009 (Zeilinger et al., 2009). Tento a další články, které byly publikovány v následujících letech, prokázaly, že některé syntetické progestiny jsou schopné velmi výrazně snižovat produkci jiker u ryb, a to už v koncentracích kolem 1 ng/l (Paulos et al., 2010, Runnalls et al., 2013). Mechanismus účinku těchto látek na ryby však nebyl známý. Cílem naší první studie, která byla zaměřena na syntetické progestiny, bylo proto zkoumat účinky jednoho ze syntetických progestinů, levonorgestrelu (LNG), na reprodukční endokrinní systém plotice obecné (*Rutilus rutilus*). Pubertální plotice byly vystaveny v

průtočném systému čtyřem koncentracím levonorgestrelu (3, 31, 312 a 3124 ng/l) po dobu 28 dnů. Podrobnosti jsou uvedeny v **Příloze 6** (Kroupova et al., 2014).

U samců a samic vystavených nejvyšší koncentraci levonorgestrelu (3124 ng/l) byla zjištěna výrazně vyšší mRNA exprese vitelogeninu a estrogenního receptoru 1 v játrech. Ve stejné koncentraci způsobil levonorgestrel významné zvýšení mRNA exprese genu kódujícího beta podjednotku luteinizačního hormonu (*lhβ*) a snížení mRNA exprese genu kódujícího beta podjednotku hormonu stimulujícího folikuly (*fshβ*) v hypofýze samců i samic. Nižší koncentrace levonorgestrelu (312 ng/l) snížila mRNA expresi *fshβ* pouze u samců. Samice exponované nejvyšší koncentraci levonorgestrelu (3124 ng/l) měly signifikantně nižší hladiny 11-ketotestosteronu (11-KT) a estradiolu (E2), zatímco jejich hladina testosteronu (T) byla vyšší v porovnání s kontrolou. Samice vystavené 312 ng/l levonorgestrelu vykazovaly signifikantně nižší koncentrace E2 v plazmě. Samci vystavení účinkům ≥ 31 ng/l levonorgestrelu měli významně sníženou hladinu 11-KT. Z histologické analýzy gonád vyplynulo, že expozice levonorgestrelu neměla významný vliv na ovaria samic, zato gonády samců vystavených koncentracím 31 a 312 ng/l obsahovaly signifikantně vyšší procento spermatogonií B ve srovnání s kontrolou. Výsledky této studie ukázaly, že levonorgestrel narušoval reprodukční systém pubertálních plotic zejména tím, že ovlivňoval expresi gonadotropinů v hypofýze a hladinu pohlavních hormonů v plasmě. Nejvyšší testovaná koncentrace levonorgestrelu (3124 ng/l) navíc vyvolala estrogenní účinky u ryb obou pohlaví. Tento efekt byl však pravděpodobně způsoben metabolity levonorgestrelu, o kterých je známo, že mají estrogenní účinky (García-Becerra et al., 2002).



Obr. 4. Vliv levonorgestrelu na reprodukční systém plotice. Osa hypothalamus-hypofýza-gonády s vyznačenými místy (zelené kroužky), kde došlo ke změnám v důsledku expozice. Cyp19b – aromatáza, E2 – 17 β-estradiol, Esr1 – estrogenní receptor 1, FSH – folikuly stimulující hormon (folitropin), GnRH – hormon uvolňující gonadotropiny, LH – luteinizační hormon (lutropin), VTG – vitelogenin.

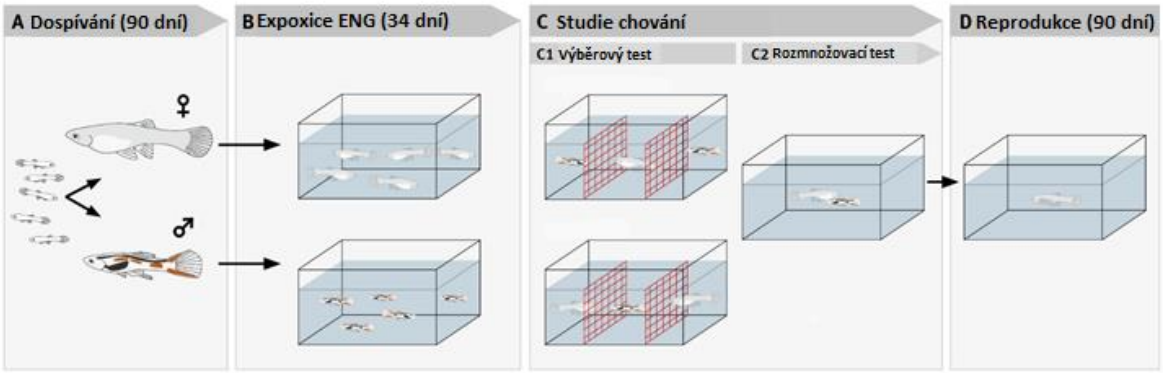
Vliv syntetického progestinu etonogestrelu na reprodukci a reprodukční chování ryb

V ekotoxikologických studiích na vodních organizmech jsou změny v chování považovány za citlivější indikátory než změny v morfologických, reprodukčních a vývojových parametrech (Melvin a Wilson, 2013). Změny reprodukčního chování jsou rovněž považovány za důležitý indikátor endokrinních poruch (Frankel et al., 2016a, Sebire et al., 2008, Zeilinger et al., 2009).

Cílem naší další studie bylo proto posoudit vliv etonogestrelu, syntetického progestinu třetí generace, na reprodukční chování, plodnost, histologii gonád a sekundární pohlavní znaky živorodek Wingeových (*Poecilia wingei*).

Ryby byly vystaveny po dobu 34 dnů dvěma koncentracím etonogestrelu, včetně jedné koncentrace, která by mohla být environmentálně relevantní (3,2 ng/l) a jedné subletální (320 ng/l). Následně po expozici byla provedena studie reprodukčního chování ryb. Tato práce byla unikátní v tom, že jsme sledovali vliv etonogestrelu na obě pohlaví, s cílem zjistit, zda se samci a samice liší v citlivosti ke studované látce. Po provedení studií reprodukčního chování jsme navíc dále zjišťovali, zda měla expozice etonogestrelu vliv i na reprodukční úspěch ryb, které se účastnily rozmnožovacího testu. Jedná se o první studii zaměřenou na progestiny, kde byly všechny výše uvedené parametry studovány společně. Schéma celého

experimentu s vysvětlivkami je zobrazeno na obrázku 5. Podrobnosti jsou uvedeny v **Příloze 7** (Steinbach et al., 2019).



Obr. 5. Schématické zobrazení jednotlivých etap reprodukční studie na živorodkách Wingeových. A – Juvenilní ryby určené pro test byly nejdříve po dobu 3 měsíců chovány v laboratorních podmínkách. Během tohoto období dosáhly pohlavní zralosti. Tyto pohlavně zralé ryby pak byly použity v testu. B – Expozice etonogestrelu trvající 34 dnů. Samci a samice byli exponováni odděleně. C – Studie chování: C1, „výběrový" test; C2, „rozmnožovací" test. D – Reprodukce (Samice, které se účastnily rozmnožovacího testu, byly drženy v izolaci po dobu 90 dnů. Během tohoto období byl zaznamenáván počet potomků, které se jim narodily). ENG – etonogestel.

Samice vystavené nejvyšší koncentraci etonogestrelu vykazovaly známky maskulinizace. Jejich řitní ploutve měli podobný tvar jako gonopodium (pohlavní orgán) samců a zbarvením těla se rovněž podobaly samcům. Samice vystavené oběma koncentracím etonogestrelu měly navíc nižší podíl zralých oocytů v gonádách v porovnání s kontrolou. Dále byla zjištěna významně nižší frekvence páření kontrolních samců s exponovanými samicemi ve srovnání s kontrolou (neexponovaní jedinci obou pohlaví), což nasvědčovalo tomu, že byly exponované samice pro samce méně atraktivní. V úplném závěru studie pak bylo zjištěno, že žádná exponovaná samice nebyla schopná reprodukce. U samců vedla expozice oběma koncentracím etonogestrelu ke snížení frekvence páření s kontrolními samicemi, jejich reprodukční úspěch tím však překvapivě ovlivněn nebyl. Studie potvrdila, že expozice etonogestrelu má výrazně závažnější důsledky pro samice než pro samce. Poměrně zajímavé bylo zjištění, že změny chování mohou (v případě samic), ale nemusí (v případě samců), vyústit v reprodukční neúspěch.

2.2. Řemenatka ptačí

2.2.1. Vliv řemenatky ptačí na reprodukční systém plotice obecné

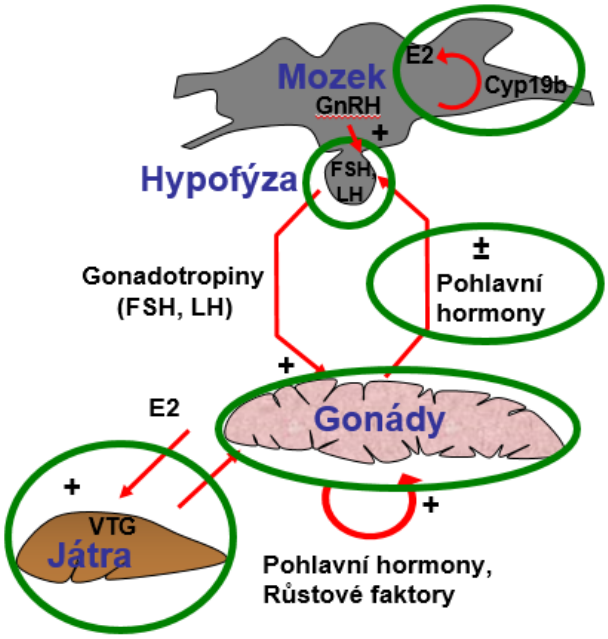
Cílem první práce (Trubiroha et al., 2010 – **Příloha 8**), na které jsem se podílela při svém pobytu na IGB v Berlíně v Německu bylo zjistit základní informace o endokrinním systému plotice obecné (*Rutilus rutilus*) infikované řemenatkou ptačí (*Ligula intestinalis*; Obr. 6) a popsat mechanismus účinku tohoto parazita na reprodukční systém infikovaných ryb.

Obr. 6. Plotice obecná infikovaná řemenatkou ptačí (*Ligula intestinalis*). Na horní fotografii je ryba s plerocerkoidy v tělní dutině. Na dolní fotografii je pak ryba s plerocerkoidy, které byly vyjmuty z její tělní dutiny. Foto: Sabrina N. Frank.

U plotice obecné infikované řemenatkou ptačí bylo pozorováno zpoždění ve vývoji gonád u obou pohlaví. Analýza pohlavních hormonů u infikovaných samic ukázala signifikantně nižší hladinu 17β-estradiolu (E2) a 11-ketotestosteronu (11-KT) v porovnání s kontrolou. U infikovaných samců byly pozorovány rovněž nižší hladiny E2 a 11-KT a dále pak testosteronu. U obou pohlaví byla zjištěna nižší exprese genů indukovaných působením estrogenů, jako je vitelogenin v hepatopankreatu a aromatáza v mozku. Infikované samice, na rozdíl od samců, vykazovaly nižší expresi estrogenního receptoru 1 v hepatopankreatu. Exprese estrogenního receptoru 2a byla naopak zvýšená u infikovaných ryb obojího pohlaví. Navzdory výraznému vlivu infekce na koncentraci pohlavních steroidů v plasmě a expresi

gonadotropinů v hypofýze (která byla zjištěna v předchozí práci – Trubiroha et al., 2009) nebyla exprese prekurzorů hormonu uvolňujícího gonadotropiny (GnRH) v mozku infekcí ovlivněna.

Výsledky této práce jasně ukázaly, že parazité mohou způsobit u ryb takové endokrinní poruchy, jaké bývají jinak zaznamenávány hlavně po expozici některým polutantům životního prostředí (Obr. 7).



Obr. 7. Vliv řemenatky ptačí na reprodukční systém plotice. Osa hypothalamus-hypofýza-gonády s vyznačenými místy (zelené kroužky), kde došlo ke změnám v důsledku infekce. Cyp19b – aromatáza, E2 – 17 β-estradiol, FSH – folikuly stimulující hormon (folitropin), GnRH – hormon uvolňující gonadotropiny, LH – luteinizační hormon (lutropin), VTG – vitelogenin.

2.2.2. Interakce řemenatky ptačí se somatotrofickou osou hostitele

V naší druhé práci (Trubiroha et al., 2011 – **Příloha 9**) jsme se zabývali otázkou, zda může neomezený přísun potravy zmírnit negativní vliv infekce řemenatkou ptačí na reprodukční systém plotice obecné.

U plotic obecných infikovaných parazitem řemenatkou ptačí, které byly chovány v laboratorních podmínkách a byly krmeny *ad libitum*, byly měřeny reprodukční parametry. Infikované plotice měly nižší GSI a histologické analýzy ukázaly zpožděný vývoj gonád u obou pohlaví v porovnání s kontrolními rybami obojího pohlaví. V gonádách infikovaných samic bylo možné nalézt folikuly ve stádiu sekundárního růstu, ale vysoké procento těchto folikulů bylo atretických. Histologická data dobře korespondovala se sníženou expresí

gonadotropinů v hypofýze a nízkými koncentracemi pohlavních steroidních hormonů v plasmě ryb. Zjištěna byla rovněž snížená hladina mRNA vitelogeninu a změněná exprese receptorů pro pohlavní steroidní hormony v játrech infikovaných ryb. Negativní vliv řemenatky na reprodukci nekoreloval se zatížením ryb parasitem (parazitickým indexem). Chov plotic v laboratorních podmínkách za neomezeného přísunu potravy tak nepatrně zmírnil, ale nezvrátil negativní vliv řemenatky na reprodukční funkce plotice, což indikuje specifickou inhibici reprodukce hostitele vyvolanou poruchou endokrinních funkcí (endokrinní disrupcí).

Cílem naší poslední práce (Kroupova et al., 2012 – **Příloha 10**) bylo charakterizovat nutriční stav plotic infikovaných řemenatkou ptačí a dále zjistit, zda tato infekce ovlivňuje expresi genů zapojených v endokrinní somatotrofické ose (tj. růstového hormonu a jeho receptorů, insulinu podobných růstových faktorů a jejich receptoru a somatolaktinů). Byly studovány dvě skupiny přirozeně infikovaných a neinfikovaných plotic, a to: 1) skupina ryb odlovených přímo z jezera Müggelsee, které byly na přirozené potravě (jezerní skupina) a skupina ryb, která byla odchovávána v laboratorních podmínkách a krmena *ad libitum* (laboratorní skupina).

Výsledky analýz energetických zásob a exprese genů, které jsou zapojeny do somatotrofické osy, ukázaly, že řemenatka ptačí nezpůsobuje hladovění svého hostitele, a zdá se tak, že díky inhibici gametogeneze dochází ke kompenzaci či minimálně zmírnění nutriční zátěže způsobené tímto parazitem. Zároveň bylo zjištěno, že přítomnost parazita má poměrně výrazný vliv na expresi některých genů somatotrofické osy. Konkrétně zvyšuje v některých případech expresi růstového hormonu, somatolaktinů a inzulinu podobných růstových faktorů. Všechny výše uvedené výsledky ukazují, že parazit manipuluje s fyziologickými funkcemi infikovaných ryb. Způsob, jakým to provádí, však zůstává zatím neobjasněn.

3. Závěry

3.1. Význam výsledků pro vědní obor a možnosti směřování dalšího výzkumu

V **Příloze 3** je prezentována poměrně unikátní metoda pro stanovení progestinů ve vodních matricích. Jedná se o metodu, která poprvé zahrnuje všechny progestiny předepisované v jedné zemi, přičemž dosud byla vždy prováděna analýza pouze několika vybraných progestinů bez jasného vztahu k jejich spotřebě. Seznam analytů poprvé zahrnuje altrenogest, etonogestrel, dienogest, nomegestrol acetát a ulipristal acetát spolu s dalšími 12 progestiny, které dosud nebyly analyzovány v jednom analytickém cyklu. Metoda je relativně citlivá, hodnoty limitu kvantifikace (LOQ) se pohybují v rozmezí od 0,02 do 0,87 ng/l.

V **Přílohách 3**, **4** a **5** jsou zahrnuta data o výskytu široké škály progestinů ve vodním prostředí České republiky. Jedná se o výsledky prvního rozsáhlejší monitoring tohoto typu nejen v České republice. Dosud byly i v celosvětovém měřítku analyzovány nesystematicky jen některé vybrané látky z této rozsáhlé skupiny. Cílenější screening se snad s výjimkou Číny dosud nikde neprováděl.

Příloha 4 obsahuje informace o příspěvku progestinů k progestagenní aktivitě v odpadních a povrchových vodách. Je to opět první komplexní analýza tohoto typu. V **Příloze 5** jsou pak uvedeny první výsledky týkající se příspěvku progestinů k (anti-)androgenním aktivitám ve vodním prostředí.

V **Přílohách 6** a **7** je zhodnocen vliv vybraných syntetických progestinů na ryby. V **Příloze 6** je prezentována jedna z prvních studií, jejímž cílem bylo odhalit mechanismus účinku jednoho ze syntetických progestinů, levonorgestelu, u ryb. Práce uvedená v **Příloze 7** hodnotí vliv syntetického progestinu třetí generace, etonogestrelu, na reprodukční chování, sekundární pohlavní znaky a reprodukci ryb. Jedná se o první práci zaměřenou na etonogestrel a jednu z prvních prací věnující se vlivu syntetických progestinů na reprodukční chování ryb obecně.

Publikace v **Přílohách 8, 9** a **10** podrobně popisují, jakým způsobem parazit, řemenatka ptačí, manipuluje s reprodukčním endokrinním systémem a somatotrofickou osou svého hostitele, plotice obecné. Tyto práce upozorňují mimo jiné i na to, že příčinnou endokrinní disrupce u ryb či jiných vodních živočichů nemusí být pouze antropogenní faktory.

K syntetickým progestinům jako nové hrozbě pro vodní prostředí byla obrácena pozornost relativně nedávno. První článek popisující negativní vliv syntetických progestinů na reprodukci ryb vyšel teprve v roce 2009. Od té doby se podařilo získat první informace o jejich výskytu ve vodním prostředí a vlivu některých z nich na vodní organismy. Pozornost však byla dosud věnována jen relativně úzké skupině syntetických progestinů, vliv některých látek z této široké skupiny na vodní živočichy byl studován jen okrajově nebo vůbec. V této oblasti je tedy ještě poměrně dost práce. Progestiny mají navíc kromě reprodukčních funkcí potenciál negativně ovlivňovat i jiné fyziologické funkce vodních organismů. To je rovněž oblast výzkumu, která by se měla dále rozvíjet.

Problematika endokrinní disrupce způsobené přírodními faktory si také zaslouží další studium. Dá se předpokládat, že tasemnice řemenatka ptačí jistě není jediným parazitem, který má schopnost manipulovat s endokrinním systémem vodních živočichů. Rozšiřování znalostí v této oblasti by mohlo pomoci správné interpretaci výsledků získaných v monitoringu vodního prostředí.

3.2. Využití dosažených výsledků při výuce

Teoretické i praktické poznatky získané při řešení prezentované problematiky byly využity při tvorbě nového studijního předmětu s názvem „Poruchy endokrinního systému vodních živočichů“ pro nový doktorský studijní program „Ochrana vodních ekosystémů“, o jehož akreditaci se naše fakulta bude v brzké době ucházet.

Na získávání některých výsledků prezentovaných v této práci se významně podíleli studenti doktorského studia Ing. Pavel Šauer a Mgr. Jitka Tumová. Dále byli zapojeni i dva studenti magisterského studia, a to Bc. Petra Beranová a Bc. Michal Pech. Všichni výše jmenovaní studenti pracovali pod vedením autorky této habilitační práce.

3.3. Využití dosažených výsledků pro praxi

Výsledky prezentované v této práci dosud, pokud je mi známo, v praxi využity nebyly, přesto potenciál k jejich využití existuje.

Naše výsledky týkající se výskytu a toxikologických vlastností syntetických progestinů by se mohly stát podkladem pro státní či evropské regulační orgány, které nastavují kritéria pro kvalitu vody. Myslím, že by bylo vhodné (velmi účelné) rozšířit soubor polutantů, které

jsou sledovány jako ukazatele jakosti povrchových vod, o některé vybrané progestiny, a to zejména v lokalitách, kde je voda využívána k vodárenským účelům

Řemenatka ptačí ovlivňuje u infikovaných ryb hodnoty parametrů, které se používají jako biomarkery endokrinní disrupce při biomonitoringu vodního prostředí. Mění například hladiny pohlavních steroidních hormonů v plasmě či expresi a výslednou koncentraci vitelogeninu. Ryby infikované tímto parazitem by měly být vyřazeny z hodnoceného souboru, jinak hrozí nebezpečí zkreslení dat. Nejen řemenatka ptačí, ale i další druhy parazitů pravděpodobně způsobují endokrinní disrupci, proto by ryby vzorkované v rámci biomonitoringu životního prostředí měly být důkladně vyšetřeny na přítomnost parazitů a tento faktor by měl být brán vždy v úvahu.

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5. Seznam prací autorky, které jsou součástí habilitační práce

Součástí habilitační práce je celkem 10 publikovaných prací. Všechny práce byly publikovány v mezinárodních vědeckých časopisech s oponentním řízením a přiděleným indikátorem IF v databázi Web of Science společnosti Thomson Reuters. Práce jsou číslovány podle odkazů v jednotlivých kapitolách průvodního textu. Uvedené hodnoty IF u publikací se vztahují k datu publikování článků v jednotlivých časopisech. U každé publikace je rovněž uveden aktuální citační ohlas (SCI) v době psaní této habilitační práce.

Příloha č. 1: Kloas, W., Urbatzka, R., Opitz, R., Würtz, S., Behrends, T., Hermelink, B., Hofmann, F., Jagnytsch, O., **Kroupova, H.**, Lorenz, C., Neumann, N., Pietsch, C., Trubiroha, A., Van Ballegooy, C., Wiedemann, C., Lutz, I., 2009. Endocrine disruption in aquatic vertebrates. Annals of the New York Academy of Sciences, 1163:187-200. (IF 2008 = 2,303; SCI 2018 = 105)

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Příloha č. 5: Šauer, P., Bořík, A., Golovko, O., Grabic, R., Vojs Staňová, A., Valentová, O., Stará, A., Šandová, M., **Kocour Kroupová, H.**, 2018. Do progestins contribute to (anti-)androgenic activities in aquatic environments? Environmental Pollution 242: 417–425. (IF 2017 = 4,358; SCI 2018 = 0)

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gonadotropin expression and sex steroid levels in pubertal roach (*Rutilus rutilus*). Aquatic Toxicology 154: 154-162. (IF 2013 = 3,513; SCI 2018 = 27)

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Příloha č. 8: Trubiroha, A., **Kroupová, H.**, Wuertz, S., Frank, S. N., Sures, B., Kloas, W., 2010. Naturally-induced endocrine disruption by the parasite *Ligula intestinalis* (Cestoda) in roach (*Rutilus rutilus*). General and Comparative Endocrinology 166(2): 234-40. (IF 2009 = 2,732; SCI 2018 = 26)

Příloha č. 9: Trubiroha, A., **Kroupová, H.**, Frank, S.N., Sures, B., Kloas, W., 2011. Inhibition of gametogenesis by the cestode *Ligula intestinalis* in roach (*Rutilus rutilus*) is attenuated under laboratory conditions. Parasitology 138: 648-659. (IF 2010 = 2,522; SCI 2018 = 10)

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6. Český abstrakt

Kocour Kroupová, H., 2019. Vybrané přírodní a antropogenní faktory způsobující endokrinní disrupci u ryb. Habilitační práce, Jihočeská univerzita v Českých Budějovicích, Fakulta rybářství a ochrany vod, Vodňany: 168 s.

Předloženou habilitační práci tvoří komentovaný soubor 10 publikací. Tematicky je práce rozdělena na dvě části. První část je věnována syntetickým progestinům, endokrinním disruptorům antropogenního původu. Druhá část práce je pak věnována vlivu infekce parazitem řemenatkou ptačí na endokrinní systém plotice obecné. Tasemnice řemenatka ptačí je zde představena jako modelový „přírodní endokrinní disruptor".

Syntetické progestiny jsou steroidní hormony, které napodobují funkci přírodního progestinu progesteronu a jsou aktivními látkami nejrůznějších hormonálních preparátů včetně hormonální antikoncepce. V první části práce je popisován vývoj unikátní analytické metody pro stanovení progesteronu a širokého spektra syntetických progestinů, které jsou distribuovány v České republice. Dále jsou sumarizována data o jejich výskytu ve vodním prostředí a hormonálních aktivitách, které mohou ve vodách způsobovat. Pozornost je věnována i mechanismu účinku syntetických progestinů a jejich vlivu na reprodukční chování ryb.

Endokrinní disrupce u vodních živočichů nemusí být způsobena pouze antropogenním faktory, zejména účinkem cizorodých látek ve vodním prostředí. Již delší dobu je známo, že například některé přírodní látky mohou vykazovat různé hormonální aktivity. Méně známý je však fakt, že s endokrinním systémem vodních živočichů mohou manipulovat například i paraziti. Příkladem je tasemnice řemenatka ptačí, která způsobuje inhibici gametogeneze (vývoje gonád) u infikovaných ryb. V habilitační práci je popsáno, jakým způsobem tento parazit ovlivňuje reprodukční endokrinní systém a somatotrofickou osu svého hostitele, plotice obecné.

Klíčová slova: (anti-)androgenní aktivita, endokrinní disrupce, LC-APCI/APPI-HRPS, paraziti, progestagenní aktivita, progestiny, reprodukce, reprodukční chování, ryby, řemenatka ptačí, steroidní hormony, tasemnice

7. Anglický abstrakt

Kocour Kroupová, H., 2019. Selected natural and anthropogenic factors causing endocrine disruption in fish. Habilitation thesis, University of South Bohemia in České Budějovice, Faculty of Fisheries and Protection of Waters, Vodňany: 168 p.

The presented habilitation thesis is an annotated set of 10 publications. The subject is divided into two parts. The first part is devoted to synthetic progestins, endocrine disruptors of anthropogenic origin. The second part of the work is devoted to the effect of the parasite *Ligula intestinalis* on the endocrine system of the roach. The tapeworm *Ligula intestinalis* is presented here as a model "natural endocrine disruptor".

Synthetic progestins are steroid hormones that mimic the function of a natural hormone progesterone and are active substances of various hormonal preparations including hormonal contraceptives. The first part of the thesis describes the development of a unique analytical method for the determination of progesterone and a wide range of synthetic progestins distributed in the Czech Republic. Data on their occurrence in the aquatic environment and on the hormonal activities they can cause in water are summarized. Attention is also paid to the mechanism of action of synthetic progestins and their effect on the reproductive behaviour of fish.

Endocrine disruption in aquatic animals may not be caused only by anthropogenic factors, in particular by the effect of xenobiotics in the aquatic environment. It has long been known that, for example, some natural substances may exhibit different hormonal activities. Less well-known, however, is the fact that the endocrine system of aquatic animals can also be affected by parasites. An example of this is the tapeworm *Ligula intestinalis* which causes the inhibition of gametogenesis (gonadal development) in infected fish. In the habilitation thesis, effect of this parasite on the reproductive endocrine system and the somatotrophic axis of its host is described.

Key words: (anti-)androgenic activity, endocrine disruption, LC-APCI/APPI-HRPS, parasites, progestagenic activity, progestins, reproduction, reproductive behaviour, fish, *Ligula intestinalis*, steroid hormones, tapeworm

8. Poděkování

Ráda bych poděkovala všem kolegům z Výzkumného ústavu rybářského a hydrobiologického Fakulty rybářství a ochrany vod ve Vodňanech a Leibnizova institutu pro vodní ekologii a sladkovodní rybářství v Berlíně (IGB Berlin), kteří mi v mé vědecké práci pomáhali.

Tato práce mohla vzniknout díky studiím finančně podpořeným následujícími poskytovateli a projekty:

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Nadace Alexandera von Humboldta

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9. Přílohy

Příloha č. 1

Kloas, W., Urbatzka, R., Opitz, R., Würtz, S., Behrends, T., Hermelink, B., Hofmann, F., Jagnytsch, O., **Kroupova, H.**, Lorenz, C., Neumann, N., Pietsch, C., Trubiroha, A., Van Ballegooy, C., Wiedemann, C., Lutz, I., 2009. Endocrine disruption in aquatic vertebrates. Annals of the New York Academy of Sciences, 1163:187-200. (IF 2008 = 2,303; SCI 2018 = 105)

Endocrine Disruption in Aquatic Vertebrates

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Environmental compounds can interfere with endocrine systems of wildlife and humans. The main sink of such substances, called endocrine disrupters (ED), are surface waters. Thus, aquatic vertebrates, such as fish and amphibians, are most endangered. ED can adversely affect reproductive biology and the thyroid system. ED act by (anti)estrogenic and (anti)androgenic modes of action, resulting in abnormal sexual differentiation and impaired reproduction. These effects are mainly driven by direct interferences of ED with sex steroid receptors rather than indirectly by impacting synthesis and bioavailability of sex steroids, which in turn might affect the hypothalamic–pituitary–gonadal axis. Recent findings reveal that, in addition to the human-produced waste of ED, natural sources, such as parasites and decomposition of leaves, also might act as ED, markedly affecting sexual differentiation and reproduction in fish and amphibians. Although the thyroid system has essential functions in both fish and amphibians, amphibian metamorphosis has been introduced as the most sensitive model to detect thyroidal ED; no suitable fish model exists. Whereas ED may act primarily on only one specific endocrine target, all endocrine systems will eventually be deregulated as they are intimately connected to each other. The recent ecotoxicological issue of pharmaceutically active compounds (PhACs) present in the aquatic environment indicates a high potential for further endocrine modes of action on aquatic vertebrates by ED derived from PhACs, such as glucocorticoids, progestins, and ß-agonists.

Key words: **endocrine disruption; aquatic vertebrates; fish; amphibians; reproductive biology; thyroid system**

Introduction

Evolution of the animal kingdom has been accompanied in parallel by chemical communication, which is the basis for all organisms to exchange information with the environment, between individuals, or between several parts of an organism. Starting at the evolutionary level of cnidarians, three major systems for such communication (immune, nervous, and endocrine) developed in parallel. The tasks of the endocrine system of all animal orders include regulation of synthesis, release, degradation, structure, and functions of hormones as well as their corresponding receptors for regulating important physiological processes, such as metabolism, osmomineral regulation, color change, reproduction, behavior, development, and metamorphosis.[1] Over the past

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few decades evidence has accumulated that environmental compounds can interfere with the endocrine system of wildlife and humans.[2] The general definition of such compounds, termed *endocrine disrupters* (ED), put forward during the Weybridge workshop in 1996[3] includes nearly all environmental pollutants, and therefore a more generalized definition of ED should be preferred, defining ED as "endocrine active compounds causing specific effects on endocrine systems at several levels without relevant toxic actions" (p. 4).[4] Surface waters are the main sink of ED, which are thought to be mainly of anthropogenic origin. Thus aquatic organisms, especially lower vertebrates, such as fish and amphibians, are the main potential targets for ED.[4,5]

From the beginning, the main focus of ED research dealt with the effects of xenoestrogens leading to feminization phenomena in several classes of vertebrates, with emphasis on assessment of estrogenic modes of action (MOA). However, more recently there has been growing evidence that ED present in the environment exhibit not only estrogenic or antiestrogenic MOA but also androgenic and antiandrogenic MOA.[6] In general, reviews about ED in the aquatic environment are numerous,[7,8] and implications for environmental risk assessment by the European Union in the framework of REACH (Registration, Evaluation and Authorisation of Chemicals)[9] have been put forward.[10]

The fact that male rainbow trout were feminized by ED in sewage effluent caused growing public concern in the effects of ED in aquatic ecosystems (e.g., surface waters).[11] As a follow up, ED research focused on determination of ED affecting reproduction of fish by xenoestrogens and led to abundant literature on endocrine disruption in fish.[5] However, although much evidence exists that ED cause adverse effects in fish kept under laboratory conditions, to what extent ED might affect wild fish populations by impairment of reproduction and development remains an open question.[12–14] Early ED research addressed possible effects of estrogenic MOA on (teleost) fish because environmental estrogenic activities correlated with sexual disruption in wild fish populations[15] biased the development of estrogenic biomarkers.[16]

Amphibians are the classical endocrine models for sex reversal and metamorphosis triggered by the thyroid system and they are, because of their obligate aquatic life style (at least during the first part of larval development), also main targets of ED. However, our scientific knowledge about the effects of ED on amphibians is limited compared to fish. This is despite the fact that ED are known to contribute potentially to the worldwide decline of amphibians[17]; the first report demonstrating feminizing sex reversal from larval exposure to ED is from 1999.[18] Among the three amphibian orders, the main ED research has been performed using anurans and to a lesser extent urodeles, whereas research in caecilians is scarce.[19]

Nevertheless, it is still a matter of debate which vertebrate models could or should be used in general for risk assessments of ED in the aquatic environment or even as sentinels for humans. Recently, amphibians have received special attention because they may provide very sensitive models for the assessment of ED effects on a wide range of physiological regulatory processes. The aim of the present paper is to provide an overview of the recent knowledge concerning endocrine disruption in aquatic vertebrates, comparing amphibians and fish and focusing on organismal and physiological aspects rather than cellular and molecular mechanisms. Our emphasis is on reproduction and thyroid actions, and we highlight the potential perspectives and ongoing directions of research in this field.

Reproductive Biology of Amphibians and Fish

Reproductive biology of lower vertebrates, such as amphibians and fish, can be divided

into two parts: the maintenance of reproductive functions in adults and the phase of sexual differentiation of the developing individuals characterized by a high degree of plasticity during early developmental phases where sex reversal may occur as a result of endocrine changes. Sex determination in vertebrates, in general, is related to genotypic, temperature-dependent, or behavioral sex determination. However, in amphibians only genotypic sex determination has been described, but to date no sex chromosomes have been identified because these chromosomes lack clear, morphological, distinctive characteristics. In most anurans the females are homogametic, belonging to the so-called XX genotype, whereas the males correspond to the XY genotype. Nevertheless, the best studied anuran model species, the South African clawed frog *Xenopus laevis*, resembles the opposite genotypic sex in having homogametic males (ZZ genotype) and heterogametic females (ZW genotype). Despite this obviously clear mode of sex determination, anurans exhibit a broad range of morphological features concerning sex from clear gender-specific gonadal appearance differentiating into male and female phenotypes to in-part obligatory or sequential hermaphroditism depending on environmental factors or developmental life stages affecting the endocrine system. In teleost fish, all types of genotypic, temperature-dependent, and behavioral sex determination occur, and, at least in some genotypic species, sex chromosomes are distinguishable.[1] Nevertheless both vertebrate classes, amphibians and teleost fish, are susceptible to exogenously administered sex steroids causing sex reversal during the sensitive window for sexual differentiation, providing in this way sentinel species for detection of ED.

Hypothalamic–Pituitary–Gonadal Axis in Amphibians and Fish

The endocrine systems of fish and amphibians are generally organized like most vertebrates, with hierarchic structures for several endocrine feedback mechanisms (Fig. 1). Hypothalamus–pituitary–endocrine gland axes exist for adrenal cortex, gonads, and thyroid system. Concerning regulation of reproduction, the hypothalamic–pituitary–gonadal (HPG) axis plays the major role for sexual differentiation and regulation of reproduction in amphibians[20] and fish,[21] providing several targets for ED. In principle, amphibians and fish have a comparable regulation of the HPG axis. The hypothalamus releases gonadotropin-releasing hormone (GnRH), stimulating the secretion of the gonadotropins, luteinizing hormone (LH), and follicle-stimulating hormone (FSH) from the pituitary. LH and FSH act on gonads, leading to synthesis and release of sex steroids, androgens, and estrogens, which in turn act on target cells and cause negative feedback on hypothalamus and pituitary for regulating homeostasis. The androgens testosterone (T) and dihydrotestosterone (DHT), found in anurans, are the same as in all higher vertebrates, whereas in urodeles and fish, 11-ketotestosterone is the predominant functioning androgen, which results in more pronounced structural differences in the corresponding androgen receptors concomitant with physiological responses. Thus (anti)androgenic MOA of anurans might be more closely related to mammals, including man, than to MOA of urodeles and fish. The natural occurring estrogen 17ß-estradiol (E2) is the only sex steroid hormone present throughout all groups of vertebrates, and estrogen receptors are remarkably similar with respect to their structures and specificities. This indicates that all vertebrates, including amphibians and fish, should be suitable for assessment of (anti)estrogenic ED. In addition, binding proteins, hormone activating and inactivating metabolic activities, and degradation and excretion processes in target organs also play important roles for regulation and bioavailability of hormones. Therefore, the potential targets of ED are not only sex steroid receptors of target cells where ED can mimic a hormone or act as a hormone antagonist.

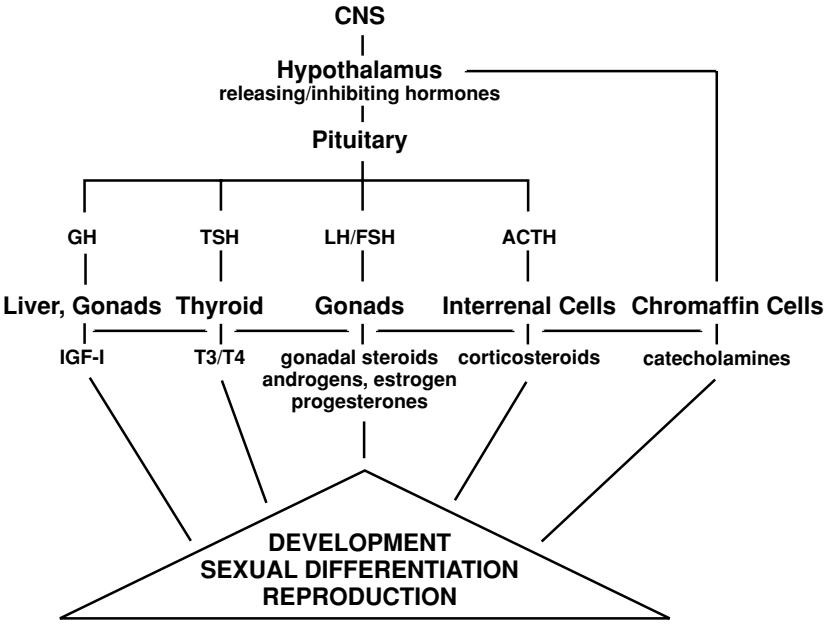

Figure 1. Endocrine systems regulating development and reproduction in amphibians and fish. Besides the main axes (hypothalamic–pituitary–gonad and hypothalamic–pituitary–thyroid), development and growth is triggered by cross talk with growth axis and with adrenal homologue comprised of two systems: the hypothalamic–pituitary–interrenal cell (adrenal cortex homologue) and the hypothalamic–autonomous nervous system–chromaffin cell (adrenal medulla homologue) axis. ACTH, adrenocorticotropic hormone; CNS, central nervous system; FSH, follicle-stimulating hormone; GH, growth hormone; IGF-1, insulin-like growth factor 1; LH, luteinizing hormone; TSH, thyroid-stimulating hormone.

Impacts of ED on Reproductive Biology of Amphibians and Fish

A comparison of ED effects on reproduction in amphibians versus fish is hampered by the fact that amphibians have not been studied in as much detail as fish and ED exposure has been examined for only a part of their life cycle. A recent survey[20] covers existing knowledge about ED affecting reproductive biology of anurans and includes a hypothesis of sexual differentiation, using the South African clawed toad *X. laevis* as model.

ED acting in amphibians and fish involve all components of the HPG axis. However, the main interferences of ED reported involve direct sex steroid receptor-mediated effects and direct or indirect effects concerning bioavailability of sex steroids. The main MOA so far determined are estrogenic, anti-estrogenic, androgenic, and anti-androgenic ones transmitted by direct binding to the binding pouch of the respective receptor or by binding to another part of the receptor and thereby changing its allosteric configuration.

In adult anurans, the impact of ED on the hypothalamic–pituitary complex has been shown in *Rana pipiens* where DHT and E2 adversely affect spermatogenesis, increase GnRH release, and decrease plasma levels of sex steroids and gene expression of LH.[22–24] Lower LH plasma levels following exposure to the estrogenic ED nonylphenol has been reported in *R. esculenta*.[25] Treatment with estrogenic, anti-estrogenic, androgenic, and anti-androgenic model compounds at physiological concentrations (10^{-8} mol/L) has been performed using adult *X. laevis*, investigating expression of

gonadotropins, liver biomarkers, sex steroid levels, and gonad histomorphology.[26–28] LH but not FSH expression decreased in response to ethinylestradiol (EE2) and methyldihydrotestosterone (MDHT). In males, T levels were decreased by EE2 as in females where the anti-estrogen tamoxifen caused an elevation of plasma E2. The most sensitive estrogenic hepatic biomarker was the increased expression of vitellogenin in males, while in females, which also showed and increased expression of vitellogenin, there was in addition a significant decreased expression occurred following tamoxifen treatment. The most sensitive parameter for detecting ED activities was gonad histomorphology; very strong histological changes were evident for all four model ED,[28] including induction of testicular oocytes by EE2 in males and spermatogenic nests in ovarian tissue of females by tamoxifen and MDHT. ED can also interfere with sex steroid-binding protein, but an impact of this phenomenon on reproductive physiology remains to be demonstrated.[4]

More recently, male *X. laevis* stimulated by human choriongonadotropin showed decreased mating call behavior as a result of the anti-androgen flutamide,[29] indicating that ED can also affect reproduction at the level of mating.

With regard to sexual differentiation that occurs during larval development of amphibians, it is generally accepted that estrogens always cause feminization[18,30]; however, reports on the impacts of androgens and anti-androgens are somewhat contradictory.[31,32] More recent findings using *X. laevis* revealed that estrogens, such as E2 and EE2, shifted sex ratios toward feminization,[33–35] as in *X. tropicalis* and *R. temporaria*[36]; the anti-estrogen tamoxifen led to neutralization causing underdeveloped gonads; the androgens MT and DHT induced significant increases of male phenotypes; and anti-androgens induced elevation of female phenotypes.[4,33] The potential basic mechanisms underlying sexual differentiation and potential impacts of ED are reviewed in detail,[20] including the ontogenetic courses of the sex steroid-synthesizing enzymes (5α-reductases and aromatase), sex steroid receptors, and gonadotropins (LH and FSH).

In fish, more standardized exposure regimes using ED exist compared to amphibians, including ecotoxicological model species, such as *Danio rerio*, *Pimephales promelas*, and *Oryzias latipes*, short-term and long-term treatments, and full life cycle or even multigenerational studies. ED addressing estrogenic MOA have been investigated extensively in fish. These generally cause feminization phenomena,[5] which are life-stage-dependent (as shown for *D. rerio)*,[37] impacting estrogenic biomarkers (including vitellogenin),[38] affecting migration behavior in Atlantic salmon,[39] or affecting courtship behavior in *D. rerio*.[40] Estrogenic responses, such as vitellogenin induction, are counteracted by anti-estrogens in Nile tilapia,[41] demonstrating in general that fish respond to estrogenic and to anti-estrogenic MOA. More recently, ED have been shown to interfere with aromatase, leading to altered estrogen availability and causing severe consequences to fish reproduction.[42] Androgenic and anti-androgenic MOA have been demonstrated in fathead minnow[43] and *O. latipes*,[44] impairing reproduction particularly of males. By far the most attention has been paid to the three-spined stickleback as an excellent model to assess (anti)estrogenic but in particular (anti)androgenic MOA[45–49] because of its unique androgenic biomarker, the androgen-dependent induction of spiggin protein in kidney. Spiggin protein is yet the only available pure androgen-dependent biomarker useful *in vivo* and *in vitro*, being the best model for studies of androgenic and anti-androgenic MOA. In addition, the three-spined stickleback also provides potential for assessing ED effects on reproductive behavior, as shown for estrogens.[50]

Recently, using advanced methodologies, estrogenic responses in fish have been investigated concerning their impacts on several other endocrine systems, such as the thyroid system, growth, and interrenal ceils.[51,52] The

existence of such cross talk between the endocrine systems has also been determined for anti-androgenic MOA (Fig. 1).[53]

The Thyroid of Amphibians and Fish

Functionally complete thyroid systems exist only in vertebrates and are represented by a common histological structure, the thyroid follicles, which are organized in a dispersed or condensed way and build up the thyroid gland.

Hypothalamic–Pituitary–Thyroid Axis in Amphibians and Fish

Endocrine feedback mechanisms regulate thyroid hormone (TH) levels via the hypothalamus–pituitary–thyroid (HPT) axis. TH, tetraiodothyronine (T4), and triiodothyronine (T3) (the latter possessing higher biological activity), trigger numerous physiological functions concerning metabolism and differentiation.[54,55] The hierarchic organization of the HPT axis is triggered by endogenous and exogenous stimuli from the central nervous system, which in turn causes the hypothalamus to secrete releasing factors acting on pituitary thyrotropes to release thyroid-stimulating hormone (TSH) into blood circulation. In contrast to higher vertebrates and adult animals where TSH-releasing hormone (TRH) is responsible for TSH secretion, amphibian larvae respond only to corticotropin-releasing hormone (CRH) as the hypothalamic stimulatory factor for pituitary TSH. In fish, marked differences exist concerning hypothalamus–pituitary regulation. Teleosts provide evidence that hypothalamic control is inhibitory by TRH and somatostatin, both inhibited hypophyseal TSH release except in salmonids where TRH and catecholamines are stimulatory. The main targets of TSH are the thyroid follicles, which respond with stimulated growth and development and TH synthesis. At the organizational level of thyroid follicles marked differences between amphibians and fish exist. In all amphibians, the follicles form a discrete thyroid gland, whereas in fish the morphological distribution of follicles has species-dependent variety with a general pattern of dispersed follicles located mainly in the pharyngeal region; however, they can also be found in other organs, such as heart, headkidney, liver, and gonad.[1,56] TSH increases enzymatic iodide uptake by follicular cells as a prerequisite for synthesizing TH. Furthermore, TSH stimulates the enzyme thyroid peroxidase, located at the apical membrane of follicular cells, which activates iodide for iodinating thyroglobulin and couples iodinated tyrosines (monoiodthyronine, diiodothyronine) to form T3 and T4. TH effects are mediated by binding to specific nuclear receptors. Thyroid receptors (TR) belong to the superfamily of nuclear hormone receptors and consist of a group of different isoforms that are localized in the nucleus. Binding of TH to TR is associated with several molecular events that lead to transactivation of TH-regulated gene expression, as described in detail by Shi.[55] In addition, the thyroid system provides further features for regulating the local bioavailability of TH by expressing different deiodinases (type 1 to 3) in various target organs for increasing or decreasing local TH concentrations. Furthermore, various enzymes involved in excretion of TH might also be involved in systemic circulation of TH. In general, ED might interfere preferentially with TH synthesizing or modifying enzymes, thus affecting local TH bioavailability rather than having direct TR-mediated effects. ED may also target TH-binding proteins, which affect systemic transport of TH in blood circulation.

The thyroid system provides the most obvious differences between amphibians and fish with respect to morphological organization and endocrine regulation; this might be related to the different significance that the endocrine system has for development in these classes of vertebrates.

Amphibian metamorphosis is the classical and unique example of endocrine regulation

of development by the thyroid system.[4,55,57,58] During this relatively short period of their life, amphibian larvae undergo a phase of extreme complex events in differentiation and growth and morphological changes, such as emergence and differentiation of limbs, resorption of tail, and reorganization of the gastrointestinal system, which are mainly under endocrine control of TH in coordination with other relevant hormones. In fish such dramatic developmental changes requiring TH are less pronounced and are dependent on species-specific life styles or life histories; examples include metamorphosis in flat fish, obligatory smoltification in anadromic fish (such as salmon), adaptation processes for changing osmoregulation, and color changes for reproduction. However, the significance and regulation of the thyroid system for developmental processes in ecotoxicological model species, such as *D. rerio*, *P. promelas*, and *O. latipes,* under normal laboratory husbandry conditions is not yet fully understood and might be underestimated because no obvious deteriorating developmental impacts (such as the arrest of metamorphosis in amphibians) can be observed as a result of the depletion of TH. In general, the amphibian model *X. laevis* possesses all components of the thyroid system as found in mammals except that during the larval phase the hypothalamic factor triggering the pituitary is CRH instead of TRH, which is used for regulation after completion of metamorphosis. Thus, amphibian metamorphosis provides the most sensitive sentinel for detection of ED affecting the thyroid system of higher vertebrates, including humans. In teleost fish, however, the large variety in appearance and regulation of the thyroid system makes it challenging to apply any findings of this system to different fish species.

Impacts of ED on the Thyroid System of Amphibians and Fish

In amphibians, ED, acting as stimulatory or inhibitory on the thyroid system of amphibian larvae, induce obvious morphological changes. Increased amounts of TH or TH mimetics accelerate metamorphosis of larvae, leading to smaller juveniles with reduced fitness. Decreased TH levels inhibit or even arrest metamorphosis. Recently, within the framework of the Organization of Economic Co-operation and Development (OECD) test guideline development, the establishment and validation of the amphibian metamorphosis assay using *X. laevis* has been a focus for screening ED affecting the thyroid system. Ongoing research in amphibians deals with the introduction of the most sensitive applicable biomarkers at different methodological levels, such as gross morphology for staging, thyroid histopathology, and TH-dependent gene expression, and corresponding proteomics. Currently, it seems likely that the histological assessment of the thyroid is the most sensitive biomarker, followed by changes in gene expression of the best established biomarkers (TRß and TSH expression) and by gross morphological observations.[59–62] Thus, the amphibian metamorphosis assay provides a unique sensitive detection system for screening ED affecting the thyroid system, and this assay has been validated for stimulatory as well as for inhibitory ED.

In fish, the impacts of thyroidal ED are less obvious and include hardly measurable parameters, for instance scale and coloration development in *P. promelas* is decreased by perchlorate, which inhibits iodine uptake for the formation of TH.[63] In *Gambusia holbrooki*, perchlorate also causes histopathological changes of thyroid follicles, which has been a much more sensitive indicator compared to measurements of whole body contents of T4.[64] Propylthiouracil, an inhibitor of thyroidperoxidase, has been assessed in zebrafish by histological and biochemical parameters, which also provides evidence for histopathology as the most sensitive tool to assess thyroid system disruption.[65] More recently, exposure to perchlorate has been shown to cause hermaphroditism and to impair reproductive behavior and swimming performance in the three-spined stickleback.[66,67] However, in light of the data concerning ED acting on

the HPT axis in fish, there are "currently no *in vitro* or *in vivo* assays in fish species that are sufficiently developed to warrant recommendation for use to efficiently screen chemicals for thyroid disruption" (p. 112).[68]

Recently, the impact of perchlorate as an antithyroidal ED has been demonstrated in wildlife, correlating thyroid histopathology with environmentally measured perchlorate concentrations. This has indicated that central stone rollers (*Campostoma anomalum*) and cricket frogs are impacted by thyroid follicle enlargement and follicle cell hypertrophy, respectively, indicating that antithyroidal ED affect wildlife populations of amphibians and fish.[69]

Naturally Derived ED Impacting Amphibians and Fish

Although there is much evidence that ED are anthropogenic in origin and contribute to environmental pollution with natural or synthetic compounds, natural conditions may also occur that act as ED without any anthropogenic causation. A large source of organic matter entering aquatic freshwater ecosystems via terrestrial–aquatic coupling is derived from leaves falling into water bodies where they start to decompose and release metabolites into surface water, contributing markedly to the concentration of dissolved organic carbon-bearing compounds with potential endocrine activities. Aqueous extracts from ground leaves of reed, beech, and oak demonstrated low estrogenic but potent anti-androgenic activities, as determined by yeast estrogen and yeast androgen screens.[70] In an investigation of the potential impact on sexual differentiation, larval *X. laevis* were exposed to the most potent leaf extract from oak at environmentally occurring concentrations of dissolved organic carbon. In male phenotypes a significant occurrence of lacunae and induction of oogonia was detected at 10 and 50 mg/L dissolved organic carbon, which is probably a result of the small estrogenic and high anti-androgenic activity of oak leaf extract. In accordance, pituitary LH-mRNA expression was elevated, indicating feedback mechanisms counteracting these anti-androgenic and estrogenic MOA of oak leaf extracts.[70]

Another natural source of ED is suspected to be parasites, such as *Ligula intestinalis* and microsporidae,[71] which has been investigated for *L. intestinalis* having low prevalence[72] and high prevalence[73] in its hosts, chub and roach. The latter study in roach revealed that ligulosis results in a block of gonadal development and reduces LH and FSH expression dramatically in the field and under laboratory conditions.

These examples demonstrate that ED are not only from anthropogenic origin, and verification is needed with respect to what extent naturally occurring ED might contribute to the overall endocrine disrupting activities present in surface waters. The abundant anti-androgenic activity of the River Lambro (Italy), for example, could not be correlated to any chemical pollutant.[6] In this case, a high concentration of dissolved organic carbon derived from a particular origin might explain such a high background level of anti-androgenic activity. We also point out that, when studying ED effects on fish in the wild, only nonparasitized animals are used because parasites can strongly impair endocrine function.

Cross Talk between Endocrine Systems by ED of Various MOA Derived from Pharmaceuticals Affecting Amphibians and Fish

The recently emerging issue of pharmaceuticals and pharmaceutically active compounds (PhACs) detected in considerable amounts in aquatic surface waters leads us to address the question whether, aside from the well-known estrogen contraceptive "pill" EE2 and androgenic anabolics (such as trenbolone), further PhACs with endocrine MOA can be found in the environment. Promising candidates for PhACs exhibiting endocrine

activities affecting aquatic vertebrates (hence ED) are corticosteroids, progestogins, ß-blockers, and ß-agonists.[74–76]

Corticosteroids, such as the synthetic dexamethasone, are used for anti-allergic and immunosuppressive treatment, and it seems reasonable that, in addition to synthetic corticosteroids, natural corticosteroids (such as cortisol, corticosterone, and aldosterone) also occur in aquatic surface waters released by sewage treatment plants. Corticosteroids are the circulating hormones of the hypothalamic–pituitary–interrenal (HPI) (adrenal cortex homologue) axis in amphibians and fish and thought to be involved in regulation of stress responses (Fig. 1).[1] However, in amphibians corticosteroids are thought to be involved in metamorphosis, interfering to some extent with the HPT axis.[4] Recently, the impacts of the natural corticosteroid corticosterone and of dexamethasone on amphibian metamorphosis have been investigated.[74] Both corticosteroids disrupt normal coordinated larval development of *X. laevis* by tissue-specific actions, leading locally to accelerating or retarding effects triggered by upregulation of prolactin mRNA and different regulation of deiodinases. Previously, corticosteroids have been thought to only cause acceleration or retardation of metamorphosis but not a severe disruption of the complex interplay of developmental processes triggered mainly by the thyroid system. Here corticosteroids interfere not only with their own HPI axis, which is important for stress and hydromineral regulation, but also with the thyroid system and prolactin, providing with dexamethasone a good example of an ED causing cross talk between endocrine systems.

Progestogins are used as a contraceptive in the "mini pill" in nearly pure form and in the classical "pill" in combination with EE2, suggesting that progestogins might be released into the environment and become even more abundant than EE2. In amphibians and fish, progesterones are thought to play an important role only for final gamete maturation, but no detailed investigations have been performed concerning their potential impact on sexual differentiation. Preliminary results[75] investigating the impact of the progestogin levonorgestrel during larval development of *X. laevis* demonstrated that levonorgestrel has no impact on the sex ratio but dramatically disrupts the testicular structure (Fig. 2) as well as ovarian tissue and suppresses gonadotropin expression concomitant with a delay of metamorphosis. Thus, in amphibians progestogins seem to be even more important for the regulation of the HPG axis, greatly affecting sexual differentiation and, in addition, cross talk with the HPT axis.

The wide use of PhACs, such as ß-blockers and ß-agonists, triggering blood pressure in humans led via excretion to their ubiquituous presence in anthropogenic impacted surface waters in a concentration of more than 1 μg/L. These PhACs act via ß-adrenoceptors, which are also present in amphibians and fish and bear a similar pharmacological profile as in mammals. ß-agonists substitute the naturally occurring catecholamines, adrenaline and noradrenaline, which are the circulating hormones released by the hypothalamic-autonomous nervous system-chromaffin cell (adrenal medulla homologue) axis. The ß-agonist isoproterenol has been used in preliminary exposures as a model ED for investigating the potential impact on *X. laevis* metamorphosis and sexual differentiation. Although there have been no obvious impacts on metamorphosis or on the sex ratio, as with the progestogin levonorgestrel a dramatic disruption of the testicular tissue has been observed (Fig. 2) while ovarian tissue appears to remain unaffected. In addition, gonadotropin expression is also diminished, indicating, again, unexpected cross talk between endocrine systems.

Discussion and Perspectives

Despite the fact that ecotoxicologists and endocrinologists tend to characterize ED preferentially by a single MOA, we have to consider that a true ED has the potential to interfere

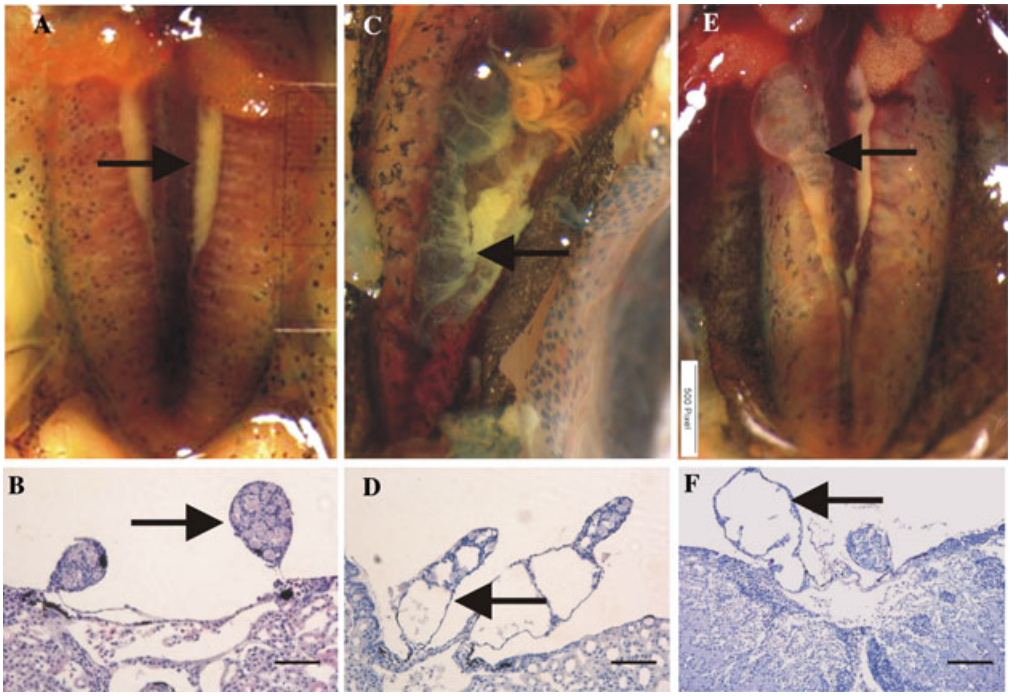

Figure 2. Gross morphology (**A, C, E**) and corresponding histology (**B, D, F**) of testes from *Xenopus laevis*. Tadpoles were treated in semistatic exposure from stage 48 onward until completion of metamorphosis of control (**A, B**), with the progestogin levonorgestrel at 5×10^{-7} mol/L (**C, D**) and with the ß-agonist isoproterenol at 5×10^{-6} mol/L (**E, F**). Both treatments revealed disruption of testicular morphology, as indicated by the appearance of "bubbles" (arrow). Bars represent 200 μm. (In color in *Annals* online.)

at several organizational levels within one endocrine system and to also affect several endocrine systems by disrupting or inducing their cross talk. It is obvious from an endocrinological point of view that endocrine systems communicate with each other. Thus, their functioning cross talk is the prerequisite for normal development and sexual differentiation during the larval phase and later on for growth, metabolism, and finally successful reproduction, triggered by gametogenesis and behavior. In order to investigate the risk of potential ED, we prefer to assess their impact during endocrine-sensitive life stages in whole animal models *in vivo*. However, *in vitro* screening assays might be very helpful for determining the exact mechanisms by which ED interfere with several parts of endocrine systems. Recent methodological approaches, such as ecotoxicogenomics[77] and proteomics, provide adequate technologies for obtaining detailed characterization of the MOA of ED involved in the cross talk between various endocrine systems and between endocrine, nervous, and immune systems.

Aquatic vertebrates, such as amphibians and fish, provide sentinel models for risk assessment of ED in aquatic ecosystems, using partial life cycle tests. Recent developments suggest that the anuran *X. laevis* provides a very sensitive model species to assess ED acting on reproduction and is likely the most useful sentinel for thyroid system disruption within the OECD test guideline framework for ED. In a similar reproducible way, at least for sexual differentiation during larval development, *X. laevis* can be used for a very sensitive established methodology.[78] The four principle MOA of sex steroids, (anti)estrogenic and (anti)androgenic ones, can be detected sensitively, but the role of progestins in amphibians and fish needs to be described concomitantly with the

detailed characterization of (anti)progestinic ED.

Fish model species can be used as sensitive sentinels because all types of (anti)estrogenic MOA, even during full life cycle or multigenerational studies, are advantageous compared to amphibians, whereas *X. tropicalis* might be the only recent candidate for full life-cycle studies. However, the only pure yet applicable (anti)androgenic biomarker has been found in fish; this is the protein spiggin, which sticklebacks produce in the kidney in response to androgens. Thus, the stickleback is a fascinating model for specifically addressing the role of ED in reproductive biology. ED assessment addressing the thyroid system in fish is hampered by several factors, including species-specific variations of the endocrine regulation of the HPT axis and the practical difficulties in determining thyroidal markers, such as thyroid histopathology or circulating TH levels.[68] Amphibian metamorphosis is, therefore, by far the best model for investigating (anti)thyroidal ED.[4]

As shown by ongoing research, more detailed investigation is needed on the cross talk between the various regulators of reproductive development and activities that lead to consequences at the population level and on other endocrine systems that trigger the thyroid system and the adrenal homologue, producing the stress hormones, catecholamines and corticosteroids. The emerging ecotoxicological issue of PhACs in the environment deals with several compounds possessing great endocrine disrupting potential on the HPG axis by direct MOA or by indirect cross talk, as shown for progestogins, corticosteroids, and ß-agonists. It is highly probable that among the PhACs many compounds exhibit ED activities that act on the endocrine systems of aquatic vertebrates and invertebrates. This requires intense ongoing research by comparative endocrinologists.

Conflicts of Interest

The authors declare no conflicts of interest.

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The Challenge Presented by Progestins in Ecotoxicological Research: A Critical Review

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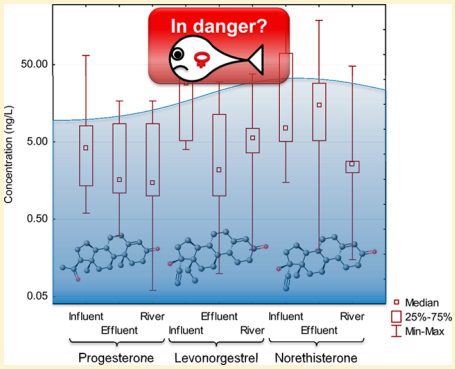
Supporting Information

ABSTRACT: Around 20 progestins (also called gestagens, progestogens, or progestagens) are used today in assisting a range of medical conditions from endometrial cancer to uterine bleeding and as an important component of oral contraception. These progestins can bind to a wide range of receptors including progestin, estrogen, androgen, glucocorticoid, and mineralocorticoid receptor, as well as sex hormone and corticosteroid binding globulins. It appears that only five of these (four synthetic and one natural) progestins have so far been studied in sewage effluent and surface waters. Analysis has reported values as either nondetects or low nanograms per liter in rivers. Seven of the progestins have been examined for their effects on aquatic vertebrates (fish and frogs). The greatest concern is associated with levonorgestrel, norethisterone, and gestodene and their ability to reduce egg production in fish at levels of 0.8−1.0 ng/L. The lack of environmental measurements, and some of the contradictions in existing values, however, hampers our ability to make a risk assessment. Only a few nanograms per liter of ethynodiol diacetate and desogestrel in water would be needed for fish to receive a human therapeutic dose for these progestins according to modeled bioconcentration factors. But for the other synthetic progestins levels would need to reach tens or hundreds of nanograms per liter to achieve a therapeutic dose. Nevertheless, the wide range of compounds, diverse receptor targets, and the effect on fish reproduction at sub-nanogram-per-liter levels should prompt further research. The ability to impair female reproduction at very low concentrations makes the progestins arguably the most important pharmaceutical group of concern after ethinylestradiol.



INTRODUCTION

The issue of endocrine disruption in fish caused by minute traces of steroidal estrogens brought to world attention the potential for harm in the environment due to the discharge of hormonal chemicals.[1] Even at very low concentrations, these steroidal hormones in natural waters can be harmful to aquatic organisms such as fish and amphibians.[2,3] Jobling et al.[4] demonstrated that both the natural and the synthetic estrogens play a major role in causing intersex in wild freshwater fish in rivers in the United Kingdom. A concentration of the synthetic estrogen, 17α-ethynylestradiol, as low as 5−6 ng/L was shown to cause the collapse of a wild fish population in a 7-year whole-lake experiment in Canada.[5] Additionally, there has been growing evidence that endocrine disrupting chemicals (EDCs) present in the environment might exhibit not only estrogenic but also antiestrogenic, (anti-)androgenic, or (anti-)progestogenic mode of action.[6] In light of these facts, recently, concern has been raised about the potential of progestins (also called gestagens, progestogens, or progestagens) to act as EDCs in aquatic wildlife.[7−10]

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The first orally active progestin "ethisterone" (pregneninolone, 17α-ethinyltestosterone) was synthesized by Inhoffen and colleagues in 1938.[11] Subsequently, the first oral contraceptive pill officially marketed in 1960 in the USA as Enovid and in the U.K. as Enavid contained the progestin norethynodrel (19-norethynodrel) together with the estrogen mestranol as active ingredients.[12] Since then a number of synthetic progestins have been introduced at the global market. Progestins are often used together with an estrogen, as active ingredients in oral contraceptives. In contrast to estrogen, there are many types of progestins used in various oral contraceptive brands and their content is usually higher (up to 100-fold) than that of estrogen, depending on the formulation and therapeutic application.[7] Besides oral contraception, progestins have a number of medical applications including hormone replacement therapy,[13] prevention of endometrial cancer,[14] treatment of dysfunctional uterine bleeding,[15] and palliative appetite stimulation for cancer patients.[16]

The worldwide consumption of progestins is not known; however, a few attempts have been made to estimate their consumptions in some European countries. For example, total annual consumption of progestins (up to 19 different molecules) in France and the United Kingdom is estimated to be about 12,800 and 1,700 kg, respectively (both for a 60 million population)[17,18] and that in the Czech Republic 2,400 kg/year (for a 10 million population).[19] The most consumed progestin in the United Kingdom was the medroxyprogesterone (MEP; 530 kg/year), whereas dihydrogesterone (745 kg/year) is the most popular in France.[17,18] With their widespread use around the globe and their potential to disrupt nontarget organisms in aquatic environment a review of their threat is needed. Therefore, the objectives of this review were as follows: (a) examine the chemical and biological properties of progestins; (b) assess the potential biological effects of progestins on aquatic organisms; (c) review different analytical approaches and their monitoring results for progestins in sewage and rivers; (d) assess the risk to aquatic organisms from predicted exposure to the progestins; (e) identify knowledge gaps and future research needs.

CLASSIFICATION AND PROPERTIES OF PROGESTINS

Synthetic progestins are derived from either progesterone (P4) or the closely related androgenic steroid, testosterone (Figure 1; Table 1). P4 derivatives further can be subdivided into 17α-hydroxyprogesterone (pregnanes) and 19-norprogesterone (norpregnanes) groups. All P4 derivatives have a $COCH_3$ group at the C17 and a double bond at the C4 position. 17α-Hydroxyprogesterone derivatives are characterized by a methyl group at the C10 position, whereas 19-norprogesterone derivatives do not have a methyl group at similar position. The 17α-hydroxyprogesterone group includes medroxyprogesterone acetate (MPA), MEP, chlormadinone acetate (CMA), and cyproterone acetate (CPA). The 19-norprogesterone group include nomegestrol acetate (NGA), nestorone (NES), and trimegestone (TRI).

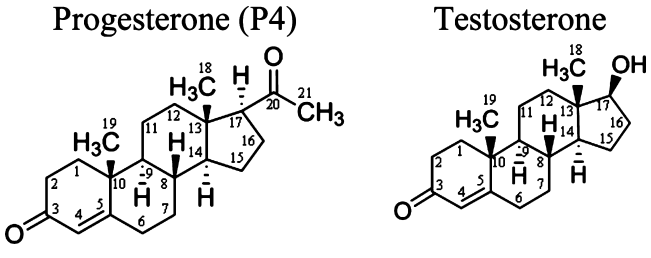

Figure 1. Basic structure of P4 and testosterone which are precursors to synthetic progestins.

Testosterone derivatives are also known as the 19-nortestosterone group and generally characterized by the lack of a methyl group at the C19 position. 19-Nortestosterone derivatives are further classified as estranes and gonanes. Estranes are characterized by a methyl group at the C13 position but no methyl group at the C10 position and no side chain at the C17 position. Ethisterone (ETH), norethisterone (NET) [also known as norethindrone] norethisterone acetate (NEA) [also known as norethindrone acetate], ethynodiol diacetate (EDA), and dienogest (DIE) belong to this group. Gonanes are generally characterized by a lack of a methyl group at the C13 position. Gonanes include levonorgestrel (LNG), etonogestrel (ENG), norelgestromin (NGMN), desogestrel (DSG), norgestimate (NTE), and gestodene (GES). A spironolactone derivative (drospirenone, DRO), which has a unique pharmacologic profile similar to that of P4, has been developed recently.

Octanol–water partitioning coefficients (log K_{ow}) of P4 and synthetic progestins range from 2.97 (NET) to 5.65 (DSG)[20] (Table 1). In terms of polarity, compounds with log K_{ow} between 1.5 and 4 would be considered to be moderately polar, whereas compounds with log $K_{ow} > 4$ would be considered as nonpolar or hydrophobic.[21] The more hydrophobic the compound, the higher the proportion of sorption to sewage particles in wastewater might be expected.

Receptor Interactions of Progestins. The biological activity of progestins is predominately mediated via nuclear progestin receptors (PRs) which are members of the nuclear receptor superfamily and act as ligand-dependent transcription factors. In humans, most rodents, and chicken, two PR isoforms (PR-A and PR-B) are transcribed from a single gene by the use of different promoters.[22] PRs have been also cloned and characterized in several other vertebrate classes including amphibians[23−25] and fish.[26−32] With the exception of Japanese eel[29,30] there is good evidence that in teleost fish, nuclear PRs are encoded by a single gene[32] which can give rise to different isoforms by means of differential splicing.[31] Although designed to interact with human PR and mimic the action of P4, progestins can bind in mammals with varying affinities to a range of other nuclear receptors such as the androgen receptor (AR), estrogen receptor (ER), glucocorticoid receptor (GR), and mineralocorticoid receptor (MR)[31,33−35] (Table 2 and Supporting Information (SI) Table S1). The greatest off-target effects of progestins have been reported for AR, GR, and MR.[36] Their metabolites arising from biotransformation can also show binding activity. For instance, metabolites of LNG and NET (SI Table S1 and Table 2) can display estrogenic activity absent with the parents.[37,38] However, in lower vertebrates little information is available about possible receptors mediating effects of progestin exposure. The few studies available so far indicate that, with the exception of DRO, most synthetic progestins do not bind to fish PRs,[36,39,40] whereas NET, MPA, LNG, ENG, and GES are able to activate the fish ARs.[36] Furthermore, activation of medaka GR and MR as well as transactivational modulation by progestins has been demonstrated recently by means of transactivation assays.[41] Besides their genomic action mediated by nuclear PR, progestins exert rapid effects in target tissues independently of *de novo* transcription.[42] The best examples for such nongenomic effects

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Table 1. Classification and Physicochemical Properties of Progestins[a,b]

Structural derivation			Compound	Abb	Molecular formula	Molecular weight	CAS	Log *K*ow	Structure
	Progesterone		Progesterone	P4	C21H30O2	314.46	57-83-0	3.67*/ 3.87**	
Structurally related to progesterone	17α-Hydroxyprogesterone		Medroxyprogesterone acetate	MPA	C24H34O4	386.52	71-58-9	4.09*	
			Medroxyprogesterone	MEP	C22H32O3	344.49	520-85-4	3.50*	
			Chlormadinone acetate	CMA	C23H31ClO4	406.94	302-22-7	3.85*	
			Cyproterone acetate	CPA	C24H29ClO4	416.94	427-51-0	4.18*	
	19-Norprogesterone		Nomegestrol acetate	NGA	C23H30O4	370.48	58652-20-3	3.55*	
			Nestorone	NES	C23H30O4	370.48	7759-35-5	3.63*	
			Trimegestone	TRI	C22H30O3	342.47	74513-62-5	3.58*	
Structurally related to testosterone	19-Nortestosterone	Estranes	Ethisterone	ETH	C21H28O2	312.45	434-03-7	3.44*/ 3.11**	
			Norethisterone	NET	C20H26O2	298.42	68-22-4	2.99*/ 2.97**	
			Norethisterone acetate	NEA	C22H28O3	340.46	51-98-9	3.99*	
			Ethynodiol diacetate	EDA	C24H32O4	384.51	297-76-7	5.22*	
			Dienogest	DIE	C20H25NO2	311.42	65928-58-7	2.34*	
		Gonanes	Levonorgestrel	LNG	C21H28O2	312.45	797-63-7	3.48*	
			Etonogestrel	ENG	C22H28O2	324.45	54048-10-1	3.89*/ 3.16**	
			Norelgestromin	NGMN	C21H29NO2	327.46	53016-31-2	3.98*	
			Desogestrel	DSG	C22H30O	310.47	54024-22-5	5.65*	
			Norgestimate	NTE	C23H31NO3	369.5	35189-28-7	4.98*	
			Gestodene	GES	C21H26O2	310.43	60282-87-3	3.26*	
	Spironolactone derivative		Drospirenone	DRO	C24H30O3	366.49	67392-87-4	4.02*	

[a]Single asterisks (*) indicate that the estimates of log K_{ow} are based on EPA's EPIWeb program [KOWWIN (v1.68)]. [b]Double asterisks (**) indicate values taken from ref 20.

are the induction of final oocyte maturation in female amphibians and fish[43] and the stimulation of sperm hypermotility in male fish.[42] Rapid progestin signaling has been attributed to membrane-localized forms of the nuclear PR as well as to membrane G-protein-coupled progestin receptors belonging to the progestin and adipoQ receptor (PAQR) vertebrate family of

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Table 2. Relative Binding Affinities (RBA) of Progestins to Various Human Steroid Receptors and Blood Plasma Proteins[a]

	RBA (%)						
progestins	PR	ER	AR	GR	MR	SHBG	CBG
P4	100[48b,49c]; *30[50d]	<0.1[50d]	9.6[49c]; **0.6[51d]	5.6[52b]; 6.3[53d]	100[54b]; 1000[48b]	<0.5[55]; *0[46]	36[46]
MPA	298[56b]; 72[49c]	<0.02[56b]	36[56b]; 4.8[49c]; **10[51d]	42[53d]; 58[56b]; 79.1[52b]	3.1[56b]	*0[46]	<0.3[57]
CPA	7.5[49c]	0[46e]	1[49c]; **2.1[51d]	5.1[53d]	8[46e]	*0[46]	0[46]
NGA	125[58e]	0[58e]	42[58e]	6[58e]	0[58e]	*0[58]	0[58]
NES	244[49c]	0[58e]	0.006[49c]	38[58e]		0[59]	
TRI	588[56b]	<0.02[56b]	2.4[56b]	13[56b]	42[56b]		
NET	134[56b]; 80[49c]	0.15[56b]	55[56b]; 19[49c]	1.4[56b]; 0.1[53d]	2.7[56b]	19.5[57]; *16[46]	0.4[57]
NEA	*20[60e]	1[60e]	**2.7[51d]	0.9[52d]			
DIE	1.2[49c]	0[46e]	0.15[49c]	1[46e]	0[46e]	0.4[57b]	0.3[57]
LNG	323[56b]; *250[50d]; 150[49c]	<0.02[56b]; <0.1[50d]	58[56b]; 49[49c]; **16[51d]	7.5[56b]; 0.4[53d]	17[56b]	87[55]; *50[46]	0[46]
ENG	325[49c]; *180[50d]	<0.1[50d]	18[49c]	1.6[53d]	0.5[54b]	51[55]; *15[46]	0[46]
NGMN	18[61b]	0[62e]	*18[63b]	8[62e]	80[62e]		
DSG	*1[46e]	0[62e]	**0.06[51d]	0.1[53d]		*0[62]	0[62]
NTE	9[61b]; 35[49c]; *15[60e]	0[46e]	0.18[49c]; *25[63b]	1[46e]	0[46e]	<0.5[55]; *0[46]	0[46]
GES	864[56b]; *70[50d]	<0.02[56b]; <0.1[50d]	71[56b]; **13[51d]	38[56b]	97[56b]; 11[54b]	202[55]; *40[46]	0[46]
DRO	19[48b]; *20[50d]	<0.1[50d]	**0.3[51d]	3[48b]	500[48b]	*0[46]	0[46]

[a]Abbreviations: PR, progestin receptor; AR, androgen receptor; ER, estrogen receptor; GR, glucocorticoid receptor; MR, mineralocorticoid receptor; SHGB, sex hormone binding globulin; CBG, corticosteroid binding globulin. Reference ligands (100% RBA) were as follows: PR, progesterone (values without asterisk (no *)) and promegestone (values marked with asterisk (*)); AR, testosterone (no *), testosterone acetate (*), and dihydrotestosterone (values with double asterisks (**); ER, estradiol-17-β (no *); GR, dexamethasone (no *) and corticosterone (*); MR, aldosterone (no *); SHBG, testosterone (no *) and dihydrotestosterone (*); CBG, cortisol (no *). [b]Human recombinant receptor (hrR). [c]Yeast-based assays with hrR. [d]Natural receptor (obtained from human cells). [e]No information available.

7-transmembrane proteins.[42] Further putative receptors (or coreceptors) associated with the cell membrane mediating nongenomic effects include progestin receptor membrane components 1 and 2.[42] Of the commonly used synthetic progestins only a few have been tested regarding their affinity to human or fish G-protein-coupled membrane PRs and most of them display a marked lower affinity than the natural ligands.[44,45]

In addition to the interaction with nuclear and membrane receptors, some progestins such as P4 display relatively high affinity to corticosteroid binding globulin (CBG) in the blood plasma,[46] whereas GES, LNG, ENG, and NET exclusively bind to sex hormone binding globulins (SHBG).[46] In fish, binding to SHBG present in the gills is thought to facilitate progestin uptake from the water, thus enhancing their biological potency.[47]

Biological Functions of Progestins. Natural progestins are steroid hormones found in all vertebrates. While in tetrapods, P4 is the major progestin, in fish P4 plasma levels are usually low and other progestins, in particular 17,20β-dihydroxypregn-4-en-3-one (17,20β-P) and 17,20β,21-trihydroxypregn-4-en-3-one (17,20β-S), predominate.[64,65] Together with estrogens and androgens, progestins are important reproductive steroid hormones in all vertebrates. In female mammals, P4 is primarily produced by the *corpus luteum* and, during pregnancy, by the placenta. It is involved in ovulation, plays a key role for ovum implantation as well as maintenance of pregnancy, and facilitates differentiation of the mammary gland.[66] P4 is also crucially involved in regulating female reproductive cyclicity by modulating gonadotropin synthesis and secretion, involving both endocrine and local actions. In that context, recent studies suggest that the estrogen-induced preovulatory surge of luteinizing hormone (LH) is particularly mediated by *de novo* (neurosteroidal) produced P4 and its metabolite allopregnanolone in the hypothalamus.[67,68] In lower vertebrates such as amphibians and fish, progestins are well-known as inducers of final oocyte maturation in females.[43] In male fish, progestins promote spermiation, increase milt production, and facilitate sperm motility.[65,69] Furthermore, in cyprinids, 17,20β-P is released by females as a pheromone triggering courtship behavior and milt production in males.[65,70] Besides their well-documented roles during final reproductive events in fish, progestins are also involved in the regulation of gonad development during early steps of gametogenesis.[71−73] In addition to their importance for reproduction, progestins are pleiotropic hormones modulating many physiological functions, e.g., the immune system[74] or the cardiovascular system.[75] Furthermore, the role of progestins for neuronal homeostasis and their neuroregenarative and neuroprotective actions received increasing attention over the past decade.[76]

EFFECTS ON AQUATIC VERTEBRATES

So far, ecotoxicological studies on progestins have been predominantly done in fish and to a lesser extent in amphibians, namely, the African clawed frog (*Xenopus laevis* (*X. laevis*)) and the tropical clawed frog (*Xenopus tropicalis* (*X. tropicalis*)). Since progestins are important reproductive hormones and synthetic progestins are used, e.g., for contraception, most studies focused on reproduction. Of the progestins of concern only P4 and 7 of the 19 synthetic molecules have been tested on aquatic wildlife. Therefore, for less than half of the synthetic progestins we have no published information about possible biological effects on aquatic vertebrates.

Of the 7 progestins so far tested (MPA, CPA, NET, LNG, DSG, GES, and P4), 6 of them have been shown to adversely affect fecundity of female fish (Table 3). The most potent of these were LNG reducing egg production in fathead minnow at 0.8 ng/L,[7] with GES[9] and NET[77] similarly reducing egg production in the fathead minnow at 1 ng/L. Japanese medaka were slightly less sensitive with reduced egg production at 25 ng/L NET.[77] A very high concentration of 10 μg/L of DSG was needed to reduce fathead minnow egg production.[9] As with fish, disrupted oocyte development in female tropical clawed frog (*X. laevis*) occurred at 1.3 ng/L LNG.[78] Occasionally, some

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Table 3. Summary of Effects Induced by Progestins (Ascending Order of Concentration) In Fish and Amphibians[a]

Trophic group	Species	Age class	Test	Effect	LOEC (ng/L)	Progestin	Reference
Fish	Fathead minnow (*Pimephales promelas*)	Adult	21-day	Females: ↓ egg production	0.8	LNG	7
			21-day	Females: ↓ egg production	1	NET	77
			7-day *in vivo* + 24-h *ex vivo*	*Ex vivo* ovarian production ↓ 11-KT	1	LNG	88
			21-day	Females: development of male secondary characteristic, ↓ egg production	1	GES	9
	Mummichog (*Fundulus heteroclitus*)	Adult (recrudescing)	14-day *in vivo* + 24-h *ex vivo*	*Ex vivo* ovarian production ↓ T and ↓ E2	1	CPA	97
	Zebrafish (*Danio rerio*)	Embryo	48-hour	↑ *ar* and *gr* mRNA expression	2	P4	8
			96-hour	↑ *pgr* and *ar* mRNA expression			
			144-hour	↑ *vtg1* mRNA expression			
			48-h	mRNA expression of ↑ *esr1* and ↑ *mr*	2	NET	
			48-h	mRNA expression of ↓ *ar*	2	LNG	
			144-h	mRNA expression of ↑ *pgr*			
		Adult	14-day	Females: mRNA expression of ↓ *vtg1* in the liver, ↓ *ccnb1*, ↓ *zp3*, ↑ *arnt2*, and ↑ *gr* in the brain, and ↓ *nr1d1* in the ovary	3.5	P4	89
	Three-spined stickleback (*Gasterosteus aculeatus*)	Adult	21-day	Females: mRNA expression of ↓ spiggin in the kidney	5.5	LNG	80
			45-day	Males: ↑ KEH	6.5		85
	Fathead minnow (*Pimephales promelas*)	Adult	21-day	Females: mRNA expression of ↓ *vtg* in the liver, ↓ egg production, ↓ fertilization	10	P4	98
			21-day	Females: ↓ plasma E2	10	NET	77
		Larvae	28-day	mRNA expression of ↓ *hsd3β*, ↓ *hsd20β*, ↓ *cyp17*, ↓ *ar*, ↓ *esrα*, ↓ *fshβ*	16.3	LNG	88
	Zebrafish (*Danio rerio*)	Adult	14-day	Females: ↑ egg production	25	P4	79
			21-day	Females: ↓ GSI			79
			21-day	Females: mRNA expression of ↓ *cyp11b*, ↓ *hsd17b3*, ↓ *pgr* in ovary ↓ *ar* in brain			99
	Japanese medaka (*Oryzias latipes*)	Adult	28-day	Females: ↓ egg production	25	NET	77
	Roach (*Rutilus rutilus*)	Pubertal	28-day	Males: ↓ plasma 11-KT and ↑ spgB (%) in the testes	31	LNG	83
	Three-spined stickleback (*Gasterosteus aculeatus*)	Adult	21-day	Females: mRNA expression of ↑ spiggin and ↓ *cyp1a* in the kidney and ↓ *vtg* in the liver, kidney hypertrophy	40	LNG	80
	Zebrafish (*Danio rerio*)	Adult	14-day	Females: ↓ proportion of late VTG oocytes, mRNA expression of ↓ *vtg1* in the liver, ↓ *nr1d1* in the brain	55	DRO	90
	Three-spined stickleback (*Gasterosteus aculeatus*)	Adult	45-day	Males: ↑ KEH, ↑ NSI, inhibition of the onset of spermatogenesis, mRNA expression of ↑ spiggin and ↓ *cyp1a* in the kidney	65	LNG	85
	Fathead minnow (*Pimephales promelas*)	Larvae	28-day	↓ growth, mRNA expression of ↓ *hsd3β*, ↓ *hsd20β*, ↓ *cyp19a*, ↓ *fshβ*	86.9	LNG	88
		Adult	7-day *in vivo* + 24-h *ex vivo*	Ex vivo ovarian production ↓ pregnenolone, ↓ 17,20-DHP, ↓ T, ↓ 11-KT in ovaries	100		
	Zebrafish (*Danio rerio*)	Embryo (F1 generation of above fish)	120-h	mRNA expression of ↓ *gr*, ↓ *pgr*, ↓ *cyp11b*, ↓ *ar*	254	P4	99
	Fathead minnow (*Pimephales promelas*)	Adult	7-day	Males: ↓ sperm motility	300	P4	86
	Roach (*Rutilus rutilus*)	Pubertal	28-day	Males: ↓ plasma 11-KT, ↑ spgB (%) in the testes, and ↓ mRNA expression of *fshβ*; Females: ↓ plasma E2	312	LNG	83
	Common carp (*Cyprinus carpio*)	Juvenile	12-hour* *in vitro*	↓ phagocytic activity of head kidney derived macrophages	314	P4	95
			3-day	↓ NO production by head kidney derived macrophages			
	Fathead minnow (*Pimephales promelas*)	Larvae	28-day	↓ survival	462	LNG	88
		Adult	4-hour *ex vivo*	Females: mRNA expression of ↓ *aqp8*, ↑ *hsd20β*, ↑ *foll*, ↑ *p38mapk*, and ↑ *zp3* in the ovary	500	P4	100
		Egg to Larvae	28-day	↓ growth	740	NET	101
	Three-spined stickleback (*Gasterosteus aculeatus*)	Adult	45-day	Males: ↑ KEH, ↑ NSI, inhibition of the onset of spermatogenesis, mRNA expression of ↑ spiggin and ↓ *cyp1a* in the kidney, ↑ *lhβ* in the pituitary	750	LNG	85
	Western mosquitofish (*Gambusia affinis*)	Adult	8-day	Males: mRNA expression of ↓ *esrα*, ↓ *esrβ*, ↓ *arβ*, and ↓ *mt* in the testis and ↓ *arβ* in the liver	1000	P4	102
	Japanese medaka (*Oryzias latipes*)	Egg to Adult	3-month	Females:↓ oogenesis; Males: ↓ spermatogenesis, ↓ mean body weight, ↓ CF	1000	CPA	103
	Roach (*Rutilus rutilus*)	Pubertal	28-day	Males: ↓ plasma 11-KT, mRNA expression of ↓ *fshβ*, ↑ *lhβ*, ↑ *vtg*, ↑ *esr1*; Females: plasma ↓ E2, ↓ 11-KT, ↑ T, and mRNA expression of ↓ *fshβ*, ↑ *lhβ*, ↑ *vtg*, ↑ *esr1*	3124	LNG	83
	Common carp (*Cyprinus carpio*)	Juvenile	96-hour	Immunosuppression (↓ NO2- formation by the head and trunk kidney derived leukocytes stimulated *in vitro*)	3900	MPA	92
	Fathead minnow (*Pimephales promelas*)	Adult	21-day	Females: ↓ egg production	6500	DRO	7
			21-day	Females: development of male secondary characteristic, ↓ egg production, plasma ↓ E2, ↓ T, and ↓ VTG	10000	DSG	9

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Table 3. continued

Trophic group	Species	Age class	Test	Effect	LOEC (ng/L)	Progestin	Reference
Amphibian	Tropical clawed frog (*Xenopus tropicalis*)	Adult	28-day	Females: ↓ oocyte development	1.3	LNG	78
	African clawed frog (*Xenopus laevis*)	Adult	96-hour	Males: mate calling behaviour - ↑ proportion of advertisement calling	31	LNG	81
	Tropical clawed frog (*Xenopus tropicalis*)	Tadpole to Adult	3-month exp. + 9-month in LNG-free water	Females: lacking oviduct, ↓ oocyte development and ↓ fertility	156	LNG	104
	African clawed frog (*Xenopus laevis*)	Tadpole	80-day	Females: mRNA expression of ↑ *tshβ*, ↑ *fshβ* mRNA in the brain-pituitary tissue; Males: mRNA expression of ↑ *tshβ* in the brain-pituitary tissue	312	LNG	84,96
			80-day	Males: inhibition of metamorphosis; inactivation of thyroid; mRNA expression of ↓ *lhβ*, ↓ *fshβ*, and ↑ *tshβ* in the brain-pituitary tissue; ↓ *star*, ↓ *p450scc*, ↓ *srd5a2* in the gonads; Females: inhibition of metamorphosis; inactivation of thyroid; mRNA expression of ↓ *lhβ* in the brain-pituitary tissue; ↓ *cyp19a*, ↑ *srd5a1* in the gonad	3124	LNG	84,96

[a]Value marked by an asterisk (*) is an estimated time (cells cultivated overnight). Abbreviations: 11-KT, 11-ketotestosterone; 17,20-DHP, 17*α*,20*β*-dihydroxyprogesterone; ah-r, aryl hydrocarbon receptor; aqp8, aquaporin 8; ar, androgen receptor; arnt2, aryl hydrocarbon receptor nuclear translocator; cat, catalase; ccnb1, cyclin B1; CF, condition factor; cyp1a, cytochrome P450, family 1, member A1; cyp11b, 11*β*-hydroxylase; cyp19a, gonad-type aromatase; d2, deiodinases type 2; d3, deiodinases type 3; E2, oestradiol; esr, estrogen receptor; foll, follistatin; fsh*β*, follicle-stimulating hormone (*β*-subunit); GSI, gonado-somatic index; gr, glucocorticoid receptor; hsd3*β*, hydroxysteroid (3-*β*) dehydrogenase; hsd17*β*3, hydroxysteroid (17-*β*) dehydrogenase 3; hsd20*β*, hydroxysteroid (20-*β*) dehydrogenase; KEH, kidney epithelium height; LOEC, lowest observed effect concentration; mpr, membrane progestin receptors; mr, mineralocorticoid receptor; mt, metallothionein; nis, sodium-iodide symporter; nr1d1, nuclear receptor subfamily 1, group D, member 1 (involved in circadian rhythm); NSI, nephrosomatic index; p38mapk, p38 mitogen-activated protein kinase; p450scc, P450 side-chain cleavage enzyme; p-gp, P-glycoprotein; pgr, progestin receptors; prl, prolactin; spgB, spermatogonia B; srd5a1(or 2), steroid-5-*α*-reductase type 1 (or 2); star, steroidogenic acute regulatory protein; *t*, testosterone; tsh*β*, thyroid stimulating hormone (*β*-subunit); vtg, vitellogenin; zp3, zona pellucida 3.

progestins showed, depending on the concentration, also stimulating effects on egg production, although further experiments are necessary to confirm such nonmonotonic dose responses.[79] In addition to reduced fecundity, progestins such as LNG, DSG, GES, and NET lead to masculinization phenomena and induced male secondary sex characteristics and androgenic biomarkers in female fish[7,9,77,80] and amphibians.[81,82] These findings are consistent with the observation that several progestins are able to activate the fish AR.[36] Furthermore, in a recent behavioral study, LNG but not P4 significantly increased the proportions of advertisement callings in male *X. laevis*, a behavior which is known to be dependent on androgens.[81] Progestin exposure also affected male reproduction by disruption of testicular differentiation and development[83−85] or by inhibiting sperm motility,[86] although these effects were observed at higher concentrations at tens or hundreds of nanograms per liter.

The mechanisms which mediate endocrine disruption by progestins are, however, poorly understood. They are likely to be very complex and not congruent for different progestins. In general, it seems that modulated gonadotropin expression in the pituitary[83−85,87,88] and changes of plasma sex steroid levels[9,77,83] underlie many of the reproductive effects such as reduced fecundity or disturbed gonad development in aquatic vertebrates. However, the progestin concentrations necessary to induce significant changes in sex steroids and gonadotropin expression are usually higher than these reported to decrease egg production. Progestins can potentially interact at all levels of the hypothalamus−pituitary−gonad axis, and several additional mechanisms might be involved including a reduction of gonadotropin plasma levels (particularly LH) or changes in the expression of genes encoding for steroid receptors or genes regulating the circadian rhythm in the brain.[89,90] In addition, some fish use steroidal pheromones to coordinate reproductive behavior and spawning.[65,70,91] Natural progestins such as 17,20*β*-P and their conjugated forms have been shown to exert a strong olfactory response mediating their pheromonal action. A detection threshold of as low as 0.3 ng/L 17,20*β*-P has been reported for the goldfish olfactory epithelium.[70] Given this high sensitivity, it might be worth exploring the potential effects of synthetic progestins on pheromonal communication and coordination of reproductive behavior in fish.

Although the majority of ecotoxicological studies focused on reproduction, there are also reports about other physiological changes after progestin exposure. These include immune suppressive effects of progestins on leukocytes from carp[92−95] as well as disruption of the thyroid system in *X. laevis* tadpoles.[96] In addition, progestins have been shown to alter the expression of a variety of genes in different tissues including GR and MR mRNA.[89,90] Together with recent findings that some progestins are able to activate/modulate medaka GR and MR transactivation, this indicates the potential of progestins to interfere with the endocrine stress axis as well.[41] With regard to nonreproductive effects, it is important also to mention here that there are no studies in lower vertebrates about any effects of progestins on tissues such as the heart or the consequences of progestin exposure on neuroprotection/neuroregenartion in the brain.

In summary, an increasing number of papers have been published recently which investigated progestin effects on various biological end points in aquatic vertebrates. Among these, the most striking observation of progestins exposure has been that on egg production in female fish and frogs at concentrations around 1 ng/L for LNG, GES, and NET. It is still unclear what effects if any NGA, NES, TRI, NEA, DIE, NGMN, and NTE would have on aquatic wildlife.

OCCURRENCE IN THE ENVIRONMENT

Analytical Approaches. Given the low and sub-nanogram-per-liter concentrations of natural and synthetic hormonal active substances in the environment, very sensitive analytical methods are vital to progress research. While some progress has been made, some analytical concerns still persist. These include the use of only a few grab samples for method development[105−107] and insufficiently low detection limits.[105,108,109] Insufficiently low detection and quantification limits of the analytical methods

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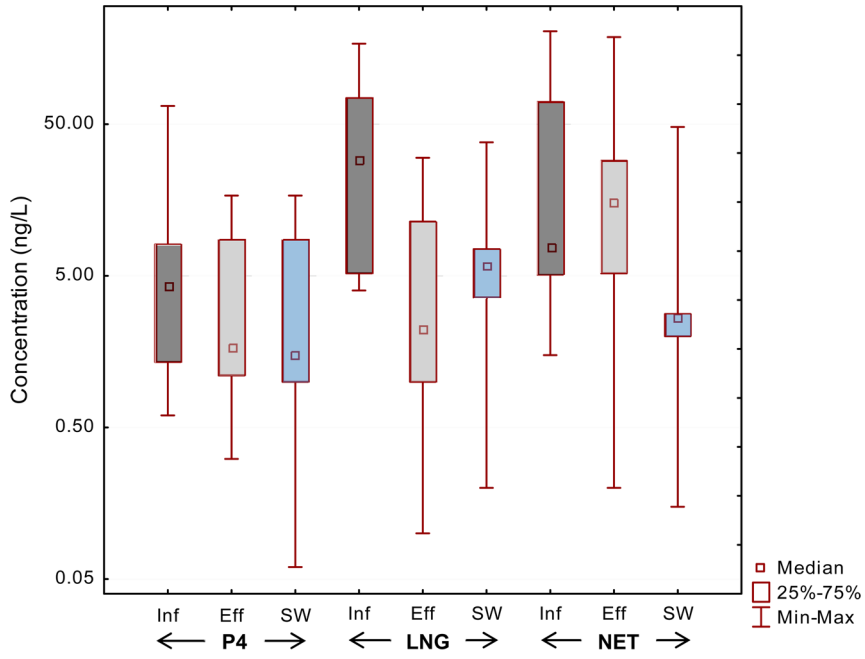

Figure 2. Box-and-whisker plot of literature positively reported concentrations of progestins in sewage influent (Inf), effluent (Eff) and surface water (SW).

(LODs and LOQs) have led to many "non-detects" in the literature, leaving researchers unsure whether the compounds are little used or possibly completely eliminated in sewage treatment? Because unknown active progestogenic chemicals may also be distributed in the aquatic environment,[110] a qualitative together with a quantitative (targeted chemical analysis) approach would be necessary. Of the 20 progestins discussed in this review, methods have only been reported for 5 of them.

Chemical Assays for Quantitative Analysis. The existing extraction methods involve mostly off line solid phase extraction (SPE) and in some cases an extract cleanup process using silica gel cartridges (SI Table S2).[106,111] Alternatively, C18 as an SPE sorbent with online coupling with LC can also be used.[107] LC-based methods have advantages over[106,111−114] GC-based methods, being more versatile, less complicated, and needing less labor intensive sample preparation (i.e., sample cleanup and derivatizations). On the other hand, GC-based methods are sensitive and have been reported for some progestins,[112,115,116] but these have been less widely used.

To adequately detect a wide range of progestins, different detectors have been used (SI Table S2). Mass spectrometry using electrospray ionization (ESI) in the positive mode is the most common detector used in progestins analyses.[105,106,108,111,113,116−119] Using single MS with LC for the trace analysis in wastewater matrix can be dangerously misleading. Relying on single MS ultimately could lead to false positive results, particularly for the environmental samples containing complex matrices of organic matter and other competing chemicals. In contrast, tandem mass spectrometric detection, coupled with GC or to LC, has substantially improved the chromatographic methods of progestins analysis by reducing their detection limits.[111,117,120] While tandem MS is an excellent technique for targeted analysis of progestins,[105,106,108,111,116,117] identification of the whole range of synthetic progestins together would be challenging for a single run method, since they have dissimilar physicochemical properties. Only if high-resolution mass spectrometry (HRMS) is connected to GC or LC might this be possible. Advancements in HRMS techniques have helped to identify unknown compounds and metabolites in environmental matrices[121,122] and could be further used as identification tools for suspected and unknown progestogenic chemicals in the aquatic environment.

In addition, diode array detector (DAD)[123,124] and the ultraviolet detector (UV) have also been used to a lesser extent to detect progestins in environmental samples.[125,126] Bioanalytical approaches such as enzyme-linked immunosorbent assay (ELISA) could provide an alternative approach for quantifying progestins in water samples. As confirmed by the HPLC, ELISA for LNG analysis can be used for real environmental samples.[125,126] However, this technique has some disadvantages that may make it difficult to adequately assess structurally similar progestins in environmental samples. For example, only one progestin can be measured from a sample unless chromatography is employed to separate the progestins. In addition, progestins having a similar structure could show cross-reactivity with the antibody used in ELISA.[127]

Biological in Vitro Assays for Qualitative Analysis. Biological assays, particularly *in vitro* systems based on mammalian, fish, or yeast cells, along with various nuclear hormone receptors are important for screening and evaluation of possible effects of endocrine disruptors.[128]

Biological assays for detecting (anti-)estrogenic and (anti-)androgenic activities have been successfully studied for various environmental contaminants. However, very few studies have evaluated progestogenic biological activity. *In vitro* methods, such as yeast progesterone reporter gene screens,[99,129,130] progestin receptors binding assays,[38,110,131] and high-throughput human progestin receptors binding assays or the CALUX assay,[132,133] can be used as screening tools. However, except for van der Linden et al.[132] and Creusot et al.,[110] no attempts have been made to validate these methods for the screening of progestins in environmental water samples. van der Linden et al.[132] detected progestogenic activity [between 0.78

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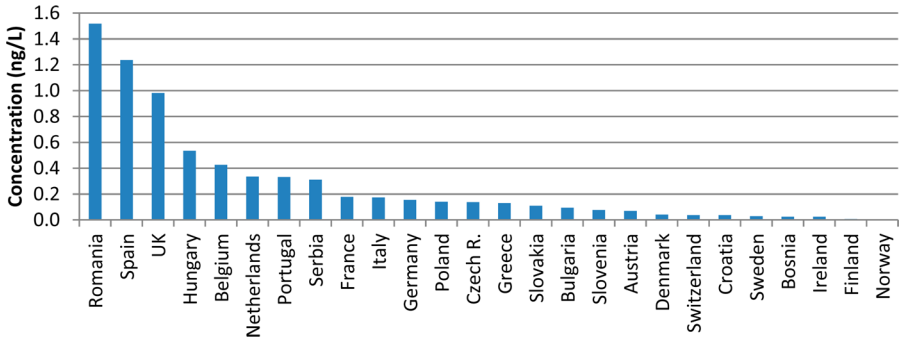

Figure 3. Predicted average river water concentrations of LNG (ng/L) throughout the European countries at 95%ile level.

and 4.5 ng/L Org2058 (a synthetic progestin) equivalent] in both effluent and surface water by using PR-CALUX, a human osteosarcoma (U2OS) cell-based reporter gene assay. However, the authors[132] provided few details on the low extraction recoveries during the sample preparation process and unknown progestogenic activities of the wastewater sample. Greater elaboration of the sampling process (passive sampling) was offered in a recent work by Creusot et al.,[110] but in the absence of parallel chemical analysis (only P4 and LNG were included for chemical analysis). Therefore, it was extremely difficult to link the reported progestogenic activities with specific substances (progestins). The screening procedures used in these studies showed promise and merit further investigation in combination with chemical analysis. However, only a few progestins can activate the fish PR at environmentally relevant concentrations,[36] and in the currently available progestogenic bioassays, the human PR is used.[129,132] Thus, the selection of best fit cell lines combined with the relevant PR would be a crucial step forward in screening environmental samples with regard to the biological significance for exposed aquatic vertebrates such as fish.

Concentrations Found in Sewage and River Samples. Of the 19 synthetic progestins and natural progestin discussed in this review, environmental measurements have only been reported for 5 of them. Thus, sewage and river water measurements have been made for P4, NET, LNG, MEP, and MPA while for the other 15 we have no information.

In municipal wastewater effluents, dissolved P4 has been reported up to 16.9 ng/L[134] with a mean P4 concentration of 1.8 ng/L and the median 1.5 ng/L (Figure 2). Despite these low effluent values, Chang et al.[135] reported 26 ng/L in Chinese rivers. Kolpin et al.[112] reported some high concentrations (P4 detection frequency was only 4.3%) with a maximum of 199 ng/L in U.S. streams during 1999−2000. Considering the high removal (98 ± 2%) of P4 during the wastewater treatment[136] such high values seem surprising. Conceivably high P4 water concentrations might be related to the immediate proximity of farm (swine or dairy) effluent[137] or pulp and paper mill effluent.[115,138]

NET, LNG, MEP, and MPA are the synthetic progestins which have been reported frequently in the environmental samples (SI Table S2). For LNG, reviewing 21 detections a median of 2.2 ng/L can be calculated for sewage effluent; however, 5.7−38.0 ng/L (median-maximum) have been reported in surface water. Considering the effluent values, these surface water values would seem questionable. NET has been reported at concentrations as high as 188 ng/L in sewage effluent in Malaysia,[139] although, in France, its concentrations has so far remained below the detection limit (varied from 1 to 5 ng/L).[140] The mean concentration of NET in effluent, calculated for all data published so far, is 29.5 ng/L and the median 15.2 ng/L (Figure 2). NET was among the most highly consumed (top 5) synthetic hormones in Malaysia, which was reflected in the sewage effluent samples.[139] Low levels of MPA have been reported in effluents with the maximal concentration of 0.4 ng/L[106] with very few surface water detections. Looking at the environmental detections reported so far it is somewhat perplexing that the surface water concentrations are very much in the same range as the effluent (Figure 2). This may reflect a preference by environmental chemists doing surface water surveys to select locations close to sewage effluents during periods of low river flow.

Concentration Predictions in the Aquatic Environment. Although consumption data can be obtained, it has not been possible to find what the human excretion rates are for the synthetic progestins or their sewage removal rates. In the absence of this crucial information, it is highly desirable to have a range of effluent values for the country where river concentrations are to be modeled. River concentration predictions starting from effluent values have been shown to be successful in the past.[141,142] Modeling river concentrations at the national or continental scale can be very helpful in assessing whether an effect threshold may be reached on a widespread basis.[143,144] In this case we know that LNG can inhibit female reproduction with an effect level reported as low as 0.8 ng/L.[7] Unfortunately, there is still some discrepancy in reported LNG in sewage effluents (SI Table S2, LNG) so in this case the median value of 2.1 ng/L was used. Thus, receiving waters would need to offer a dilution factor greater than 2.6 to remain below 0.8 ng/L. Or to put it another way, the receiving water would need to contain at least 38% effluent to reach the 0.8 ng/L effect concentration. Therefore, the process used to derive national specific water concentrations for LNG was as follows: (a) use literature measured LNG effluent concentrations (median) as the starting point; (b) assume no loss of LNG from the water column; (c) use previously derived national sewage effluent dilution factors to predict river water concentrations.

A recent study has attempted to calculate national sewage effluent dilution factor values for European countries[145] to predict widespread river concentrations. This geography-related gridded approach divides continents with their river networks into approximately 50 × 50 km grid cells. Flow in the grids with river networks is predicted from a 30 year climate data set. The model also incorporates the human population in the grid and assumes a wastewater per capita discharge (based on national water use rates). Thus, dividing the natural flow by the wastewater discharge gives a dilution factor. So each cell will have a series of dilution factors based on its geography and the 30 year climate/flow data. A country may have 100 cells with each

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cell having a range of dilution factors. Thus, for a nation it is possible to select a series of different statistics. In this case only when the 95%ile dilution factors for European countries (dilution exceeded 95% of the time) is chosen can countries be identified where LNG values would exceed 1 ng/L on a widespread basis (Figure 3).

This overview of LNG concentrations across Europe indicates that 0.8 ng/L would be reached only in Romania, Spain, and U.K. for brief periods in some locations. Thus, LNG does not appear to pose a widespread threat to European fish reproduction, but some local areas of concern will exist. Finer resolution modeling would be appropriate to examine this in more detail at the regional or catchment scale.

Comparing these predicted LNG values for Europe at very low flows (0.03−1.5 ng/L) with those reported measurements not detected in Spain but 4−7 ng/L in France (Figure 2, SI Table S2) might at first suggest the model is underestimating. However, the model simply added dilution factors to reported effluent concentrations. So the high surface water LNG concentrations reported in France seem surprising. More measurements would be helpful.

Bioconcentration Factor and Predicted Effect Concentration. Bioconcentration factor (BCF) is generally referred to as a concentration of a specific chemical inside biological tissues compared to that of the surrounding environment. BCF is often considered a useful parameter to evaluate chronic risks of a chemical to nontarget organisms. However, in the absence of BCFs (except NET[146] and MPA[147]) calculated from real water and tissue measurements, predicted BCFs of the chemicals (using the (log K_{ow})-based models) can be examined instead.[102,148,149]

Predicted BCF values can be used to derive the effect concentration of progestins in water.[118,150,151] BCF values for predicting critical environmental concentrations of 500 pharmaceuticals has been attempted before.[148] This approach is based on the concept of a "therapeutic dose". Thus, the dose of a pharmaceutical prescribed to treat a human patient has been carefully calculated to achieve a certain plasma concentration in order to have an effect. The theory is if the concentration in the plasma of a fish were to reach this level so similar effects in the fish could be expected. If the plasma concentrations of the particular progestin and BCFs (either measured or predicted) are known, the environmental concentration of progestins in surrounding water for fish could be back-calculated:

$$PEC_W = \frac{C_{FP}}{BCF_{FP}}$$

where PEC_W = predicted effect concentration of progestin in surrounding water at steady state (ng/L), C_{FP} = concentration of progestin in fish plasma [assuming that the fish plasma therapeutic dose is equal to human plasma therapeutic dose] (ng/mL), and BCF_{FP} = bioconcentration factor in fish plasma, and BCF_{FP} can be derived from log BCF = 0.73 log K_{ow} − 0.88.[118,150,151]

When this approach was applied to the progestins in this study, it suggested some would have a tendency to accumulate in fish, when present in the surrounding water at low nanogram per liter levels (Table 4). Of all of the progestins, derived PEC_W values suggested that EDA and DSG were most likely to be bioconcentrated in fish up to human plasma therapeutic levels since only an environmental concentration of 2−3 ng/L in the surrounding waters was required.

Table 4. Predicted Effect Concentrations (PEC_W) of Progestins Based on Bioconcentration Factor in Fish Plasma (BCF_{FP}) and Human Plasma Therapeutic Level (C_{FP})[a]

progestins	BCF_{FP}	C_{FP} (ng/mL)[b]	PEC_W (ng/L)	median concen in surface water (ng/L)
EDA	852	1.6[c]	2	NA
DSG	1756	5.8[d]	3	NA
MPA	128 (8.9*)	1[b]	8 (11**)	NA
NGMN	106	1.1[b]	11	NA
CMA	85	1.6[e]	19	NA
MEP	47	1[b]	21	NA
ENG	27	0.8[b]	29	NA
GES	32	1[d]	31	NA
LNG	46 (12000*)	2.4[f]	52 (0.2**)	5.7
NEA	108	9.8[g]	91	NA
CPA	148	15.2[h]	103	NA
NGA	52	7.2[i]	138	NA
P4	88	17.3[b]	197	2.1
DRO	113	30.8[j]	273	NA
TRI	54	25[k]	463	NA
NET	19 (10.6*)	9.8[g]	516 (924**)	2.8
NES	59	124.9[l]	2116	NA
DIE	7	85.2[b]	12171	NA
ETH	25	NA	NA	NA

[a]Values marked with an asterisk (*) were measured. Values with a double askerisk (**) were estimated from measured BCFs. NA: Not available. [b]See http://dailymed.nlm.nih.gov/dailymed/about.cfm. [c]Reference 157. [d]See http://pubchem.ncbi.nlm.nih.gov/. [e]Reference 152. [f]Reference 118. [g]Reference 156. [h]Reference 153. [i]Reference 154. [j]Reference 158. [k]Reference 58. [l]Reference 155.

NET,[146,159] MPA,[147] and LNG[83,118] are the only progestins which have been studied for fish tissue bioaccumulation so far. Nallani et al.[146] reported tissue specific BCF for NET ranging between 2.6 and 40.8 in channel catfish and fathead minnow exposed for 28 days under laboratory conditions which compares well to the 19 predicted here (Table 4). For MPA reported BCF values ranged between 4.3 and 37.8 in common carp exposed for 7 days,[147] which is less than the 128 predicted. The BCF ranged between 17 and 53 in the liver, brain, and gills of roach exposed to LNG for 28 days[83] while that predicted here was 46. On the other hand, rainbow trout exposed to undiluted, treated sewage effluents containing 1 ng/L LNG for 14 days were found to have the LNG concentrations in blood plasma 8.5−12 μg/L, i.e., values exceeding the human therapeutic plasma level by a factor of 4.[118] The BCF of LNG calculated for rainbow trout in this study was as high as 8,500−12,000. The authors suggested that the ability of fish to concentrate LNG was due to active sequestration by SHBG which controls the flux of natural and synthetic steroids between the blood and aquatic environment.[47] Furthermore, unique experimental design (treated effluent in tanks with a flow-through system) could have led to such a high BCF. However, since this finding has not been replicated in other studies yet, ecotoxicologists may need to be cautious about applying these values until there is more evidence.

CONCLUSIONS

A surprisingly wide range of progestins are used in modern medicine and healthcare. However, environmental ecotoxicologists have only so far reported on effects on aquatic organisms of 7 of the 19 synthetic progestins. Thus, until further ecotoxicology

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studies are completed, we cannot build a complete picture on hazards to wildlife. The greatest concerns are associated with LNG, NET, and GES impairing female reproduction affecting fathead minnow at 1 ng/L or less. If such effects could be replicated in common cyprinid fish, then it would be clear we have a major issue.

From a biological point of view there is still much to learn about the target sites and mechanisms of progestin action in fish and amphibians. For example, nothing is known about the threshold concentrations for interference of synthetic progestins with pheromonal communication in fish and the consequences for successful reproduction. Furthermore, the information about interactions of synthetic progestins with steroid receptors in nontarget organisms is very limited. Additional studies are needed which thoroughly characterize the activity of different synthetic progestins in reporter gene assays using recombinant nuclear as well as membrane steroid receptors of fish and amphibians. Future studies should also investigate specifically end points which are not directly related to changes in reproductive physiology such as the immune system, the stress axis, the thyroid hormone system, or effects on the heart or the neuroprotection/neurodegeneration in the brain.

So far environmental chemists have caught up with only 4 of the 19 synthetic progestins (NET, MEP, MPA, and LNG) plus P4. Thus, we have no idea about the contamination levels of the other progestins. By comparing modeling with measurements we suspect that the reported surface water concentrations may be giving a misleading impression of levels being higher than might be generally the case. The lowest effect level reported for progestins so far is 0.8 ng/L (LNG). More effluent measurements would be helpful, but if 2.1 ng/L was typical, then the receiving water needs to offer at least a dilution factor of 2.6 to avoid harm. Therefore, it is possible at some hot spots for brief periods this could be exceeded. Moreover, several types of synthetic progestins are expected to be present in the environment simultaneously, and it is possible that they would have additive effects.[90] When reviewing modeled BCFs and the associated water concentration needed for reaching a human plasma therapeutic level in fish, only a couple of nanograms per liter of EDA and DSG would be needed to achieve this. However, there is some evidence of unexpectedly high bioaccumulation such as with LNG and trout mediated probably by active uptake.

Other than direct toxicity, a chemical which impairs female reproduction is arguably the great concern for survival of fish populations. A case could be made for the synthetic progestins as being the pharmaceuticals of greatest concern, following ethinylestradiol, given the very low nanogram and sub-nanogram-per-liter effect levels reported.

■ ASSOCIATED CONTENT

Supporting Information

Tables of biological activity of progestins to various human steroid receptors, and reported concentrations of progestins in sewage influent, effluent, and surface water and accompanying references. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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Příloha č. 3

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Determination of progestogens in surface and waste water using SPE extraction and LC-APCI/APPI-HRPS

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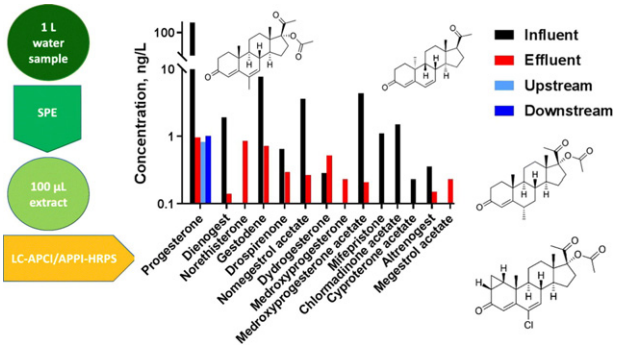
HIGHLIGHTS

- 17 progestogens were analysed using SPE extraction with LC-APCI/APPI-HRPS.
- Newly: altrenogest, etonogestrel, dienogest, nomegestrol acetate and ulipristal acetate
- The method is very selective and sensitive with LOQs ranging from 0.02 to 0.87 ng/L.
- In influent waste water samples, most of the progestogens were detected above LOQs.
- Megestrol acetate, medroxyprogesterone acetate, and dienogest detected most often.

GRAPHICAL ABSTRACT



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ABSTRACT

The aim of this study was to develop a reliable analytical method for the measurement of 17 selected progestogens in waste water and surface water. Automated whole water solid phase extraction (SPE) was used for sample concentration. Liquid chromatography tandem atmospheric pressure chemical ionization/atmospheric pressure photoionization with hybrid quadrupole/orbital trap mass spectrometry operated in high resolution product scan mode (LC-APCI/APPI-HRPS) was applied for the analyses. The whole-method recoveries ranged from 60% to 140% for all analytes at two different spike levels (5 and 50 ng/L) in the studied matrices. The method is very sensitive with LOQs ranging from 0.02 to 0.87 ng/L. The developed method was used for the determination of progestogens in real samples of waste water from three waste water treatment plants (WWTPs) and in surface water from the corresponding recipients. Progesterone was detected in all samples with concentrations in the range of 0.82 to 1.1 ng/L in surface water and 0.11 to 110 ng/L in waste water samples. Three synthetic progestogens, namely, megestrol acetate, medroxyprogesterone acetate, and dienogest, were detected most frequently in effluents; therefore, further attention should be paid to the monitoring of these compounds.
To the best of our knowledge, this study is the first to present analysis of altrenogest, etonogestrel, dienogest, nomegestrol acetate and ulipristal acetate in waste water and surface water using a solid-phase extraction method.

1. Introduction

Endocrine disrupting compounds (EDCs) have received increasing attention from the international scientific community during the last several decades (Schröder et al., 2016; Fent, 2015). Recently, synthetic

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progestogens, which are widely used in human and veterinary therapies, received attention as a new EDC group of concern (Fent, 2015; Kumar et al., 2015; Liu et al., 2011). Synthetic progestogens are used primarily in contraceptive pills but also in the promoting regular menstrual cycles, treating abnormal uterine bleeding, controlling the symptoms of menopause, and preventing endometrial cancer (Apgar and Greenberg, 2000).

However, only limited data on a narrow range of progestogens have been reported in waste waters (Kolodziej et al., 2003; Jenkins et al., 2001; Fernandez et al., 2007; Chang et al., 2011) and surface waters (Jenkins et al., 2001; Chang et al., 2011; Vulliet et al., 2008).

Steroid hormones are excreted by humans and animals and subsequently reach the surface waters due to direct discharge or their incomplete removal in waste water treatment plants (WWTPs), which have been reported to be the primary source of contamination of the aquatic environments (Kumar et al., 2015). In recent studies, it has been demonstrated that synthetic progestogens can affect sexual development and reduce egg production in fish at concentrations similar to those detected in aquatic environments (Svensson et al., 2016; Zeilinger et al., 2009; Runnalls et al., 2013).

The development of fast, reliable and sensitive analytical methods for the determination of progestogens in water matrices is of crucial importance for the assessment of the concentration levels of these compounds and their related ecological risk (Kumar et al., 2015).

Due to the low concentration levels of progestogens in surface water and waste water together with the complexity of environmental matrices in which these compounds are dispersed, a pre-concentration and clean-up step (Chang et al., 2011) or large volume injection (Fayad et al., 2013) are usually performed.

For instrumental analysis, gas chromatography - mass spectrometry (GC–MS) has been applied in the determination of steroids due to its high separation and good identification capability (Kolodziej et al., 2003; Labadie and Budzinski, 2005). However, complicated cleanup and derivatization steps are required before instrumental analysis. Liquid chromatography (LC-MS) is more convenient than GC–MS because direct analysis is possible without derivatization.

Three different ionization sources, electrospray ionization (ESI), atmospheric pressure chemical ionization (APCI), and atmospheric pressure photoionization (APPI) are available for LC-MS. ESI has demonstrated detection capabilities for steroid hormones, but its applicability is limited by selectivity (Jeannot et al., 2002; Rodriguez-Mozaz et al., 2004). In addition, co-extracted matrix compounds can cause significant suppression or enhancement of the signal and, consequently, analytical error in electrospray ionization (Schlüsener and Bester, 2005). APPI may be useful as an alternative for sensitive and selective detection of steroid hormones that are difficult to ionize by either electrospray or APCI (Schlüsener and Bester, 2005; Robb et al., 2000). In present study APCI and APPI analyses were performed to use more selective ionization in order to reduce interferences from matrix, increase selectivity and consequently increase the signal to noise ratio for target compounds in matrix-rich aqueous samples.

The most popular sample preparation technique is solid-phase extraction (SPE), and it has been widely employed for analysis of progestogens in water samples (Vulliet et al., 2008; Al-Odaini et al., 2010; Liu et al., 2014; Liu et al., 2015). A recent development in this technology is the automated SPE system, which has the following advantages: extraction of whole water samples without any pretreatment step, lower amount of solvent needed, low waste and controlling costs; increased safety by reducing exposure to solvents; better reproducibility between technicians; reliable handling of most samples and less attention required for good results.

The aim of this study was to develop a reliable analytical method to measure the selected 17 progestogens in waste water and surface water by SPE extraction followed with liquid chromatography tandem atmospheric pressure chemical ionization/atmospheric pressure photoionization with/atmospheric pressure photoionization with a hybrid quadrupole/orbital trap system operated in high resolution product scan mode (LC-APCI/APPI-HRPS). In the present study, the SPE system works with whole water samples and sample filtration and extraction is included in one step.

The progestogens of interest were selected according to their consumption (Fig. SI 1) and concern over their possible effect on aquatic organisms (Kumar et al., 2015). Target compounds, synthetic progestogens, progesterone and selective progesterone modulators (SPRMs) were selected according to their annual consumption which was calculated from raw data freely available at website of Czech State Institute for Drug Control, Fig. SI 1 (State Institue for Drug Control, 2017). Only those compounds that are consumed in the Czech Republic were further assessed by LC-APCI/APPI-HRPS analysis. The only exception was medroxyprogesterone because it has previously been detected in the aquatic environment (Kolodziej et al., 2003; Macikova et al., 2014). Some progestogens are precursors of other progestogens; therefore, the consumption of precursors and corresponding active substances was summed up. Namely, desogestrel is a precursor of etonogestrel, and consumption of etonogestrel was calculated as the sum of consumption of desogestrel and etonogestrel. Similarly, norethisterone acetate and lynestrenol are precursors of norethisterone and norgestimate and norelgestromin are metabolized into levonorgestrel.

In our study we aimed to develop simple multi-residue method for simultaneous determination of 17 progestogens selection which was based on consumption data survey. Most studies focus only on several progestogens, such as progesterone and megestrol acetate (Chang et al., 2011; Vulliet et al., 2008; Liu et al., 2014; Sun et al., 2009), and only Liu et al. investigated the occurrence of 21 progestogens in seawater (Liu et al., 2015). Our study is the first to analyse altrenogest, etonogestrel, dienogest, nomegestrol acetate and ulipristal acetate in waste water and surface water together with other 12 progestogens which have not been analysed in one analytical run. Compared to previous studies we aimed to develop rapid extraction method followed with highly selective and sensitive detection method LC-APCI/APPI-HRPS.

The applicability of the method for the analysis of environmental samples was verified on real WWTP effluents and recipient samples.

2. Materials and methods

2.1. Chemicals and reagents

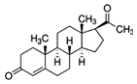
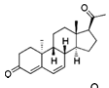
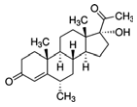
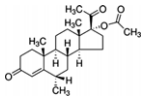
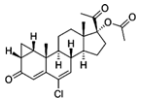
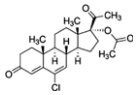
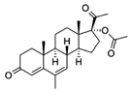
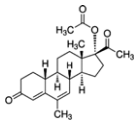
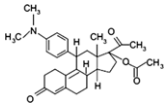
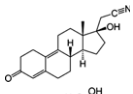
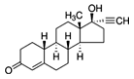
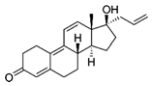
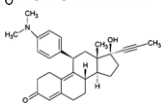
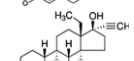
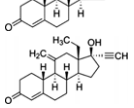
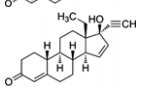
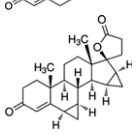
Methanol and acetonitrile (LiChrosolv® Hypergrade) were purchased from Merck (Darmstadt, Germany). Ultra-pure water was produced using an Aqua-MAX-Ultra System (Younglin, Kyounggi-do, Korea). All analytical standards were of high purity (mostly 98%).

Medroxyprogesterone, progesterone, dydrogesterone, dienogest, norethisterone, gestodene, drospirenone, nomegestrol acetate, mifepristone, ulipristal acetate, altrenogest, medroxyprogesterone acetate, megestrol acetate, levonorgestrel, etonogestrel, chlormadinone acetate and cyproterone acetate were purchased from Sigma Aldrich (Steinheim, Germany). The classification and the main physical chemical properties of the selected progestogens are presented in Table 1.

The internal standards (ISs) for synthetic progestogens, Norethindrone-D6, Gestodene-D6, Drospirenone-13C6, Medroxyprogesterone-D3, Chlormadinone acetate-D6, Megestrol acetate-D3, and Progesterone-D9, were obtained from Toronto Research Chemicals, Inc. (Toronto Research Chemicals, ON, Canada). All internal standards were of analytical grade (>98% purity).

Individual stock solutions of the standards were prepared at 1 mg/mL concentration in methanol and stored at −20 °C. A spiking mixture of ISs was prepared by diluting the stock solutions with methanol to a final concentration of 1 μg/mL for each compound. Working standard mixtures (0.01–10 μg/mL) of the native compounds were prepared monthly in methanol.

Table 1
The classification and the main physical chemical properties of the selected progestogens.

Structural derivation			Compound	Molar mass	CAS number	Purity (%)	log K_{ow}	Water solubility (mg/L)	Structure
Natural hormone			Progesterone	314.47[a]	57-83-0	99.9	3.67[a]	5.0[a]	
Progesterone derivative			Dydrogesterone	312.46[a]	152-62-5	99.5	3.45[b]	3.7[b]	
Structurally related to progesterone	17α-OH-progesterone derivatives		Medroxyprogesterone	344.50[a]	520-85-4	98.5	3.50[b]	3.0[b]	
			Medroxyprogesterone acetate	386.54[a]	71-58-9	≥97	4.09[b]	1.2[b]	
			Cyproterone acetate	416.95[a]	427-51-0	≥98	3.10[b]	51.7[b]	
			Chlormadinone acetate	404.94[a]	302-22-7	99.7	3.95[b]	0.3[b]	
	19-Norprogesterone derivatives		Megestrol acetate	384.52[a]	595-33-5	≥99	4.00[b]	2.0[b]	
			Nomegestrol acetate	370.49[a]	58652-20-3	≥98	3.55[a]	4.3[a]	
			Ulipristal acetate	475.63[a]	126784-99-4	≥98	5.07[a]	0.1[a]	
Structurally related to testosterone	19-Nortestosterone derivatives	Estranes	Dienogest	311.43[a]	65928-58-7	99.9	2.34[a]	57.9[a]	
			Norethisterone	298.43[a]	68-22-4	≥98	2.97[b]	7.0[b]	
			Altrenogest	310.44[a]	850-52-2	≥99	3.94[a]	14.8[a]	
			Mifepristone	429.61[a]	84371-65-3	≥98	5.40[b]	0.1[a]	
		Gonanes	Levonorgestrel	312.46[a]	797-63-7	≥99	3.48[b]	2.1[b]	
			Etonogestrel	324.47[a]	54048-10-1	≥98	3.16[b]	57.1[a]	
			Gestodene	310.44[a]	60282-87-3	≥98	3.26[b]	8.1[b]	
17α-Spironolactone derivative			Drospirenone	366.50[a]	67392-87-4	99.9	4.02[a]	1.8[a]	

[a] Estimation Programs Interface Suite™ for Microsoft® Windows, KOWWIN v. 1.68 estimate. United States Environmental Protection Agency, Washington, DC, USA.
[b] (Liu et al., 2011).

2.2. Sampling and sample preparation

Sampling of water was performed in November 2016 and January and February 2017. Grab waste water samples (influent and effluent) from WWTP in Vodňany (Czech Republic, 7000 inhabitants and light industry, Table 2) and surface water samples from Blanice River (Czech Republic) (7 replicates from each site) were used for method validation. Seven replicates (1 L aliquot) were spiked with ISs to achieve a concentration of 10 ng/L. Other sets of samples (seven replicates each) were also spiked with a mixture of progestogens at two concentration levels (5 and 50 ng/L for waste water and surface water) to check the recovery of target compounds.

Furthermore, to evaluate the effectiveness of the developed method, 24-hour composite samples (time proportional sampling with 15 min. intervals) were collected from influents and effluents of two WWTPs. The characteristics of the WWTPs sampled are shown in Table 2. Surface water grab samples were collected upstream and downstream of the corresponding WWTPs. The sampling region is located in the Vltava River basin. All samples were collected into amber glass bottles that were pre-cleaned with acetonitrile and distilled water.

Samples were transported to the laboratory immediately after collection, stored in darkness at 4 °C and extracted within 48 h.

2.3. Solid-phase extraction and sample evaporation

Waste and surface water samples were extracted on an SPE-DEX 4790 automated solid-phase extraction system (Horizon Technology, Salem, NH, USA).

For the extraction method, we checked two extraction solvents for the selected progestogens: acetonitrile (ACN) and methanol (MeOH). Extraction was performed as follows: the system was purged, and Atlantic C18 SPE disks (Horizon Technology, Salem, NH, USA) were conditioned with ACN and LC/MS grade water. One liter of whole water sample was loaded into the extraction system. The particles were trapped at 5 and 1 μm glass fibre filters (Horizon Technology, Salem, NH, USA). Glass fibre filtration material appears to be the best choice for extraction of progestogens because high recoveries are reached using these filters (Fayad et al., 2013). Filtered samples passed through the C18 disks, and target compounds were retained onto the sorbent. C18 disks were chosen due to their known ability to extract mid and non-polar compounds (Horizon Technology, Salem, NH, USA), such as synthetic progestogens, which are slightly polar or non-polar compounds (Kumar et al., 2015). When samples passed through the disks, the system, including sample bottle, was washed with demineralised water. Filters and C18 disks were air dried for 15 min to remove residual water. Subsequently, the sample bottle was rinsed twice with ACN to wash off residual analytes from the bottle walls. C18 disks containing adsorbed target compounds were eluted with the total volume of 10 mL of ACN into glass collection vessels. Soak time of the ACN on C18 disks was 1 min and 30 s per rinse for elution. The first rinsing was followed by 15 s of air dry, while the latter air drying after rinsing lasted 1 min. The eluates were transferred into glass vials with a conical bottom. To ensure that all target compounds were transferred, eluate collection vessels were rinsed three times with 2 mL of ACN. Combined extract was evaporated by a gentle nitrogen stream to dryness in a nitrogen sample concentrator Termovap TV10+ (ECOM, the Czech Republic). The sample residues were re-dissolved in 2 × 50 μL of ACN for further analysis. The same extraction procedure was performed using methanol as the extraction solvent for comparison purpose. The sample extract was transferred into an autosampler vial with a glass insert with a volume of 200 μL and sealed with a cap. All extracts were subsequently stored at −20 °C until analysis, which was performed within one week.

2.4. LC-MS instrumentation

An Accela 1250 LC pump (Thermo Fisher Scientific, San Jose, CA, USA) coupled with a hybrid quadrupole/orbital trap Q-Exactive mass spectrometer (Thermo Fisher Scientific) and an HTS XT-CTC autosampler (CTC Analytics AG, Zwingen, Switzerland) were used to separate and detect target analytes. An analytical Hypersil Gold column (50 mm × 2.1 mm ID × 3 μm particles; Thermo Fisher Scientific) preceded by the same phase pre-column (10 mm × 2.1 mm ID × 3 μm particles) were used for the chromatographic separation of the target analytes.

2.5. LC-APCI/APPI-HRPS analysis

Ultra-pure water and methanol were used as the mobile phases. The LC gradient for the elution of target compounds is presented in Supplementary Materials Table SI 1. The elution conditions were programmed as follows: 350 mL/min 30% methanol in water for 1 min, isocratically followed by a gradient change to 20/80 water/methanol at a flow of 400 mL/min in 8 min and a final gradient change to 100% of methanol at a flow of 400 mL/min in 10 min. These parameters were held for 2 min and then changed to the starting conditions and held for 1.5 min to equilibrate the column for the next run.

An atmospheric pressure photoionization (APPI) in positive mode was used to ionize target compounds. The instrument was calibrated daily (mass calibration) in negative modes using a standard procedure proposed by Thermo Scientific.

The APCI/APPI parameters were set as follows: capillary temperature (300 °C), vaporizer temperature (300 °C), sheath gas pressure (40 arbitrary units), auxiliary gas (15 arbitrary units) and discharge current (4 μA in positive ionization mode). UV krypton lamp (10 eV) was used in the source.

The MS/MS conditions were optimized for each compound by infusion of individual standard solution at a concentration level of 1 μg/mL at a rate of 10 μL/min to the mobile phase stream (50/50 MeOH/water at 300 μL/min). All progestogens were detected in positive ionization mode as protonated molecules [M + H]+.

Most progestogens undergo fragmentation with many non-specific fragments. Selection of the proper fragments usually is based on intensity and selectivity of fragments not only in the standard but in the real

Table 2
Wastewater treatment plant characterization.

WWTP	Sampling technique	Water flow at influent (m^3/d)	Catchment population (capacity)	Influent (mg/L)			Effluent (mg/L)			Main producers of wastewaters (except for households)
				BOD	COD-Cr	TSS	BOD	COD-Cr	TSS	
Vodňany	Grab	2151	28,500	294[a]	516[a]	125.5[a]	3.5[a]	25.6[a]	3.45[a]	Poultry industry, research institute and instrumentation manufacturing plant
Strakonice	24-h composite	15,000	75,000	250	431	245	9	38.3	7.2	Hospital, brewery, textile and engineering industry
České Budějovice	24-h composite	37,791	375,000	247	597	250	1.9	19	<2.0	Hospitals, breweries, paper mill, dairy, heating and food manufacturing plants

Abbreviations: BOD – biochemical oxygen demand, COD-Cr – chemical oxygen demand using potassium dichromate, TSS – total suspended solids.
[a] Data taken from year 2014.

matrices, as well. The high resolution product scan (HRPS) analysis was used with the mass inclusion list and expected retention times of the target analytes with a 1 min time window. Collision energy values were optimized for all the compounds of interest and are presented in Table SI 3. General MS parameters were set up as follows: orbital trap resolution 17,500 FWHM; product scan range of 50 to 600 *m/z*; AGC target of 1e6; maximum filling time of 30 ms; and an isolation window at the quadrupole of 1 *m/z*. The advantage of HRPS is not that single transitions are monitored but that a full scan spectrum is recorded. This even allows picking up new suspicious fragment ions using old data without re-measuring. The MS/MS parameters for the Q-Exactive mass spectrometer are presented in Table SI 3.

Data analysis was performed with TraceFinder 3.3 software (Thermo Fisher Scientific).

2.6. Method validation

The performance of the method was assessed regarding linearity, limits of quantification (LOQs), trueness and repeatability. Two matrices were used for method validation: waste water (influent and effluent) and surface water.

The linearity of the calibration curve was tested in the range from 0.1 ng/L to 200 ng/L. Calibration curves were measured at the beginning and at the end of the sequence to check instrument stability. The calibration was prepared in water/MeOH (1/1). LOQs were calculated as one quarter of the lowest calibration point in the calibration curve where the relative standard deviation of the average response factor was <30% (in some cases one or two points at low concentration levels had to be removed). The peak area corresponding to this concentration was used to calculate LOQ for each individual compound in each sample.

The matrix effect was assessed for each compound. Corrections of ion suppression or enhancement were performed using matrix-matched standards for quantification if this effect exceeds 30% in a given matrix. Each matrix-matched standard was prepared from corresponding water sample extract that was spiked with ISs at concentration levels of 10 ng/L and native compounds at concentration levels of 200 ng/L. The matrix effect was evaluated as the difference between the matrix-matched standards' relative response factor and average relative response factor obtained from the calibration curve. Repeatability of the method was evaluated as the relative standard deviation (RSD) of the seven replicates at corresponding fortification levels.

In an effort to generate quality data, several quality control samples were included during sample analysis. No target analytes were detected in method blanks. The method blank was ultra-pure water. The blank was prepared and extracted in the same way as the samples.

3. Results and discussion

3.1. Efficiency of extraction procedure

The extraction solvent is one of the critical parameters influencing the extraction efficiency of the analytes and method performance. In this method, we tested two extraction solvents for the selected progestogens, ACN and MeOH. The recovery achieved by each extraction procedure for each progestogens was evaluated at a fortification level of 10 ng/L and was expressed as the ratio between the determined concentration and the nominal concentration ($n = 3$). The recovery results for progestogens and two extraction solvents are shown in Table SI 3. The most consistent results were achieved using ACN, where median recovery ranged between 62% and 130%. Extraction procedures with MeOH provided highly variable results across the selected hormones, ranging from 4% to >135%. According to the literature data, elution with ACN solvent provides better recoveries of progestogens than with MeOH, ethyl acetate or dichloromethane (Sun et al., 2009). Consequently, ACN was selected for further validation because of its consistent recoveries for most target compounds.

3.2. Method validation

The linearity, limit of quantification (LOQ), trueness and repeatability of the method were evaluated under the optimum extraction conditions for each sample matrix.

One of the analytical challenges of progestogens quantification in aquatic samples is the need to analyse very low concentration levels. Average LOQs together with linearity parameters are presented in Table 3. All progestogens showed good linear response in the range of 0.1 to 200 ng/L with R squared coefficients (R^2) higher than 0.997. The proposed analytical method resulted in very low LOQs in the range of 0.02 to 0.87 ng/L without additional purification or derivatization steps.

Varying composition of the sample matrix can suppress or enhance analyte signal and consequently may influence ion-ratio and overall method performance, as well as producing false positives, even when an IS or isotope dilution method is used (Fedorova et al., 2013). Significant matrix effect (over 30%, Fig. 1) was observed in influent waste water samples for most of the studied compounds. The dominant matrix effect in this case was ionization suppression, which varied from −33% to −101%. For the rest of the samples (surface and effluent waste water), matrix effects were between −30 and +30% for most of progestogens with the exceptions of dienogest (−31%) and dydrogesterone (−54%) in effluent samples. This finding is in accordance with our previous study, where APCI/APPI showed a lower matrix effect than ESI (Lindberg et al., 2014).

As seen in Table 4, for most target compounds, the recoveries were in the satisfactory range from 60% to 140%. Dienogest showed the lowest recoveries in all matrices (30–41% for surface water, 56–80% for waste water). This result could be attributed to the fact that dienogest is more polar compared to the rest of the studied progestogens (log Kow = 2.34, Table 1), and the C18 SPE disk might not be the best choice for extraction of this kind of compound. However, the multiresidual method is a compromise between analysing a wide range of analytes and the method performance for border compounds. Repeatability of the method for dienogest was good, and the method can also be used for this compound, at least as a semiquantitative method.

Recoveries slightly over 140% were obtained in the effluent waste water samples for levonorgestrel, norethisterone and etonogestrel. It should be mentioned that the calculation of influent recoveries for progesterone was problematic because of the presence of this hormone in these samples at high concentrations (110 ng/L). For this reason, data for progesterone in influent samples had to be omitted for method development.

Table 3
R square coefficients (R^2) and average limit of quantification (LOQ), ng/L of selected hormones measured in surface water and wastewater (influent and effluent).
Number of samples:
- surface water (Blanice River) = 7 replicates;
- wastewater Vodňany (influent and effluent) = 7 replicates of each site.

Compound	R^2	Surface water	Effluent	Influent
Dienogest	0.9992	0.10	0.08	0.04
Norethisterone	0.9989	0.04	0.11	0.05
Gestodene	0.9992	0.05	0.44	0.19
Drospirenone	0.9983	0.87	0.07	0.03
Levonorgestrel	0.9996	0.08	0.51	0.23
Etonogestrel	0.999	0.07	0.55	0.25
Nomegestrol acetate	0.9988	0.07	0.09	0.04
Dydrogesterone	0.9977	0.65	0.41	0.21
Medroxyprogesterone	0.999	0.06	0.04	0.02
Medroxyprogesterone acetate	0.9988	0.10	0.06	0.03
Mifepristone	0.9986	0.59	0.36	0.27
Ulipristal acetate	0.9996	0.6	0.37	0.28
Chlormadinone acetate	0.9983	0.44	0.32	0.24
Cyproterone acetate	0.9983	0.32	0.38	0.20
Altrenogest	0.9996	0.06	0.05	0.03
Megestrol acetate	0.9994	0.07	0.05	0.03
Progesterone	0.9991	0.15	0.06	0.03

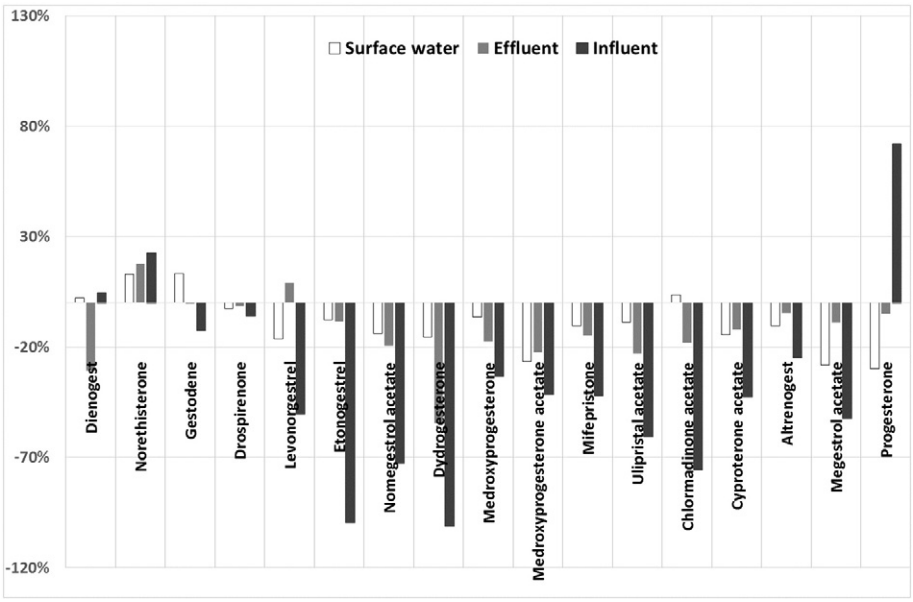


Fig. 1. Progestogens quantification MS signal suppression/enhancement in surface water from Blanice River and wastewater samples (influent and effluent) from WWTP in Vodnany (Czech Republic). Positive values correspond to ion enhancement, negative - to ion suppression.

Method repeatability was tested for both fortified and native samples, and the data are presented in Table 4. For all matrices, repeatability values were satisfactory, and the RSDs in all samples at different concentration levels were lower than 30% for most of the progestogens. Slightly higher RSDs were found for dienogest (31%), gestodene (34%), dydrogesterone (31%) and chlormadinone acetate (33%) in influent water samples at a fortification level of 5 ng/L.

3.3. Consumption of progestogens. Application of the method. Analysis of progestogens in surface and waste water samples

Data on consumption of progestogens are presented on Fig. SI 1. Six compounds (progesterone, drospirenone, megestrol acetate, cyproterone acetate, medroxyprogesterone acetate, dienogest) reached or exceeded consumption of 100 kg per year in the Czech Republic in year 2014. Progesterone was the most consumed progestogen and is also naturally produced in human body. Indeed, this compound has been detected in all studied influents (Table 5). Up to date, consumption rates of progestogens have been reported only for few times (Besse and Garric, 2009; Ji et al., 2016; Runnalls et al., 2010). Thus, the present results may provide useful addition.

The optimized method was used for the identification and determination of target hormones in real samples of waste water from three WWTPs and in samples of recipient streams taken down-stream and up-stream of the respective WWTPs.

In the waste water samples, there were higher concentrations in the influent than in the effluent samples for most of the studied compounds, as presented in Table 5. The concentration range for influent was from 0.23 ng/L (cyproterone acetate in Vodňany WWTP) to 110 ng/L (progesterone in Vodňany WWTP). Because of incomplete elimination by WWTPs, certain of the studied progestogens can be observed in effluent samples in lower concentration levels compared to influent samples:

Table 4
Trueness (% of added amount) and repeatability of selected hormones measured in surface water and wastewater (influent and effluent)-in brackets (relative standard deviation; % RSD) at different concentration levels.

Compounds	Surface water		Wastewater			
			Effluent		Influent	
	5 ng/L	50 ng/L	5 ng/L	50 ng/L	5 ng/L	50 ng/L
Dienogest	30 (16)	41 (26)	59 (15)	58 (20)	80 (31)	56 (17)
Norethisterone	66 (10)	75 (11)	125 (16)	150 (19)	106 (30)	106 (16)
Gestodene	64 (9)	70 (11)	122 (12)	121 (8)	66 (34)	81 (11)
Drospirenone	64 (13)	77 (9)	134 (11)	133 (8)	121 (16)	109 (5)
Levonorgestrel	82 (13)	85 (11)	164 (13)	152 (10)	103 (19)	88 (14)
Etonogestrel	81 (11)	91 (14)	150 (10)	132 (9)	94 (14)	90 (12)
Nomegestrol acetate	86 (9)	95 (20)	123 (12)	115 (13)	80 (24)	73 (7)
Dydrogesterone	87 (27)	85 (22)	112 (27)	109 (18)	68 (31)	73 (13)
Medroxyprogesterone	76 (9)	82 (9)	111 (11)	108 (10)	100 (14)	96 (7)
Medroxyprogesterone acetate	93 (6)	99 (11)	116 (15)	108 (10)	58 (11)	71 (6)
Mifepristone	65 (9)	78 (11)	83 (14)	92 (14)	122 (20)	106 (19)
Ulipristal acetate	68 (9)	82 (18)	78 (11)	84 (13)	114 (25)	90 (25)
Chlormadinone acetate	64 (15)	75 (10)	105 (12)	108 (7)	82 (33)	113 (30)
Cyproterone acetate	79 (20)	88 (9)	116 (12)	112 (15)	91 (10)	88 (10)
Altrenogest	63 (15)	78 (16)	107 (15)	96 (18)	111 (17)	83 (11)
Megestrol acetate	95 (8)	94 (10)	119 (8)	116 (11)	102 (11)	88 (7)
Progesterone	80 (8)	76 (8)	119 (7)	114 (11)	NA	NA

NA – not analysed.

Table 5
Average concentration ng/L (n = 3) of 17 target compounds detected in surface water, influent and effluent waste water samples from three WWTPs.

Compounds	Surface water (Blanice River)	České Budějovice				Strakonice				Vodňany	
		Upstream	Downstream	Effluent	Influent	Upstream	Downstream	Effluent	Influent	Effluent	Influent
Dienogest	<0.09	<0.08	<0.11	**1.0**	**7.0**	<0.05	<0.05	<0.05	**11**	**0.14**	**1.9**
Norethisterone	<0.04	<0.03	<0.05	<0.03	<0.02	<0.05	<0.05	<0.04	<0.17	**0.85**	<0.06
Gestodene	<0.05	<0.57	<0.78	<0.49	<0.38	<0.48	<0.36	<0.29	**5.5**	**0.71**	**7.7**
Drospirenone	<0.85	<0.71	<1.1	<0.62	<0.77	<0.24	<0.25	<0.18	<0.66	**0.29**	**0.64**
Levonorgestrel	<0.08	<0.83	<1.3	<0.83	<2.1	<0.27	<0.28	<0.22	<1.4	<0.53	<0.26
Etonogestrel	<0.07	<0.93	<1.4	<0.89	<1.4	<0.25	<0.26	<0.21	<1.1	<0.57	<0.28
Nomegestrol acetate	<0.07	<0.06	<0.09	<0.05	<0.08	<0.04	<0.04	<0.03	<0.21	**0.26**	**3.6**
Dydrogesterone	<0.63	<0.58	<0.86	<0.55	<1	<0.22	<0.24	<0.18	<1.5	**0.51**	**0.28**
Medroxyprogesterone	<0.06	<0.05	<0.08	<0.05	<0.06	<0.04	<0.04	<0.03	<0.13	**0.23**	<0.02
Medroxyprogesterone acetate	<0.1	<0.09	<0.14	<0.09	<0.15	<0.67	<0.76	**0.58**	**2.6**	**0.21**	**4.4**
Mifepristone	<0.61	<0.5	<0.81	**0.5**	**3.0**	<0.34	<0.36	<0.26	<1.3	<0.41	**1.1**
Ulipristal acetate	<0.61	<0.51	<0.84	<0.43	<1.6	<0.2	<0.21	<0.15	<0.56	<0.42	<0.27
Chlormadinone acetate	<0.45	<0.4	<0.64	<0.36	<0.63	<0.26	<0.28	<0.2	<1.3	<0.36	**1.5**
Cyproterone acetate	<0.32	<1.4	<2.2	**2.8**	**6.7**	<0.73	<0.74	<0.59	<1.8	<0.44	**0.23**
Altrenogest	<0.06	<0.05	<0.08	<0.06	<0.10	<0.05	<0.06	<0.04	<0.16	**0.15**	**0.35**
Megestrol acetate	<0.07	<0.06	<0.09	<0.06	**4.8**	<0.07	<0.07	**0.4**	**6.3**	**0.23**	<0.03
Progesterone	**1.1**	**0.82**	**1.0**	**0.59**	**47**	<0.09	<0.05	**0.11**	**14**	**0.95**	**110**

The values above LOQs are marked bold.

the concentration range for effluent was from 0.11 ng/L (progesterone in Strakonice WWTP) to 2.8 ng/L (cyproterone acetate in České Budějovice WWTP).

Eight hormones (dienogest, gestodene, drospirenone, nomegestrol acetate, dydrogesterone, altrenogest, medroxyprogesterone acetate and progesterone) were found above LOQ in both influent and effluent samples from Vodňany WWTP. Four target compounds (dienogest, mifepristone, cyproterone acetate and progesterone) were found both in influent and effluent samples from WWTP in České Budějovice, and megestrol acetate was found only in influent. Approximately the same number of hormones were found in Strakonice WWTP, but they were different compounds (progesterone, medroxyprogesterone acetate and megestrol acetate were in both waste water, but dienogest and gestodene were only in influent). The differences among cities can be assigned to presence of the regional centre for cancer treatment in České Budějovice, as well as different residence time in the sewer system. It should be mentioned that the highest positive finding in Vodňany occurred only in grab samples, which indicates that sampling technique might have an influence, as well.

Levonorgestrel, etonogestrel and ulipristal acetate were below LOQs in all studied samples, although high concentrations of levonorgestrel, 150–170 ng/L in influent and 30 ng/L in effluent, were shown in a study by Viglino et al. (Viglino et al., 2008). In a recent study by (Liu et al., 2014), the concentrations of progesterone and dydrogesterone in influent were 10.1 and 35.1 ng/L, respectively. Usually, the presence of hormones in waste water is associated with consumption rates, as well as transformation rates of conjugated to the un-conjugated form.

In our study, the concentrations of medroxyprogesterone acetate in influent samples ranged from 2.6 to 4.4 ng/L. The concentration of medroxyprogesterone acetate was reported in influent samples in two recent studies (Chang et al., 2011; Liu et al., 2014), and the concentration levels were 2.4 ng/L and 18–58 ng/L, respectively.

Despite the lowest recovery among investigated compounds, dienogest was detected in waste water, although at sub-ng/L concentrations. Medroxyprogesterone was not detected in the influent of WWTP Vodňany, but it was present in the effluent, which might be explained by biotransformation of medroxyprogesterone acetate into medroxyprogesterone.

Despite the lack of data on the biotransformation of steroids in waste water, certain progestogens (progesterone and levonorgestrel) have already been reported to rapidly undergo biotransformation to other steroids in surface waters and sediments (Ojoghoro et al., 2017; Peng et al., 2014; Sangster et al., 2016). Several compounds analysed in the present study, namely, medroxyprogesterone, norethisterone, dydrogesterone, and megestrol acetate (in WWTP Vodňany), could have undergone such a biotransformation during the waste water treatment process (Table 5) because either they were present in the effluent samples only or their concentration in the effluent samples was higher than in the influent samples.

The major point source of progestogens into the aquatic environment is WWTPs because they are not completely capable of removing progestogens during treatment processes. Therefore, treated effluents discharged into receiving water bodies (river or surface water) may still contain substantial progestogen residues (Vulliet et al., 2008; Al-Odaini et al., 2010; Liu et al., 2014; Macikova et al., 2014; Chang et al., 2008; Kuster et al., 2008; Vulliet and Cren-Olivé, 2011), see Table SI 4. Similar results were shown in work by Liu et al. (Liu et al., 2014) for progesterone in surface water samples. In a recent study by (Torres et al., 2015), the concentrations of progesterone in surface water ranged from 0.58 to 26 ng/L. For example, Liu et al. (Liu et al., 2014) detected progesterone, megestrol acetate and norethisterone in concentrations ranging from 0.6 to 1.7 ng/L, as well as up to 9.6 ng/L in the case of dydrogesterone in the river downstream of a WWTP. In our study, only progesterone was observed in surface water at concentrations ranging from 0.82 to 1.1 ng/L, and it was the most frequently detected hormone in the studied samples.

4. Conclusions

We developed a reliable analytical method for simultaneous determination of 17 progestogens in environmental samples by combining simple high sample volume SPE extraction with LC-APCI/APPI-HRPS. This method enables very low LOQs (0.02–0.87 ng/L) to be obtained without additional purification or derivatization steps. The method could be used for regular monitoring of progestogens in the aquatic environment because it uses a whole water sample.

The method has been applied for the analyses of progestogens in different environmental samples. To the best of our knowledge, this study is the first to analyse altrenogest, etonogestrel, dienogest, nomegestrol acetate and ulipristal acetate in waste water and surface water.

In influent waste water samples, most of the studied progestogens were detected above the quantification limits but with high spatial variability.

The most frequently detected compound was progesterone, with its highest levels being observed in influent waste water. Three synthetic progestogens, namely, megestrol acetate, medroxyprogesterone acetate, and dienogest, were detected most frequently in effluents, and further attention to the monitoring of these compounds should therefore be paid. Only progesterone was detected in surface water samples.

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Supplementary data

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Příloha č. 4

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Two synthetic progestins and natural progesterone are responsible for most of the progestagenic activities in municipal wastewater treatment plant effluents in the Czech and Slovak republics

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ABSTRACT

Vast numbers of xenobiotics are known still to be present in treated municipal wastewater treatment plant (WWTP) effluents. Some of these possess endocrine-disrupting potency and pose risks for exposed aquatic animals. We searched for 17 potential environmental contaminants having affinity to the progesterone receptor. Relative potency values of these progesterone receptor-active chemicals were obtained. On the basis of relative potencies and measured environmental concentrations, the contribution of progestins to measured progestagenic activities was evaluated. Wastewaters (influent and effluent) and surrounding surface waters (upstream and downstream) at six municipal WWTPs were screened using instrumental chemical analysis and *in vitro* reporter gene bioassay. We showed the presence of target compounds and (anti-)progestagenic activities in municipal wastewater and surface water. Nine and seven progestins were identified in influent and effluent wastewaters, respectively. Only two compounds, progesterone and medroxyprogesterone were found in surface waters. Progestagenic agonistic activities in influents were partially masked by strong anti-progestagenic activities that were detected in all influents and ranged from 2.63 to 83 ng/L of mifepristone equivalents (EQs). Progestagenic activities were detected in all effluents and ranged from 0.06 to 0.47 ng/L of reference compound ORG 2058 EQs (a synthetic progestin equivalents), thus indicating incomplete removal of progestins during wastewater treatment processing. This activity poses a continuing risk for the aquatic environment. By contrast, anti-progestagenic activities showed better removal efficiency in WWTPs compared to progestagenic agonistic activities. Anti-progestagenic activities were found in only three of six effluents and ranged from 0.26 to 2.1 ng/L mifepristone EQs. We explained most of the progestagenic activity in municipal WWTP effluents by the presence of synthetic progestins and progesterone, which contributed 65–96% of such activity in samples where no antagonistic activity was found. The progestins medroxyprogesterone acetate, megestrol acetate and progesterone contributed most to the progestagenic activity detected in municipal effluents. Anti-progestagenic activities were found in some municipal effluents, but no causative agents were revealed because two analysed selective progesterone receptor modulators (SPRMs) with anti-progestagenic activities, mifepristone and ulipristal acetate, were not present in the effluents.

1. Introduction

It is well known that many chemicals are daily discharged into sewage and that some of them pass through municipal wastewater treatment plants (WWTPs). Therefore, WWTP effluents still contain certain amounts of xenobiotics that subsequently enter the aquatic environment. Some of these environmental contaminants may impair the endocrine systems of exposed organisms (Colborn et al., 1993; Jobling and Tyler, 2003; Lange et al., 2001; Sumpter, 1998).

One group of endocrine-disrupting pollutants, synthetic progestins, have recently come under suspicion of causing a significant

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toxic burden and substantial risk to the aquatic environment (Fent, 2015; Kumar et al., 2015). Synthetic progestins are mainly used as the active ingredients in women's contraceptives, but also in other hormonal preparations (Sitruk-Ware, 2004; Zeilinger et al., 2009). As a result, they are consumed in relatively large amounts ranging from 0.34 to 9864 kg/year as reported from various European countries (Besse and Garric, 2009; Runnalls et al., 2010; Zhao et al., 2015), including the Czech Republic (Golovko et al., 2018). Therefore, municipal WWTPs might be important sources of synthetic progestins (Chang et al., 2009). The naturally occurring progestin progesterone originates from farmed animals, such as swine, cattle, and chickens, as well as from humans (Shore and Shemesh, 2003). In addition, progesterone is an active ingredient of several widely prescribed drugs (Golovko et al., 2018). Some synthetic progestins and progesterone already have been detected in WWTP effluents (Fan et al., 2011; Liu et al., 2014; Viglino et al., 2008), thereby indicating insufficient elimination of these compounds during wastewater treatment processes. Accumulating evidence indicates that municipal WWTP discharges may also contaminate surface waters (Kumar et al., 2015). Several analytical surveys conducted to date have confirmed the presence of synthetic progestins at concentrations up to tens of ng/L in aquatic environments throughout the world (Al-Odaini et al., 2010; Chang et al., 2011; Kolpin et al., 2002; Liu et al., 2011; Viglino et al., 2008; Vulliet et al., 2007) and progesterone even at concentrations as high as 199 ng/L (Kolpin et al., 2002). Nevertheless, only limited knowledge about environmental levels of synthetic progestins appears in the literature (Fent, 2015; Kumar et al., 2015).

This topic deserves greater attention, as it has been demonstrated that exposure of aquatic organisms to progestins at even low ng/L levels affects their reproduction (Paulos et al., 2010; Runnalls et al., 2013; Zeilinger et al., 2009; Zucchi et al., 2012). Namely, levonorgestrel, norethisterone, and gestodene have been shown to decrease egg production and diminish development of secondary male characteristics in fathead minnow (*Pimephales promelas*) at concentrations as low as 0.8, 1.0, and 1.0 ng/L, respectively (Paulos et al., 2010; Runnalls et al., 2013; Zeilinger et al., 2009). Furthermore, levonorgestrel was shown to negatively affect oocyte development in western clawed frog (*Xenopus tropicalis*) after sub-chronic exposure to concentration of 1.3 ng/L (Säfholm et al., 2012).

Although not all synthetic progestins are structurally similar to the natural progestin progesterone (Africander et al., 2011), they were designed to be progesterone receptor (PR) agonists and thus mimic progesterone function (Stanczyk et al., 2013). Most synthetic progestins are even more potent than natural progesterone (Besse and Garric, 2009). Surprisingly, there is a paucity of information regarding the contribution of progestins to progestagenic activity in municipal wastewaters and surface waters (Creusot et al., 2014).

Estrogenic activities have been studied very thoroughly and androgenic activities also have attracted a certain interest, but the other hormonal activities have not been given the proper research focus. Compounds exhibiting these activities, such as progestins, are nevertheless known environmental contaminants and endocrine disruptors (Kumar et al., 2015). Therefore, there exists a real need for further investigation of progestagenic activity in combination with thorough chemical analysis. To the best of our knowledge, only one study has been directed to revealing causative compounds of progestagenic activity downstream from a pharmaceutical factory (Creusot et al., 2014).

The first goals of the present study were to determine whether: 1) progestins are present in wastewaters and surface waters, 2) municipal wastewaters contain progestagenic activities, 3) progestagenic activities are removed during wastewater treatment processes, and 4) progestagenic activities emerging from the studied WWTPs affect receiving surface waters. Subsequently, we aimed to discover the extent to which detected progestins contribute to progestagenic activities in municipal WWTPs' effluents and in receiving surface waters. Because investigation of antagonistic activities should always be included into such an analysis (Ihara et al., 2014; Weiss et al., 2009), we also assessed each sampling locality in parallel for the presence of anti-progestagenic activities and for two selective progesterone receptor modulators, mifepristone and ulipristal acetate. SPRMs are synthetic compounds that block PR in certain tissues.

2. Material and methods

2.1. Chemicals

Methanol and acetonitrile (LiChrosolv® Hypergrade) were purchased from Merck (Darmstadt, Germany). Ultrapure water was produced using an Aqua-MAX-Ultra System (Younglin, Kyounggi-do, Korea).

Selection of analytes was made based upon the consumption of these compounds in the Czech Republic as reported elsewhere (Golovko et al., 2018). The native standards (Table S1) were purchased from Sigma-Aldrich (Czech Republic). The chemicals for which tests were made are listed in Supplementary material (Table S1). The internal standards (ISs): Altrenogest 19,19,20,21,21-d5; Chlormadinone-d6 Acetate; Cyproterone Acetate-13C2,d3; Medroxy Progesterone-d3; 6-epi-Medroxy Progesterone-d3 17-Acetate; Megestrol Acetate-d3; Mifepristone-d3; Progesterone-d9; and Ulipristal Acetate-d3 (purity ≥98%) were obtained from Toronto Research Chemicals (Canada). All chemicals tested in the *in vitro* reporter gene bioassay were dissolved in dimethyl sulfoxide of ≥99.5% purity. Individual stock solutions of the standards were prepared for chemical analysis at 1 mg/mL concentration in methanol and stored at −20 °C. A spiking mixture of ISs was prepared by diluting the stock solutions with methanol to a final concentration of 1 μg/mL for each compound. PR-CALUX cells, ORG 2058 standards in dimethyl sulfoxide, illuminate mix, and lysis mix were purchased from BioDetection Systems (BDS, Amsterdam, Netherlands).

2.2. Collection of samples, solid-phase extraction, and sample evaporation

Samples were taken from wastewaters and surface waters in the Czech and Slovak republics during January to June 2017. The study was performed at five Czech (Tábor-Klokoty, Strakonice, Prachatice, České Budějovice, and Brno) and one Slovak (Bratislava–Petržalka) municipal WWTPs and those watercourses receiving discharges from the WWTPs. Time proportional (15 min interval) composite wastewater samples (3–4 L) were collected from WWTP influents and effluents and cooled at 4 °C. Selected WWTPs employ mechanical-biological treatment technology with activated sludge-based secondary biological treatment in which they differ slightly as it is summarized in Supplementary material (Table S2). Grab surface water samples were collected up- and downstream from the respective WWTPs at 50 m distance from the WWTP effluent outlets at the same river side and at the same time as sampling of wastewater. Grab samples were taken close to the riverbank by submerging a 1 L amber glass bottle fastened to a stick.

Solid-phase extraction was carried out on an SPE-DEX 4790 automated solid-phase extractor (Horizon Technology, Salem, NH, USA) using a method reported by Golovko et al., 2018. Briefly, the collected samples (1 L each) were filtered through 5 and 1 μm glass fibre filters (Horizon Technology, Salem, NH, USA). Subsequently, the volume of filtered sample was passed through Atlantic C18 solid-phase extraction (SPE) discs (Horizon Technology, Salem, NH,

USA). Analytes were then retained on discs. The analytes were eluted from the discs with 10 mL of acetonitrile. SPE extracts were evaporated under gentle nitrogen stream to dryness at 37 °C on a Termovap TV10 + evaporator (ECOM, Czech Republic). After evaporation, wastewater and surface water extracts were redissolved either in 100 μL of acetonitrile or in 40 μL of dimethyl sulfoxide for chemical or biological analyses, respectively.

2.3. LC-APCI/APPI-HRPS analysis

An Accela 1250 LC pump (Thermo Fisher Scientific, San Jose, CA, USA) coupled with a hybrid quadrupole/orbital trap Q-Exactive mass spectrometer (Thermo Fisher Scientific) and HTS XT-CTC auto-sampler (CTC Analytics AG, Zwingen, Switzerland) were used to separate and detect target analytes. An analytical Hypersil Gold column (50 mm × 2.1 mm ID × 3 μm particles; Thermo Fisher Scientific) preceded by the same phase pre-column (10 mm × 2.1 mm ID × 3 μm particles) was used for chromatographic separation of the target analytes. Our analytical method for the analysis of a wide range of compounds with affinity to PR in wastewaters and surface waters using SPE followed by liquid chromatography tandem atmospheric pressure chemical ionization/atmospheric pressure photoionization with hybrid quadrupole/orbital trap mass spectrometry operated in high resolution product scan mode (LC-APCI/APPI-HRPS) had been validated for linearity, repeatability, quantification limit (LOQ), and recovery (Golovko et al., 2018). In the present study, the LC-APCI/APPI-HRPS method was applied to determine concentration of 15 progestins and 2 SPRMs in extracts from influent and effluent of six WWTPs and in respective up- and downstream recipient surface waters. The sample preparation is described in detail in a paper by Golovko et al., 2018. Samples from each sampling site were stored at 4 °C and analysed within 72 h. Prior to extraction for each site, a procedural blank (demineralized water) was extracted and analysed to distinguish between positive detections and potential sample contamination. Matrix matching standards corresponding to analysed matrices were used for correction of matrix effects. Briefly, the matrix standards were prepared by adding both IS and native compounds at amount of 10 ng and 200 ng into extract of corresponding matrix prepared the same way as real samples but without addition of IS. The peak area/internal standard ratio determined in non-spiked samples was subtracted from the peak area/internal standard ratio in matrix-matched standards to achieve the matrix-affected response factor. If matrix effect was lower than 20% we used response factors derived from calibration curve.

2.4. PR-CALUX and resazurin reduction assays

To detect (anti-)progestagenic activities either in water extracts or pure chemicals, PR-CALUX *in vitro* reporter gene bioassay was carried out as described elsewhere (Sonneveld et al., 2005). PR-CALUX bioassay was selected, because it is a highly sensitive *in vitro* bioassay and responds selectively to (anti-)progestagenic compounds (Sonneveld et al., 2011). The reference compound for progestagenic activity was ORG 2058, while mifepristone was the reference compound for assessing anti-progestagenic activity. All samples, including pure chemicals, were tested for cytotoxicity using resazurin reduction assay. First, PR-CALUX cells exposed to samples were visually inspected for cytotoxicity under a microscope. Subsequently, we carried out resazurin reduction assay using a resazurin-based *in vitro* toxicology assay kit (Sigma-Aldrich) to evaluate the potential effect of the sample on cell viability (O'Brien et al., 2000). The procedures of PR-CALUX assay and cytotoxicity testing are described in detail in the Supplementary material (chapters 1 and 2).

2.5. Data analysis

Total activities determined by a PR-CALUX bioassay (bio-TEQ, ng/L ORG 2058 EQs) were compared with the sum of the potencies of the individual compounds identified by chemical analysis (chem-TEQ) in order to estimate the degree to which analysed substances account for the biological activity. Chem-TEQ_i of a single compound was calculated using the following equation: chem-$TEQ_i = C_i \times REP_i$, where C_i is concentration of a compound (data from LC-APCI/APPI-HRPS chemical analysis) and REP_i is relative potency of a compound (derived from PR-CALUX). Chem-TEQ of a whole extract was calculated as the sum of chem-TEQs of individual compounds found in the sample: chem-TEQ = Σ chem-TEQ_i. Contribution of a single compound to bio-TEQ was calculated as follows: % contribution = (chem-TEQ_i/bio-TEQ) × 100. The same calculation was used for anti-progestagenic activity.

3. Results and discussion

3.1. Measured concentrations of progestins and SPRMs

Municipal WWTP influents contained nine progestins (cyproterone acetate, dienogest, drospirenone, gestodene, medroxyprogesterone, medroxyprogesterone acetate, megestrol acetate, nomegestrol acetate, and progesterone) in the range of 0.19–48 ng/L (Tables 1 and 2). Three compounds – dienogest, megestrol acetate, and progesterone – were found in all assessed influents. There was only one positive detection of an SPRM all through the sampling period and all studied types of matrices. This was mifepristone, at a concentration of 0.65 ng/L, in influent of České Budějovice's WWTP.

Seven progestins (cyproterone acetate, dienogest, drospirenone, megestrol acetate, medroxyprogesterone, medroxyprogesterone acetate, and progesterone) were detected in effluents at concentrations ranging from 0.11 to 3.2 ng/L (Tables 1 and 2). Those three compounds most widespread in influents (dienogest, megestrol acetate, and progesterone) were also those most frequently present in effluents, with four (megestrol acetate and progesterone) and three (dienogest) positive detections out of six attempts through the sampling campaign (Tables 1 and 2).

Only medroxyprogesterone and progesterone occurred even in surface waters. Progesterone was found three times both up- and downstream in concentration ranges of 0.20–0.42 ng/L (median 0.23 ng/L) and 0.17–1.2 ng/L (median 0.21 ng/L), respectively. The concentrations of progesterone are comparable with data reported from China, where progesterone was found upstream and downstream at concentrations of 0.5 and 2.5 ng/L, respectively (Liu et al., 2011). In the present study, most WWTP discharges did not significantly increase progesterone levels in surface waters. The exception was the WWTP at Bratislava–Petržalka, where progesterone was found at considerably higher concentration (Table 2) downstream (1.2 ng/L) than upstream (0.42 ng/L). Medroxyprogesterone is the only synthetic progestin found in surface water. It was detected only once (0.12 ng/L), that being in the River Svratka downstream from WWTP Brno. Occurrence of medroxyprogesterone in surface water has once been reported in the United States (Kolodziej et al., 2003) and in municipal WWTP effluent in the Czech Republic (Golovko et al., 2018). Medroxyprogesterone appears to be a specific pollutant among synthetic progestins for WWTP Brno, because this compound has already been detected in untreated wastewater from a hospital in Brno (Macikova et al., 2014). Therefore, the main source of medroxyprogesterone is likely this local hospital. Medroxyprogesterone is not prescribed in the Czech Republic and it may occur as a product of biotransformation from medroxyprogesterone acetate (Golovko

Table 1
Concentration of (anti-)progestins, chemical toxic equivalents (chem-TEQs), and biological toxic equivalents performed in agonistic and antagonistic modes (bio-TEQs and mifepristone EQs) in wastewater and receiving surface water of three wastewater treatment plants (WWTPs) with small catchment populations. "inf" = influent; "eff" = effluent; "usw" = upstream of WWTP; "dsw" = downstream of WWTP; "ND" = not determinable; "<LOQ" = results of biological method below the limit of quantification; "[a]" = mean of two replicates; "[b]" = masking effect occurred. Positive detections in chemical analysis are highlighted. Chem-TEQs were not calculated when concentrations of target compounds were below LOQ.

	Concentration (ng/L)											
	Tábor-Klokoty				Prachatice				Strakonice			
	inf	eff	usw	dsw	inf	eff	usw	dsw	inf	eff	usw	dsw
Progesterone	**9.5**	**0.63**	**0.20**	**0.17**	**48**	**0.15**	**0.23**	**0.21**	**27**	<0.04	<0.07	<0.09
Dydrogesterone	<0.18	<0.13	<0.18	<0.07	<0.29	<0.26	<0.28	<0.3	<0.49	<0.30	<0.46	<0.26
Medroxyprogesterone	<0.02	<0.01	<0.02	<0.01	<0.01	<0.01	<0.01	<0.01	<0.04	<0.02	<0.04	<0.02
Medroxyprogesterone acetate	<0.20	<0.14	<0.20	<0.08	<0.83	<0.35	<0.08	<0.05	**0.88**	**0.38**	<0.02	<0.01
Cyproterone acetate	<0.14	<0.10	<0.14	<0.06	<0.91	<0.39	<0.87	<0.54	<0.29	<0.24	<0.32	<0.19
Chlormadinone acetate	<0.19	<0.15	<0.18	<0.08	<0.33	<0.27	<0.25	<0.23	<0.49	<0.28	<0.41	<0.25
Megestrol acetate	**0.52**	**0.14**	<0.02	<0.01	**8.0**	**1.0**	<0.84	<0.52	**8.0**	**0.28**	<0.07	<0.07
Nomegestrol acetate	**5.3**	<0.01	<0.02	<0.01	<0.02	<0.02	<0.02	<0.02	**10**	<0.02	<0.03	<0.02
Ulipristal acetate	<0.18	<0.15	<0.18	<0.08	<0.33	<0.27	<0.25	<0.23	<0.42	<0.25	<0.36	<0.22
Dienogest	**2.8**	**0.51**	<0.24	<0.24	**1.3**	**0.31**	<0.33	<0.39	**12**	<0.04	<0.06	<0.04
Norethisterone	<0.02	<0.02	<0.02	<0.01	<0.03	<0.03	<0.03	<0.04	<0.10	<0.05	<0.10	<0.04
Altrenogest	<0.20	<0.14	<0.20	<0.08	<0.12	<0.05	<0.11	<0.07	<0.02	<0.02	<0.02	<0.02
Mifepristone	<0.19	<0.15	<0.18	<0.08	<0.29	<0.24	<0.23	<0.20	<0.06	<0.04	<0.05	<0.03
Levonorgestrel	<0.26	<0.18	<0.21	<0.09	<0.35	<0.28	<0.31	<0.34	<0.07	<0.03	<0.05	<0.03
Etonogestrel	<0.25	<0.18	<0.20	<0.09	<0.31	<0.24	<0.27	<0.30	<0.62	<0.32	<0.46	<0.26
Gestodene	<0.41	<0.19	<0.23	<0.09	**5.0**	<0.25	<0.31	<0.34	**6.3**	<0.29	<0.52	<0.27
Drospirenone	**0.89**	<0.18	<0.20	<0.09	<0.34	<0.27	<0.30	<0.33	**3.0**	**0.11**	<0.04	<0.04
chem-TEQ (ng/L ORG 2058 EQs)	1.9	0.11	0.02	0.01	16	0.33	0.02	0.02	20	0.29	ND	ND
bio-TEQ (ng/L ORG 2058 EQs)	<LOQ	0.04	<LOQ	<LOQ	0.62	0.47	0.04	0.06	<LOQ	0.3	<LOQ	<LOQ
antagonism (ng/L mifepristone EQs)	5.6	0.77	<LOQ	<LOQ	9.7	<LOQ	<LOQ	<LOQ	83[a]	<LOQ	<LOQ	<LOQ
contribution of compounds to bio-TEQ (%)	ND[b]	>100[b]	ND	ND	>100[b]	70	45	28	ND[b]	96	ND	ND

Table 2
Concentration of (anti-)progestins, chemical toxic equivalents (chem-TEQs), and biological toxic equivalents performed in agonistic and antagonistic mode (bio-TEQs and mifepristone EQs) in wastewater and receiving surface water of three wastewater treatment plants (WWTPs) with large catchment populations. "inf" = influent; "eff" = effluent; "usw" = upstream of WWTP; "dsw" = downstream of WWTP; "ND" = not determinable; "<LOQ" = results of biological method below the limit of quantification; "[a]" = mean of two replicates; "[b]" = masking effect occurred. Positive detections in chemical analysis are highlighted. Chem-TEQs were not calculated when concentrations of target compounds were below LOQ.

	Concentration (ng/L)											
	České Budějovice				Brno				Bratislava-Petržalka			
	inf	eff	usw	dsw	inf	eff	usw	dsw	inf	eff	usw	dsw
Progesterone	**27**	<0.04	<0.07	<0.07	**4.3**	**0.31**	<0.20	<0.42	**14**	**3.2**	**0.42**	**1.2**
Dydrogesterone	<0.31	<0.26	<0.47	<0.45	<0.37	<0.31	<0.20	<0.50	<1.1	<0.81	<0.30	<0.16
Medroxyprogesterone	<0.03	<0.03	<0.04	<0.04	**0.19**	**0.95**	<0.02	**0.12**	<0.53	<0.16	<0.23	<0.09
Medroxyprogesterone acetate	<0.04	<0.04	<0.07	<0.07	**8.1**	**0.13**	<0.17	<0.40	<1.1	<0.18	<0.24	<0.10
Cyproterone acetate	**2.9**	<0.44	<0.44	<0.45	**12**	**0.50**	<0.16	<0.37	<0.07	<0.02	<0.03	<0.01
Chlormadinone acetate	<0.25	<0.23	<0.39	<0.36	<0.28	<0.14	<0.08	<0.21	<1.3	<0.40	<0.25	<0.19
Megestrol acetate	**3.4**	**0.13**	<0.06	<0.06	**13**	<0.38	<0.20	<0.48	**4.2**	<0.21	<0.29	<0.11
Nomegestrol acetate	<0.07	<0.06	<0.10	<0.10	<0.34	<0.22	<0.15	<0.36	<1.3	<0.23	<0.31	<0.11
Ulipristal acetate	<0.28	<0.26	<0.44	<0.40	<0.34	<0.24	<0.11	<0.28	<0.22	<0.07	<0.04	<0.03
Dienogest	**9.6**	**0.62**	<0.06	<0.05	**6.1**	<0.22	<0.18	<0.55	**3.9**	<4.0	<0.32	<0.72
Norethisterone	<0.04	<0.05	<0.07	<0.07	<0.36	<0.20	<0.17	<0.54	<0.91	<4.1	<0.33	<0.74
Altrenogest	<0.04	<0.04	<0.06	<0.07	<0.05	<0.03	<0.02	<0.05	<1.1	<0.25	<0.20	<0.15
Mifepristone	**0.65**	<0.26	<0.43	<0.40	<0.06	<0.03	<0.02	<0.06	<0.10	<0.08	<0.03	<0.02
Levonorgestrel	<0.36	<0.32	<0.58	<0.47	<0.32	<0.18	<0.21	<0.49	<1.2	<0.18	<0.26	<0.10
Etonogestrel	<0.38	<0.35	<0.62	<0.50	<0.38	<0.26	<0.26	<0.59	<1.2	<0.94	<0.35	<0.19
Gestodene	**7.0**	<0.25	<0.43	<0.37	<0.22	<0.15	<0.14	<0.34	<0.79	<3.5	<0.29	<0.64
Drospirenone	**1.9**	<0.07	<0.07	<0.05	**6.7**	<0.25	<0.25	<0.57	<0.91	<0.29	<0.30	<0.11
chem-TEQ (ng/L ORG 2058 EQs)	18	0.06	ND	ND	9.81	0.13	0.26	0.03	3.1	0.26	0.03	0.09
bio-TEQ (ng/L ORG 2058 EQs)	0.57	0.08	0.03	<LOQ	0.71	0.2	<LOQ	<LOQ	0.09	0.09	0.04	<LOQ
antagonism (ng/L mifepristone EQs)	8.3	<LOQ	<LOQ	<LOQ	5.4	<LOQ	<LOQ	<LOQ	2.6	0.89	<LOQ	<LOQ
contribution of compounds to bio-TEQ (%)	>100[a]	76	ND	ND	>100[a]	65	ND	ND	>100[a]	>100[a]	83	ND

et al., 2018).

3.2. (Anti-)progestagenic activity of pure compounds

It was possible to obtain complete dose-response curves for all studied progestins (Fig. 1) in order to derive EC_{50}, PC_{10}, and relative potency (REP) values (Table S3). Ulipristal acetate showed a full antagonistic dose-response curve (Fig. 2), and this was used to derive IC_{50}, PC_{20} and antagonistic REP value for the SPRM (Table S4).

Six of the 17 compounds analysed in this study (cyproterone acetate, dydrogesterone, levonorgestrel, medroxyprogesterone, mifepristone, norethisterone, and progesterone) have already been

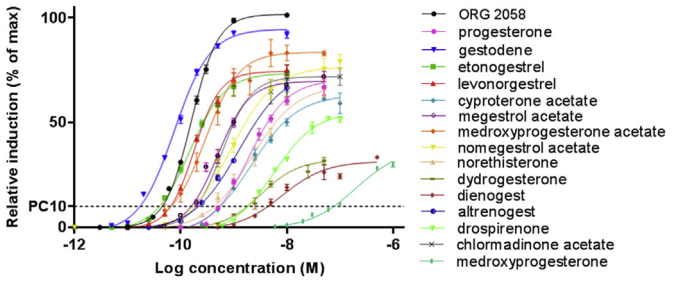

Fig. 1. Dose-response curves of synthetic progestins and progesterone in PR-CALUX cells. Maximum relative induction corresponds to 100% induction caused by reference compound ORG 2058. Dotted line indicates positive control 10 (PC_{10}) that is effect of a compound corresponding to 10% induction caused by reference compound ORG 2058. Values are expressed as means ± standard error of the mean.

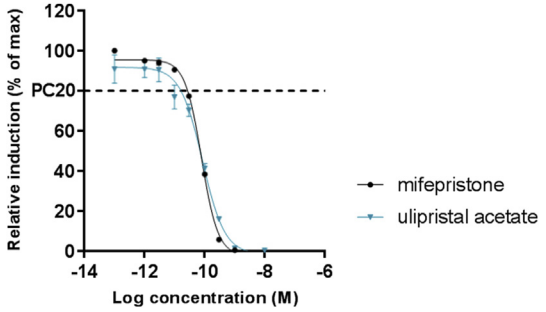

Fig. 2. Dose-response curves of two selective progesterone receptor modulators in PR-CALUX carried out in antagonistic mode. Assay medium contained EC_{50} concentration of ORG 2058. Dotted line indicates positive control 20 (PC_{20}) that is effect of a compound corresponding to 20% inhibition caused by reference compound mifepristone. Values are expressed as means ± standard error of the mean.

tested by other authors using PR-CALUX assay (Houtman et al., 2009; Rižner et al., 2011). To the best of our knowledge, this is the first systematic and comprehensive *in vitro* profiling of progestagenic activity of progestins that are relevant for the aquatic environment. In addition, medroxyprogesterone has been tested in the present study in *in vitro* reporter gene bioassay for the first time.

Ulipristal acetate was the only compound tested for anti-progestagenic activity. It has shown similar potency as a reference compound (mifepristone) for anti-progestagenic activity (Table S4). Our results together show that ulipristal acetate is the first environmentally relevant compound with anti-progestagenic activity similar to that of mifepristone.

3.3. (Anti-)progestagenic activity in wastewater and surface water

Unlike estrogenic activities, there have been only a few attempts to date to detect (anti-)progestagenic activities in aquatic environments (Table S5). In the present study, progestagenic agonistic activities were found in four of six WWTP influents in the range of 0.09–0.6 ng/L ORG 2058 EQs (median 0.38 ng/L ORG 2058 EQs). The PR-CALUX assay revealed also the presence of progestagenic activity ranging from 0.04 to 0.47 ng/L ORG 2058 EQs (median 0.15 ng/L ORG 2058 EQs) in all six WWTP effluents. That roughly corresponds to 0.5–6.1 ng/L progesterone EQs. Such contamination is still of considerable concern when bearing in mind that ORG 2058 is approximately 13 times more potent than is progesterone (Table S3; van der Linden et al., 2008). Activities found in the present study were in the same order of magnitude as those reported for effluents in the Netherlands (van der Linden et al., 2008) and India (Viswanath et al., 2008). Higher concentrations reaching to 5.4 ng/L ORG 2058 EQs have been found in effluents in Australia (Bain et al., 2014; Leusch et al., 2014). Surprisingly, no progestagenic activities were found in worldwide inter-laboratory screening (Escher et al., 2014) and at some sites in Australia (Scott et al., 2014), China (Rao et al., 2014), France (Bellet et al., 2012), and Tunisia (Mnif et al., 2010). Some non-detects, however, might be attributed to high limits of detection, such as 5 ng/L ORG 2058 EQs (Scott et al., 2014).

Progestagenic activities detected in surface water were one order of magnitude lower than those found in effluents. They were detected at three sampling points and ranged from 0.03 to 0.06 ng/L ORG 2058 EQs (median 0.04 ng/L ORG 2058), which corresponds to approximately 0.4–0.8 ng/L progesterone EQs. Surprisingly, weak progestagenic activities, at 0.03 and 0.04 ng/L ORG 2058 EQs, occurred twice in upstream but not in downstream surface waters (at the České Budějovice and Bratislava–Petržalka sampling sites). To sum up, Czech and Slovak surface waters did not seem to be seriously affected by progestagenic activity from municipal WWTP discharges in most cases. Only once did the progestagenic activity persist in surface water 50 m downstream, and then at a concentration of 0.06 ng/L ORG 2058 EQs. Such concentration is approximately equivalent to 0.8 ng/L of progesterone. The highest reported concentration of progestagenic activity has been as high as 4.5 ng/L ORG 2058 EQs in a Netherlands stream (van der Linden et al., 2008).

Anti-progestagenic activities were detected in all six influents and at three out of six investigated WWTP effluents, with concentrations ranging between 2.63 and 83 ng/L mifepristone EQs (median 6.97 ng/L mifepristone EQs) and 0.77 to 1.01 ng/L mifepristone EQs (median 0.89 ng/L mifepristone EQs), respectively. Strong anti-progestagenic activities in effluent and surface water (up to 31.5 and 121 µg/L mifepristone EQs, respectively) had previously been detected in China (Rao et al., 2014). Potent anti-progestagenic activities up to 32 µg/L mifepristone EQs were also reported in Australian surface water (Scott et al., 2014). Somewhat weaker anti-progestagenic activities were found in France with maximal concentration of 3.8 ng/L mifepristone EQs (Bellet et al., 2012). High anti-progestagenic loads detected at all six studied influents were completely or significantly removed during the treatment process. In the case of effluents at WWTP Tábor-Klokoty and WWTP Bratislava–Petržalka, anti-progestagenic activities co-occurred with progestagenic agonistic activities. No anti-progestagenic activity has been found in surface water samples (Tables 1 and 2).

3.4. Comparison of chem-TEQs and bio-TEQs in effluents and surface water

Predicted chem-TEQs of synthetic progestins and progesterone have accounted to measured bio-TEQs in effluents with 65, 70, 76 and 96% on the basis of REPs calculated from EC_{50} and 58, 64, 78 and 114% based on the latter approach with REPs calculated from PC_{10}. The unexplained portions of progestagenic activities (remaining to bio-TEQ, Table 3 and Table S6) might be attributed either to the presence of unknown compounds or to synergy between compounds (Table 3). When anti-progestagenic activities suppress the signal of agonists, the chem-TEQs are higher than the bio-TEQs (Tables 1 and 2). This phenomenon is known as masking effect (Creusot et al., 2014; Ihara et al., 2014; Weiss et al., 2009). All influent samples exhibited masking effects, while among WWTP effluents only those at Tábor-Klokoty and Bratislava–Petržalka were observed to have masking effects (Tables 1 and 2).

Additionally, contributions of the individual compounds to bio-TEQs in effluents and surface waters were estimated (Table 3). In effluents, medroxyprogesterone acetate, megestrol acetate, and

Table 3
Contribution of progesterone receptor-active compounds to measured progestagenic activity (bio-TEQs) in effluents at six municipal wastewater treatment plants on the basis of EC_{50}-derived relative potencies. Those compounds contributing most are highlighted. Values of contributing compounds were rounded to nearest whole number.

					Masking effect occurred	
Contribution to bio-TEQs (%)	Prachatice	Strakonice	České Budějovice	Brno	Tábor-Klokoty	Bratislava-Petržalka
Cyproterone acetate	0	0	0	18	0	0
Dienogest	2	0	22	0	34	0
Drospirenone	0	1	0	0	0	0
Medroxyprogesterone	0	0	0	<1	0	0
Medroxyprogesterone acetate	0	**67**	0	**34**	0	0
Megestrol acetate	**66**	29	**54**	0	108	0
Progesterone	3	0	0	12	**124**	**288**
Unknown compounds or synergy	29	3	24	36	0	0

progesterone were the main contributors to progestagenic activities (in two out of six studied WWTP effluents). Interestingly, progesterone was the greatest contributor in those effluents where masking effects were observed. It is noteworthy that the main contributors to progestagenic activity were progesterone derivatives (cyproterone acetate, medroxyprogesterone, medroxyprogesterone acetate, and megestrol acetate), while there was only one contributing testosterone (dienogest) and one spironolactone (drospirenone) derivative. To date, only one study has described a contribution of progestins to progestagenic activities. In that case, it was found that a single progestin, levonorgestrel, can contribute as much as approximately 50% to progestagenic activities in surface water (Creusot et al., 2014).

Regarding surface waters, progestins contributed as much as 83% to overall progestagenic activities. There was only one case in which progestagenic activity was detectable in surface water contaminated with progestins. The recipient of effluent from WWTP Prachatice, the Živný Brook, manifested progestagenic activities of 0.06 ng/L ORG 2058 EQs, which is equivalent to almost 1 ng/L of progesterone. This can be attributed to the fact that Živný Brook is a small stream and so the dilution is less than in the other studied recipients. Moreover, we revealed that progesterone was responsible for 45% of progestagenic activity upstream but only for 28% downstream from WWTP Prachatice. This indicates discharging of some unknown PR-active compounds in the effluent. Co-occurrence of progestagenic activity (0.04 ng/L ORG 2058 EQs) and the natural progestin progesterone (0.5 ng/L) has also been recorded upstream from WWTP Bratislava–Petržalka, and progesterone was responsible for most of the progestagenic activity (83%).

In the present study, we revealed only one substance contributing to anti-progestagenic activity of wastewater and that was mifepristone (in influent of WWTP České Budějovice). It was responsible, however, for only 7.8% of detected mifepristone EQ. As no SPRM was revealed in effluents and surface waters, the causative anti-progestagenic compounds remained unknown. We can speculate that it might originate from various industrial by-products and compounds included in personal care products, such as UV-filters or polycyclic musks, that are present in the aquatic environment and known to possess anti-progestagenic activities (Hamers et al., 2006; Schreurs et al., 2005).

3.5. Potential adverse effects in aquatic animals

Of the three compounds (dienogest, megestrol acetate, and progesterone) that occurred most frequently in effluents, effects on aquatic animals only of progesterone are reported in the literature (Kumar et al., 2015). The lowest observed effect concentration (LOEC) for progesterone in fish has been reported to be as low as 2 ng/L (Zucchi et al., 2012) which amount was exceeded in effluent of WWTP Bratislava–Petržalka and is quite close to the concentration of 1.2 ng/L detected even in surface water downstream from this WWTP. Among the compounds detected less frequently in effluents (cyproterone acetate, drospirenone, and medroxyprogesterone acetate), only the concentration of cyproterone acetate (0.5 ng/L) was close to its LOEC value, reported by Sharpe et al. (2004) to be 1 ng/L. Medroxyprogesterone acetate, which was detected twice, and drospirenone, which was found once, have LOECs (Zhao et al., 2015; Zucchi et al., 2014) much higher than the respective concentrations in effluents. If, however, the compounds occur in the water together, they may act in synergy and their LOEC values can then be significantly lower than determined under laboratory conditions. Zhao et al. (2015) describe such a synergy in the case of dydrogesterone and medroxyprogesterone acetate. Particular attention should be devoted to medroxyprogesterone, which was the only synthetic progestin detected in surface water. Unfortunately, there are no toxicological data available for this compound.

Progestins have been found to be very weak (drospirenone, gestodene, and progesterone) or non-agonists (e.g. levonorgestrel or etonogestrel) of PR in fish (Bain et al., 2015). Nevertheless, some of them do considerably transactivate fish androgen receptor (Bain et al., 2015; Ellestad et al., 2014). The mode of action in fish is likely different from what we know in humans (Kumar et al., 2015), and using PR-CALUX, which is based on human PR, may not be appropriate. Regarding aquatic vertebrates, activity detected by PR-CALUX assay seems to be a better risk indicator for amphibians than for fish, as amphibian PR is believed to have high amino acids sequence similarity (within the hormone binding domain) with PR in humans (Bayaa et al., 2000). Moreover, adverse effects of exposure to progesterone and synthetic progestins in amphibians seem to be similar to the effects in higher vertebrates, including humans (Kvarnryd et al., 2011; Säfholm et al., 2014, 2016).

4. Conclusions

Results of the present study show that synthetic progestins together with progesterone constitute the majority of causative agents of progestagenic activity in municipal WWTP effluents, with medroxyprogesterone acetate, megestrol acetate, and progesterone being the most important. Of the progestins that occurred most frequently in water, only four have been tested on aquatic vertebrates. Even in these cases, knowledge as to their possible adverse effects is very limited. Although strong anti-progestagenic activities were detected in two effluents, no causative agents were revealed. Despite that anti-progestagenic activities have been detected by various authors and were detected also in this study, *in vivo* anti-progestagenic effects on aquatic biota are practically not known and should therefore be studied.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.watres.2018.02.065.

Abbreviations

APCI	atmospheric pressure chemical ionization
APPI	atmospheric pressure photoionization
bio-TEQ	measured progestagenic activity equivalents (ng/L ORG 2058 EQs)
C	concentration
CALUX	*in vitro* bioassay called chemically activated luciferase gene expression
chem-TEQ	predicted progestagenic activity equivalents (ng/L ORG 2058 EQs)
EQ	equivalent
HRPS	high resolution product scan
LC	liquid chromatography
LOEC	lowest observed effect concentration
LOQ	limit of quantification (ng/L)
PR	progesterone receptor
REP	relative potency
SPE	solid-phase extraction
SPRM	selective progesterone receptor modulator
UV	ultraviolet light
WWTP	wastewater treatment plant

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Do progestins contribute to (anti-)androgenic activities in aquatic environments?☆

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ABSTRACT

Unknown compounds with (anti-)androgenic activities enter the aquatic environment via municipal wastewater treatment plants (WWTPs). Progestins are well-known environmental contaminants capable of interfering with androgen receptor (AR) signaling pathway. The aim of the present study was to determine if 15 selected progestins have potential to contribute to (anti-)androgenic activities in municipal wastewaters and the respective recipient surface waters. AR-specific Chemically Activated LUciferase gene eXpression bioassay in agonistic (AR-CALUX) and antagonistic (anti-AR-CALUX) modes and liquid chromatography tandem atmospheric pressure chemical ionization/atmospheric photoionization with hybrid quadrupole/orbital trap mass spectrometry operated in high resolution product scan mode (LC-APCI/APPI-HRPS) methods were used to assess (anti-)androgenic activity and to detect the target compounds, respectively. The contribution of progestins to (anti-)androgenic activities was evaluated by means of a biologically and chemically derived toxicity equivalent approach. Androgenic (0.08–59 ng/L dihydrotestosterone equivalents – DHT EQs) and anti-androgenic (2.4–26 μg/L flutamide equivalents – FLU EQs) activities and progestins (0.19–75 ng/L) were detected in selected aquatic environments. Progestins displayed androgenic potencies (0.01–0.22 fold of dihydrotestosterone) and strong anti-androgenic potencies (9–62 fold of flutamide). Although they accounted to some extent for androgenic (0.3–29%) and anti-androgenic (4.6–27%) activities in influents, the progestins' contribution to (anti-)androgenic activities was negligible (≤2.1%) in effluents and surface waters. We also tested joint effect of equimolar mixtures of target compounds and the results indicate that compounds interact in an additive manner. Even if progestins possess relatively strong (anti-)androgenic activities, when considering their low concentrations (sub-ng/L to ng/L) it seems unlikely that they would be the drivers of (anti-)androgenic effects in Czech aquatic environments.

1. Introduction

Mixtures of many chemicals are continuously discharged by wastewater treatment plants (WWTPs) into aquatic environments. Some of these compounds may adversely affect the endocrine system of exposed organisms via androgen receptor (AR)-mediated signaling pathway (Gray et al., 2001; Kelce et al., 1998; Sohoni and Sumpter, 1998). To date, natural and synthetic estrogens have drawn great eco-toxicological interest due to their ability to induce intersex and feminization in freshwater fish (Leusch et al., 2017; Sumpter, 2005). Widespread feminization of male fish living downstream from WWTPs has been revealed to be caused not only by estrogens, however, but also by anti-androgenic compounds (Jobling et al., 2009). Moreover, androgenic contaminants of surface water can cause masculinization of resident female fish (Howell et al., 1980; Parks et al., 2001). (Anti-)androgenic activities have frequently been reported in aquatic environments worldwide (Bain et al., 2014; Boehler et al., 2017; Escher et al., 2014; Kinani et al., 2010; König et al., 2017; Urbatzka et al., 2007; van der Linden et al., 2008; Zhao et al., 2011). Compounds responsible for these activities often remain unidentified (Chen and Chou, 2016; Kinani et al., 2010; Leusch et al., 2014; Urbatzka et al., 2007).

Recently, progestins have come to be one of the groups of

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emerging pollutants drawing attention (Fent, 2015; Kumar et al., 2015). The group of substances termed progestins includes not only such natural hormones as progesterone but also synthetic substances designed to have biological activity similar to that of progesterone. Progestins are contained in contraceptives and other hormonal preparations (Sitruk-Ware, 2004). Both progesterone and synthetic progestins act primarily as progesterone receptor (PR) agonists (Africander et al., 2011), but they also cause off-target modulation of other steroid receptors (Bain et al., 2015; Besse and Garric, 2009; Stanczyk, 2003). Among other activities, progestins are known to act as both potent agonists and antagonists of human AR (Bain et al., 2015; Schindler et al., 2008). Androgenicity of some progestins has recently been observed also *in vivo* (Hua et al., 2015; Runnalls et al., 2013; Svensson et al., 2013, 2016; Zeilinger et al., 2009) and *in vitro* (Bain et al., 2015; Ellestad et al., 2014) within fish. Moreover, some of these progestins have been shown to inhibit synthesis of androgens *in vivo* (Fernandes et al., 2014) and possess anti-androgenic activity *in vitro* (Siegenthaler et al., 2017) in fish.

Surprisingly, no study to date has investigated whether environmental levels of progestins reach sufficient concentrations and have relative potencies strong enough to cause a substantial part of (anti-)androgenic activity observed in aquatic environments. The aim of the present study was to discover the extent to which progestins are responsible for (anti-)androgenic activities in Czech aquatic environments associated with municipal WWTPs. In parallel, we also assessed each sampling locality for the presence of a PR antagonist mifepristone and a selective PR modulator ulipristal acetate, because these compounds are suspected to be novel environmental contaminants (Golovko et al., 2018; Liu et al., 2010; Šauer et al., 2018). (Anti-)androgenic activities of mifepristone and ulipristal acetate and their contribution to overall sample activities were determined, as well.

2. Material and methods

2.1. Chemicals and material

All progestins, mifepristone and ulipristal acetate prescribed in the Czech Republic (Golovko et al., 2018) were chosen as target compounds. In addition, medroxyprogesterone was included because it has recently been found in Czech aquatic environments (Macikova et al., 2014a; Šauer et al., 2018). All tested compounds were of high purity as follows: altrenogest (≥99%), chlormadinone acetate (99.7%), cyproterone acetate (≥98%), dienogest (99.9%), drospirenone (99.9%), dydrogesterone (99.5%), etonogestrel (≥98%), flutamide (≥99%), gestodene (≥98%), levonorgestrel (≥99%), medroxyprogesterone (98.5%), medroxyprogesterone acetate (≥97%), megestrol acetate (≥99%), mifepristone (≥98%), nomegestrol acetate (≥98%), norethisterone (≥98%), progesterone (99.9%), and ulipristal acetate (≥98%). All were purchased from Sigma-Aldrich (Czech Republic). Classification of the studied compounds and their physicochemical properties are described in more detail in the Supplementary Material (Table S1). As internal standards, Altrenogest 19,19,20,21,21-d5, Chlormadinone-d6 Acetate, Cyproterone Acetate-13C2,d3, Medroxy Progesterone-d3, 6-epi-Medroxy Progesterone-d3, 17-Acetate, Megestrol Acetate-d3, Mifepristone-d3, Progesterone-d9, and Ulipristal Acetate-d3 were purchased from Toronto Research Chemicals (Canada). Individual stock solutions of native and internal standards were prepared for chemical analysis at 1 mg/mL concentration in methanol and stored at −20 °C. A spiking mixture of internal standards was prepared by diluting the stock solutions with methanol to a final concentration of 1 μg/mL for each compound. AR-CALUX cells, illuminate mix, lysis mix, and dihydrotestosterone standards prepared in dimethyl sulfoxide (≥99.5% purity) were purchased from BioDetection Systems (the Netherlands). Ultrapure water was produced using an Aqua-MAX-Ultra System (Younglin, Kyounggi-do, Korea).

2.2. Collection of samples, sample preparation, and solid-phase extraction

Samples were collected from wastewaters (influents and effluents) of four WWTPs located in the Czech Republic and from the receiving surface waters (upstream and downstream). The WWTPs receive domestic and industrial wastewaters and at two sites, WWTPs at Prachatice and České Budějovice, hospital wastewaters. While all the studied WWTPs are based on mechanical–biological treatment with activated sludge secondary treatment, they differ slightly in their biological treatment (Table S2). Grab or time proportional (15-minute interval) 24 h composite samples (3–4 L) were collected (Table S2). Grab surface water sampling was performed up- and downstream from the respective WWTPs at a distance of 50 m from WWTP outlets at the same side of the point of discharge. Grab samples were taken using a 2 L bottle fastened to a stick and then poured into 1 L amber glass bottles. Surface water samples were collected at the same time as were samples of effluents. The collected samples were transported to the laboratory and stored at 4 °C in darkness until extraction, which was carried out within 24 h.

In order to preconcentrate the target compounds, a recently developed protocol for solid-phase extraction (SPE) and sample evaporation was used (Golovko et al., 2018), albeit with a slight change wherein some samples were acidified prior to extraction to find out the influence of acidification on the extraction efficiency of SPE (see section 2.3.). Briefly, an SPE-DEX 4790 automated solid-phase extractor (Horizon Technology, Salem, NH, USA) was employed to extract 1 L water samples. Atlantic C18 SPE disks (Horizon Technology) were used as a sorbent and preconditioned with acetonitrile for liquid chromatography mass spectrometry (Sigma-Aldrich, Czech Republic) and demineralized water. The samples were filtered through Atlantic Fast Flow glass fiber filters of pore sizes 5 and 1 μm (Horizon Technology). After a sample had been passed through the Atlantic C18 SPE disks, the entire extraction system was rinsed with demineralized water. The Atlantic C18 SPE disks were air dried for 15 min. The retained target compounds were then eluted with total volume of 10 mL acetonitrile. The SPE extracts thus obtained were evaporated by gentle nitrogen stream until dryness at 37 °C using a Termovap TV10 + sample concentrator (ECOM, Czech Republic). The extracts were redissolved either in 2 × 50 μL of acetonitrile for chemical or in 2 × 20 μL of dimethyl sulfoxide for biological analyses.

2.3. pH test

Because sample pH is an important factor influencing extraction efficiency (Kuster et al., 2009; Vulliet et al., 2008), we tested the effect of sample acidification prior to SPE. An advantage of sample acidification prior to SPE is that it inhibits biological activity of microorganisms potentially present in samples and thereby may help in preventing biotransformation and bioconcentration of target compounds. Sample acidification also may influence the dissociation of ionizable compounds, however, and thus cause problems with retention of analytes on SPE sorbents. C18 SPE sorbents such as Atlantic disks best retain neutral forms of polar compounds, but some analytes may be affected due to sample acidification depending upon their dissociation constants (Table S3). Thus, we assessed whether sample pH adjustment had an effect on retention of progestins, mifepristone and ulipristal acetate on Atlantic C18 SPE disks. The effect was evaluated

according to the recoveries of target analytes, assessing whether recoveries were within a satisfactory range of 60–130%. Detailed description of pH test can be found in Supplementary material (see section 1).

2.4. Chemical analysis

An Accela 1250 LC pump (Thermo Fisher Scientific, San Jose, CA, USA) coupled with a hybrid quadrupole/orbital trap Q-Exactive mass spectrometer (Thermo Fisher Scientific) and HTS XT-CTC auto-sampler (CTC Analytics AG, Zwingen, Switzerland) were used for analysis of water extracts. An analytical Hypersil Gold column (50 mm × 2.1 mm ID × 3 μm particles; Thermo Fisher Scientific) preceded by the same phase pre-column (10 mm × 2.1 mm ID × 3 μm particles) was used for chromatographic separation of the target analytes. Our analytical method for the analysis of a wide range of progestins, mifepristone and ulipristal acetate in wastewaters and surface waters using SPE followed by LC-APCI/APPI-HRPS had previously been validated for linearity, repeatability, limit of quantification (LOQ), and recovery (trueness) (Golovko et al., 2018). In the present study, the LC-APCI/APPI-HRPS method was applied to determine the concentration of 15 progestins, mifepristone and ulipristal acetate in extracts from influent and effluent of the studied WWTPs and in respective up- and downstream recipient surface waters. The sample preparation is described in detail in the paper by Golovko et al. (2018). Samples from each sampling site were stored at 4 °C and analyzed within 48 h. Prior to extraction for each site, a procedural blank (demineralized water) was extracted and analyzed to distinguish between positive detections and potential sample contamination. Each sample was analyzed simultaneously with matrix matching standards for the determination of matrix effects.

Data analysis and calculation was performed using TraceFinder 3.3 software (Thermo Fisher Scientific).

2.5. In vitro reporter gene bioassays and cytotoxicity testing

AR-CALUX and anti-AR-CALUX (BioDetection Systems, the Netherlands) *in vitro* reporter gene bioassays were performed as previously described (Sonneveld et al., 2005; van der Burg et al., 2010) to detect (anti-)androgenic activities of either pure compounds or in environmental water extracts. In addition, PR- and anti-PR-CALUX bioassays were carried out using methods described elsewhere (Sonneveld et al., 2005, 2011). They were used for determining progestagenic and anti-progestagenic activities in water extracts as we wanted to find out if there is any relationship among occurrence of (anti-)androgenic and (anti-)progestagenic activities in aquatic environments (section 2.6.).

Briefly, AR- or PR-CALUX cells were seeded in a 100 μL volume of cell suspension in assay medium (phenol-red free 1:1 mixture of Dulbecco's modified Eagle's medium with Ham's F12 medium supplemented with 5% dextran coated charcoal stripped fetal calf serum, 0.2% penicillin–streptomycin solution, and 1% nonessential amino acids) into 96-well white plates with transparent bottom (Corning, the Netherlands) at density of 10^4 cells per well. Assay medium for antagonism testing contained in addition reference agonist compound at its EC_{50} value (3.5×10^{-10} M of dihydrotestosterone for anti-androgenicity or 2.0×10^{-10} M ORG 2058 for anti-progestagenicity). After 24 h of incubation at 37 °C and in a humidified atmosphere with 5% CO_2, the cells were exposed to serial dilutions of either pure compounds or environmental water extracts dissolved in either assay medium for agonism (AR- and PR-CALUX assays) or in respective assay medium for antagonism (anti-AR- and anti-PR-CALUX assays) testing and returned to the incubator. Each sample was tested at least in two independent experiments, with each calibration point performed in triplicate. A calibration row of reference agonist (dihydrotestosterone for AR-CALUX and ORG 2058 for PR-CALUX) or antagonist (flutamide for anti-AR-CALUX and mifepristone for anti-PR-CALUX) were included in each 96-well plate and tested in triplicate. Solvent control (0.1% dimethyl sulfoxide) was included at least in triplicate on each plate. Calibration rows of pure compounds and serial dilutions of water extracts were prepared in dimethyl sulfoxide of ≥99.5% purity (Sigma-Aldrich, Czech Republic). To assess joint (anti-)androgenic effects of target compounds in mixtures, we combined pure compounds at equimolar ratios. These mixtures consisted of all active compounds in the respective modes (agonism and antagonism). All the mixtures were serially diluted and tested using (anti-)AR-CALUX bioassays.

Luciferase signal was quantified using an Infinite M200 Plus luminometer (Tecan, Switzerland). Potential cytotoxic effects of samples were tested using resazurin reduction assay (O'Brien et al., 2000) and samples evaluated as cytotoxic were excluded from further analysis in CALUX assays (see section S1 in Supplementary Material and Figs. S1–S10).

2.6. Data analysis

Luciferase signal in (anti-)AR- and (anti-)PR-CALUX assays was recorded as relative light units. To normalize data, relative induction of samples was derived after subtracting a background signal (solvent control). Principal component analysis (PCA) was used to determine possible relationships among detected activities. Results below the LOQ were constrained at 0.

The data obtained from measurement of pure compounds were fitted by nonlinear regression with four parameters (robust fit) using Prism 7 software (Graph Pad, San Diego, CA, USA). The maximum response (efficacy) induced by a tested compound (at top plateau in agonism and bottom plateau in antagonism testing) was reported (= RPCmax). We determined if tested compounds possess (anti-)androgenic activity on the basis of cutoff criterion (RPCmax values reach or exceed a specific effect) as proposed by the Organisation for Economic Co-operation and Development (OECD, 2016). A compound was evaluated as active (androgenic) in AR-CALUX assay if its RPCmax value was ≥10% of relative induction in at least two out of two or two out of three experiments. A compound assigned as active (anti-androgenic) in anti-AR-CALUX assay had to have RPCmax value ≤ 80% of relative induction in at least two out of two or two out of three experiments. Estimates of potency were determined for active compounds: half-maximal effective (EC_{50}s) and inhibitory (IC_{50}s) concentrations, positive controls 10 (PC_{10}s) for agonism, and positive controls 20 (PC_{20}s) for antagonism were derived from the dose-response curves. EC_{50} is a concentration of a chemical that produces 50% stimulating efficacy in agonistic mode, while IC_{50} produces 50% inhibiting efficacy in antagonistic mode. PC*i* is the concentration of a tested chemical that produces the same effect *i* as does the reference compound. PC_{10} and PC_{20} values were calculated for agonists and antagonists of AR, respectively. Determining multiple toxicological estimates had been proposed by Villeneuve et al. (2000) to provide robust results against deviation from parallelism of curves and non-equal maximal efficacies, and thus relative potencies (REPs) of progestins were calculated using two approaches. In the first, $E(I)C_{50}$ of the reference compound was divided by $E(I)C_{50}$ of a tested compound. In the second, $PC_{10(20)}$ of the reference compound was divided by $PC_{10(20)}$ of a tested compound. The measured androgenic (DHT EQs) and anti-androgenic (FLU EQs) equivalents in extracts of water samples were derived according to the licensor's (BioDetection Systems, NL) instructions, as described elsewhere (Sonneveld et al., 2005). Predicted (anti-)androgenic activity of a

target compound was derived by multiplying its determined concentration by its relative potency based on E(I)C_{50} values.

Joint effects measured in AR-CALUX cells are mediated through the same mechanism of action (AR-mediated activities). Thus, tested individual compounds are supposed to interact in mixtures according to concentration addition model (Loewe and Muischnek, 1926), which assumes that tested compounds behave as dilution of each other. To evaluate the joint effects of individual chemicals in mixture, toxic unit method has been used as described elsewhere (Brown, 1968). Briefly, dose-response curves of single compounds and mixtures were used to calculate the concentrations of individual chemicals causing a specific effects (PC*i* values) and the concentration of individual chemicals causing the same effects in a mixture. Toxic unit value of a substance = d_A/D_A, where d_A is concentration of compound A in mixture causing specific effect (PC*i*) and D_A is concentration of the compound A needed to produce the same effect (PC*i*) on its own. Toxic units of individual compounds derived from response PC*i* were summed to derive toxic unit of a mixture at PC*i* level. We determined toxic units at four PC*i* levels (PC_{10}, PC_{20}, PC_{30} and PC_{40} for agonistic, and PC_{20}, PC_{30}, PC_{50} and PC_{70} for antagonistic mixtures, respectively) for each tested curve. If the toxic unit value equals to 1, there is additivity between compounds. Toxic unit smaller than 1 indicates synergism, while toxic unit higher than 1 indicates antagonism between compounds. Following a study by Rossier et al. (2016), we used a margin of error of ±0.5 of the toxic value for additivity to account for possible variability.

3. Results and discussion

3.1. pH test

The pH value of samples has been reduced prior to SPE in approximately 48% of those studies determining the presence of progestins in aquatic environments, albeit without testing the influence of sample pH on the recoveries of target compounds (Table S4). Recently, there have been a few reports investigating the effect of sample pH on the efficiency of extracting progestins using different solid-phase extraction sorbents (Chen et al., 2017; Huysman et al., 2017; Kuster et al., 2009; Shen et al., 2018; Vulliet et al., 2008; Table S5). Our results showed that acidification of samples resulted in slightly poorer recoveries (mean values did not lie within the satisfactory range of 60–130%) for three progestins (gestodene, levonorgestrel, and nomegestrol acetate) and PR antagonist mifepristone. In non-acidified samples, all analytes fell within this range (Fig. S11). Similarly to our observation, the results of Huysman et al. (2017), Kuster et al. (2009), and Shen et al. (2018) indicate that adjusting sample pH does not play a significant role in extraction efficiency for progestins (Table S5). In the present study, no difference was observed between detected activities in samples with and without pH acidification in (anti-)AR-CALUX assays (Fig. S12). Thus, both sample pH pretreatment (pH adjustment) and no such pH adjustment appear to be applicable for the determination of progestins in water samples using SPE that employs Atlantic C18 sorbent. Even if there were no significant differences, however, we would recommend not using sample acidification in order to better simulate real environmental conditions.

3.2. Occurrence of progestins in water samples

The results of chemical analysis are summarized in Table S6 and are comparable to those reported by other authors (reviewed in Golovko et al., 2018). Concentrations of seven progestins (cyproterone acetate, dienogest, drospirenone, gestodene, medroxyprogesterone acetate, megestrol acetate, and progesterone) in influents ranged from 1.0 to 75 ng/L. Progesterone and dienogest were found in all studied influents. PR antagonist mifepristone was detected once in the influent of the České Budějovice WWTP at concentration of 0.32 ng/L. Only two progestins (megestrol acetate and progesterone) reached effluents, and those were found in the range of 0.19–2.7 ng/L.

Progesterone was the only progestin present in surface waters at concentrations ranging from 0.47 to 1.3 ng/L. Widespread occurrence of progesterone has been observed also in other studies (Houtman et al., 2018; Liu et al., 2015; Šauer et al., 2018), and this compound has been proposed as a chemical indicator for the presence of steroids in environmental water samples (Liu et al., 2015). The lowest observed effect concentration of 2 ng/L of progesterone for fish (Zucchi et al., 2012) has been exceeded in several effluents (Fan et al., 2011; Liu et al., 2014; Šauer et al., 2018; Table S6) and even in surface waters (Kolpin et al., 2002; Liu et al., 2014; Macikova et al., 2014a; Vulliet et al., 2008). Thus, we propose to assess further the risk posed by progesterone for aquatic environments.

Some progestins that we found in the present study (Table S6) have been shown to undergo biotransformation in aquatic environments (Ojoghoro et al., 2017; Peng et al., 2014; Sangster et al., 2016). The occurrences and fates of progestin, mifepristone and ulipristal acetate metabolites deserve greater attention, because some studies have indicated that metabolites of progestins and mifepristone may be also strong activators (Schoonen et al., 2000; Houtman et al., 2009) and inhibitors (Attardi et al., 2004) of steroid receptors, respectively.

3.3. In vitro reporter gene bioassays with pure compounds

Seven progestins were active androgens and dose-dependent response curves in AR-CALUX assay could be constructed for them (Table 1; Fig. S13). Nine progestins, mifepristone and ulipristal acetate showed AR antagonism and produced full dose-response curves in the anti-AR-CALUX assay (Table 2; Fig. S14). All androgenic progestins were weaker than reference compound dihydrotestosterone (Table 1). On the contrary, all anti-androgenic progestins, mifepristone and ulipristal acetate showed stronger relative potencies than did the reference compound flutamide (Table 2).

Previous studies have tested (anti-)androgenicity for some of our target compounds using (anti-)AR-CALUX bioassays and their results have been comparable with our observations (Tables S7 and S8; Houtman et al., 2009; Sonneveld et al., 2005). We have newly identified (anti-)androgenic activity of medroxyprogesterone (Tables 1 and 2) and anti-androgenic activity of ulipristal acetate (Table 2). Particularly noteworthy is that megestrol acetate and chlormadinone acetate had similar potencies as did cyproterone acetate, a well-known strong anti-androgen (Table 2). To the best of our knowledge, this is the first study to show (anti-)androgenic potencies (EC_{50}s, IC_{50}s, PC_{10}s, and PC_{20}s) of multiple environmentally relevant progestins, mifepristone and ulipristal acetate measured in a single *in vitro* bioassay.

3.4. Joint effects of individual compounds in mixtures

Toxic units for mixture of androgenic compounds ranged from 0.93 to 1.01, while toxic units for mixture of anti-androgenic compounds ranged from 0.70 to 0.87. All these values fall within the range of 1 ± 0.5 of toxic unit which indicates that both androgenic and anti-androgenic compounds tested in the present study act in an additive manner. Up to date, *in vitro* studies have focused on joint effects of binary mixtures of progestins and observed mostly additivity (Rossier et al., 2016; Siegenthaler et al., 2017).

Table 1
Androgenic potencies of progestins in the AR-CALUX assay.

Compound (number of replicates)	Active or non-active compound	RPCmax (%)	log PC_{10} (M)	log EC_{50} (M)	REP	
					based on PC_{10}	based on EC_{50}
dihydrotestosterone (reference compound, n = 50)	active	100	−10.3	−9.6	1.00	1.00
levonorgestrel (n = 3)	active	59	−9.4	−8.9	0.14	0.22
gestodene (n = 3)	active	47	−9.2	−8.8	0.08	0.17
altrenogest (n = 3)	active	31	−8.9	−8.7	0.04	0.14
etonogestrel (n = 3)	active	55	−8.9	−8.4	0.05	0.07
medroxyprogesterone acetate (n = 3)	active	51	−8.6	−8.1	0.02	0.04
norethisterone (n = 3)	active	47	−8.3	−7.7	0.01	0.01
medroxyprogesterone (n = 3)	active	11	−6.8	−7.5	<0.01	0.01
megestrol acetate (n = 2)	non-active	9	NA	NA	NA	NA
cyproterone acetate (n = 2)	non-active	4	NA	NA	NA	NA
progesterone (n = 2)	non-active	4	NA	NA	NA	NA
dydrogesterone (n = 2)	non-active	<1	NA	NA	NA	NA

Cyproterone acetate, dydrogesterone, megestrol acetate, and progesterone did not produce response greater than PC_{10} and therefore they were designated as non-active. RPCmax = maximum response induced by the tested chemical. PC_{10} = positive control 10, the concentration of a compound eliciting effect equal to 10% induction caused by the reference compound dihydrotestosterone. EC_{50} = half-maximal effective concentration. REP = relative potency. NA = not analyzed.

Table 2
Anti-androgenic potencies of progestins, a progesterone antagonist and a selective progesterone receptor modulator in the anti-AR-CALUX assay.

Compound (number of replicates)	Active or non-active compound	RPCmax (%)	log PC_{20} (M)	log IC_{50} (M)	REP	
					based on PC_{20}	based on IC_{50}
flutamide (reference compound, n = 50)	active	4	−6.6	−6.2	1	1
megestrol acetate (n = 3)	active	24	−8.3	−8.0	52	62
chlormadinone acetate (n = 3)	active	15	−8.3	−7.9	49	48
cyproterone acetate (n = 3)	active	14	−8.3	−7.8	51	44
nomegestrol acetate (n = 3)	active	11	−8.3	−7.8	43	40
progesterone (n = 3)	active	15	−8.2	−7.7	38	34
medroxyprogesterone (n = 3)	active	19	−8.1	−7.7	30	30
ulipristal acetate (n = 3)	active	17	−7.9	−7.7	19	33
drospirenone (n = 3)	active	15	−7.7	−7.3	12	14
dydrogesterone (n = 3)	active	4	−7.7	−7.3	10	13
dienogest (n = 3)	active	19	−7.5	−7.3	8	12
mifepristone (n = 3)	active	12	−7.5	−7.2	7	9
altrenogest (n = 2)	non-active	>80	NA	NA	NA	NA

RPCmax = maximum response induced by the tested chemical. PC_{20} = positive control 20, the concentration of a compound eliciting effect equal to 20% attenuation of the signal of dihydrotestosterone by the reference compound flutamide. IC_{50} = half-maximal inhibitory concentration. REP = relative potency. NA = not analyzed.

Nevertheless, the interactions of various environmentally relevant progestins in mixtures still need to be elucidated by complementary *in vitro* and *in vivo* experiments.

3.5. In vitro reporter gene bioassays with water extracts

Androgenic and anti-androgenic activities were detected at most of our sampling localities with 75% and 85% frequency of occurrence, respectively. The greatest androgenic and anti-androgenic activities were observed in influents and reached up to 59 ng/L DHT EQs and 26 μg/L FLU EQs, respectively (Table 3). The androgenic and anti-androgenic activities determined fall within the range reported by other authors (Fang et al., 2012; Leusch et al., 2014; Roberts et al., 2015; Thomas et al., 2002; van der Linden et al., 2008; Zhao et al., 2011). Anti-androgenic activities are believed to pose even greater risk for aquatic environments than do androgenic activities (Weiss et al., 2009; Zhao et al., 2011), and they are more commonly found in environmental samples (Macikova et al., 2014b; Urbatzka et al., 2007). Recently, the effect-based trigger (EBT) value for anti-androgenic activity has been estimated at 25 μg/L flutamide EQs. Activities lying below this value are presumed to present a low risk for the environment. In our sampling campaign, the EBT for anti-androgenic activity was exceeded only in the influent of WWTP Prachatice but not in environmental samples (Table 3). In another study as one example, however, EBT was exceeded in 29% of surface water samples taken in the Netherlands (van der Oost et al., 2017).

3.6. Contribution of compounds to (anti-)androgenic activities

Androgenic activities in most of the samples were masked by anti-androgenic activities (Table 3). This phenomenon can occur in samples containing both receptor agonists and antagonists (Weiss et al., 2009) and has been reported also in other studies (Creusot et al., 2014; Ihara et al., 2014; König et al., 2017; Šauer et al., 2018; Weiss et al., 2009).

Androgenic activities in aquatic environments related to municipal wastewater discharges have been found mostly to be caused by natural steroid androgens (Jenkins et al., 2001; König et al., 2017; Thomas et al., 2002) but also with a contribution of synthetic androgenic steroids (Hashmi et al., 2018). Progestins were recently suggested to be one of the groups of compounds possibly contributing to androgenic activity in aquatic environments (Hashmi et al., 2018). In the present study, two progestins (gestodene and medroxyprogesterone acetate) accounted for 0.26–29% of androgenic activities measured within influents (Fig. 1), while neither progestins nor mifepristone and ulipristal acetate contributed to androgenic activities in effluents and surface waters (Table 3).

To date, the frequently occurring anti-androgenic activities in the aquatic environment have been explained by presence of the antimicrobial agent triclosan (Liscio et al., 2014; Ma et al., 2017;

Table 3
Predicted and measured (anti-)androgenic activities in aquatic environments and total contributions of target compounds to measured (anti-)androgenic activities.

Wastewater treatment plant	Sample	Androgenic activity (ng/L DHT EQs)		Anti-androgenic activity (μg/L FLU EQs)		Contribution to measured activities (%)	
		predicted	measured	predicted	measured	androgenic	anti-androgenic
Vodňany	inf	**3.9**	**47**	**1.4**	**5.2**	**8.3**	**27**
	eff	ND	**0.34**	**0.09**	**4.4**	ND	**2.1**
	usw	ND	**0.23**	**0.04**	**2.8**	ND	**1.6**
	dsw	ND	**0.18**	**0.04**	**3.4**	ND	**1.3**
Tábor	inf	**13**	**44**	**0.7**	**14**	**29**	**4.6**
	eff	ND	<LOQ	**0.02**	**8.9**	ND	**0.28**
	usw	ND	<LOQ	**0.03**	**5.5**	ND	**0.54**
	dsw	ND	<LOQ	**0.02**	**5.9**	ND	**0.31**
Prachatice	inf	**2.0**	**54**	**2.0**	**26**	**3.8**	**8.8**
	eff	ND	**0.42**	**0.02**	**3.5**	ND	**0.46**
	usw	ND	**0.08**	ND	<LOQ	ND	ND
	dsw	ND	**0.12**	**0.02**	**2.4**	ND	**0.77**
České Budějovice, 1st sampling campaign	inf	**3.7**	**59**	**2.0**	**18**	**6.4**	**11**
	eff	ND	**0.72**	**0.02**	**5.4**	ND	**0.40**
	usw	ND	<LOQ	**0.02**	<LOQ	ND	ND
	dsw	ND	**0.45**	**0.02**	**4.4**	ND	**0.46**
České Budějovice, 2nd sampling campaign	inf	**0.13**	**50**	**3.2**	**15**	**0.3**	**23**
	eff	ND	**0.79**	**0.04**	**4.8**	ND	**0.73**
	usw	ND	<LOQ	ND	<LOQ	ND	ND
	dsw	ND	**0.31**	**0.02**	**3.4**	ND	**0.48**

Abbreviations: ND = not determined, predicted (anti-)androgenic activities and contribution of compounds were not determined when concentrations of target compounds were below limit of quantification. LOQ = limit of quantification. DHT = dihydrotestosterone. FLU = flutamide. EQ = equivalent. inf = influent. eff = effluent. usw = upstream. dsw = downstream. Activities exceeding LOQs and estimated contributions are marked in bold; measured activities are expressed as median (n = 2–3).

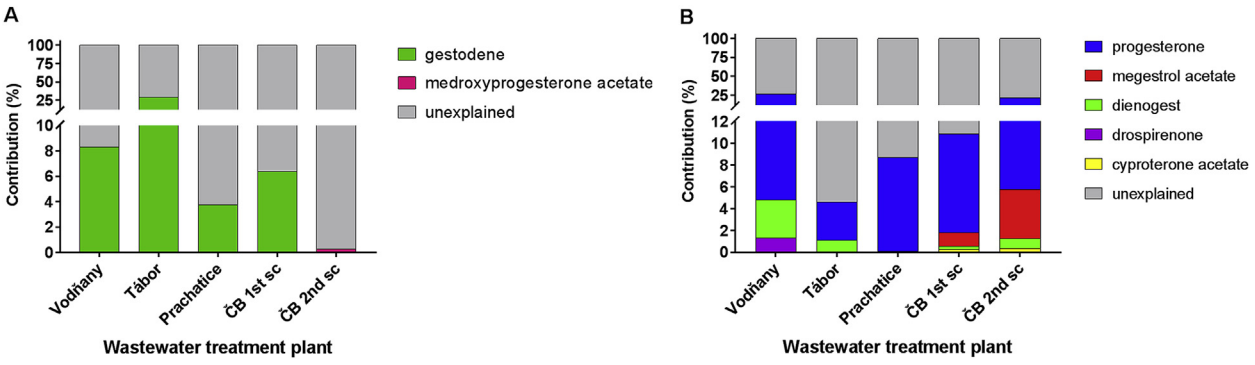

Fig. 1. Contributions of individual progestins to androgenic (A) and anti-androgenic (B) activities in influents to wastewater treatment plants. ČB = České Budějovice, sc = sampling campaign.

Rostkowski et al., 2011), dibutyl phthalate (Ma et al., 2017), phytoestrogens (König et al., 2017), and 4-methyl-7-diethylaminocoumarin (Muschket et al., 2018). Several studies have reported also co-occurrence of anti-androgenic activities and compounds with anti-androgenic potencies, such as pesticides, flame retardants, pharmaceuticals (Liscio et al., 2014), alkylphenols (Shi et al., 2016), naphthenic acids (Thomas et al., 2009), and polycyclic aromatic hydrocarbons (Weiss et al., 2011) in aquatic environments. On the other hand, no dominant causative anti-androgenic agents have been revealed in Taiwanese (Chen and Chou, 2016), Italian (Urbatzka et al., 2007), and French (Kinani et al., 2010) aquatic environments. To sum up, it appears that anti-androgenic activities are caused by various groups of chemicals in the aquatic environments. One group of possible contributors to these activities are progestins, mifepristone and ulipristal acetate, which possess considerable anti-androgenic activities (Table 2). A total of five anti-androgenic progestins (cyproterone acetate, dienogest, drospirenone, megestrol acetate, and progesterone) and a PR antagonist (mifepristone) were found in WWTP influents. They were responsible for 4.6–27% of anti-androgenic activities. Only two of these (megestrol acetate and progesterone), however, reached effluents, where they accounted for just 0.28–2.1% of anti-androgenic activities. Natural progestin progesterone was the only target compound found in surface water, contributing 0.34–1.6% of anti-androgenic activity. The contribution of individual progestins to (anti-)androgenic activities in wastewater is given in Fig. 1. Progesterone was by far the main contributor to anti-androgenic activities (up to 21%) among target substances. Of the synthetic progestins, megestrol acetate was the greatest contributor to anti-androgenic activities in influents (up to 4.5%) and the only one in effluents (0.25%). In the inter-week monitoring (1st and 2nd sampling campaigns in influent to the WWTP at České Budějovice), the extent to which compounds contributed to anti-androgenic activities changed only slightly (Fig. 1). Progestins seem to be important causative agents of anti-androgenic activities in sewage, but not in treated effluents and surface waters. This is logical considering their concentrations found there. If, however, a wastewater treatment process is ineffective in eliminating some strong androgenic (gestodene, etonogestrel, and levonorgestrel) or anti-androgenic (e.g., megestrol acetate, chlormadinone acetate or cyproterone acetate, and progesterone) progestins, and if they occur in units or tens of ng/L or

higher concentrations in effluents, then their contribution to anti-androgenic activities may be much greater due to their strong relative potencies (Tables 1 and 2). Indeed, the synthetic progestin cyproterone acetate recently found in treated wastewater in the Netherlands at a concentration of 20 ng/L was the main contributor (71%) to the detected anti-androgenic activities (Houtman et al., 2018). Moreover, metabolites of progestins might be present in an aquatic environment and also possess some anti-androgenic activity. This, too, is a topic deserving further attention.

4. Conclusions

The majority of samples in the present study exhibited relatively low (anti-)androgenic activities. Progestins occurred mostly in influents and accounted for as much as 29% of (anti-)androgenic activities. Most of them were eliminated to below LOQs during the wastewater treatment process, however, and thus did not contribute significantly to (anti-)androgenic activities in effluents and surface waters. In the light of our results, it seems unlikely that progestins are capable of inducing (anti-)androgenic activities that could pose a high risk to aquatic organisms in Czech surface waters. Our results of joint action testing indicate additive effect of individual (anti-)androgenic target compounds in mixtures. Therefore using the REPs of progestins obtained in our study, it will be possible to estimate (anti-)androgenic activity caused by progestins just on the basis of the results from chemical analysis (concentration of progestins) in future studies.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.envpol.2018.06.104.

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The progestin levonorgestrel disrupts gonadotropin expression and sex steroid levels in pubertal roach (*Rutilus rutilus*)

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ABSTRACT

The aim of the present study was to investigate the effects of the synthetic progestin levonorgestrel (LNG) on the reproductive endocrine system of a teleost fish, the roach (*Rutilus rutilus*). Pubertal roach were exposed for 28 days in a flow-through system to four concentrations of LNG (3, 31, 312, and 3124 ng/l). Both males and females treated with 3124 ng/l LNG exhibited the upregulated levels of vitellogenin and oestrogen receptor 1 mRNA in the liver. At the same concentration, LNG caused a significant upregulation of the mRNA expression of the gene encoding luteinising hormone β-subunit (*lhβ*) and the suppression of the mRNA expression of the gene encoding follicle-stimulating hormone β-subunit (*fshβ*) in the pituitary of both male and female roach. A lower LNG concentration (312 ng/l) suppressed mRNA expression of *fshβ* in males only. Females treated with 3124 ng/l LNG exhibited significantly lower plasma 11-ketotestosterone (11-KT) and oestradiol (E2) concentrations, whereas their testosterone (T) level was higher compared with the control. Females exposed to 312 ng/l LNG presented significantly lower plasma E2 concentrations. Males exposed to ≥31 ng/l LNG exhibited significantly reduced 11-KT levels. As determined through a histological analysis, the ovaries of females were not affected by LNG exposure, whereas the testes of males exposed to 31 and 312 ng/l LNG exhibited a significantly higher percentage of spermatogonia B compared with the control. The results of the present study demonstrate that LNG disrupts the reproductive system of pubertal roach by affecting the pituitary gonadotropin expression and the sex steroid levels. This disruption was determined to occur in males after exposure to an environmentally relevant concentration (31 ng/l). Moreover, the highest tested concentration of LNG (3124 ng/l) exerted an oestrogenic effect on fish of both sexes.

1. Introduction

Pharmaceutical products and metabolites present in the aquatic environment have become an increasingly important issue in environmental protection (Fent et al., 2006; Corcoran et al., 2010). In particular, considerable attention has been paid to endocrine disruption associated with the oral contraceptive ingredient 17β-ethinyloestradiol (EE2; Desbrow et al., 1998; Jobling et al., 2002). Recently, however, studies have also investigated synthetic progestins (Zeilinger et al., 2009; Paulos et al., 2010), which are ingredients of oral contraceptives and other hormonal medicines (Africander et al., 2011). Synthetic progestins (also called gestagens, gestogens, progestogens, and progestagens) to some extent mimic endogenous progesterone but also exhibit a wide range of biological activities that differ from those of progesterone (Stanczyk, 2003; Besse and Garric, 2009; Paulos et al., 2010; Africander et al., 2011). These compounds are known to interact not only with progesterone receptors (PR) but also with androgen (AR), oestrogen (ESR), glucocorticoid (GR), and mineralocorticoid (MR) receptors (Africander et al., 2011). In the past, when data on the environmental concentrations of progestins were scarce, it was thought that synthetic progestins are found in sub-nanogram per litre concentrations or in the very low ng/l range in surface waters (Zeilinger et al., 2009; Scott et al., 2010). However, since then, more analytical

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surveys have been conducted, and these have reported concentrations ranging from several ng/l (Vulliet and Cren-Olive, 2011) to tens of ng/l (Al-Odaini et al., 2010; Liu et al., 2011). These findings suggest that progestins in surface water may pose a substantial risk to aquatic organisms. Indeed, recent studies have reported the negative effects of progestins at the low ng/l range on the reproductive functions of fish (Zeilinger et al., 2009; Paulos et al., 2010; Runnalls et al., 2013) and amphibians (Säfholm et al., 2012).

One of the commonly used pharmaceutical progestins is levonorgestrel (LNG), a synthetic steroid hormone structurally related to testosterone that has not only progestagenic but also androgenic activity (Besse and Garric, 2009). In addition, metabolites of LNG exhibit oestrogenic activity (García-Becerra et al., 2002). It was recently demonstrated that fish exposed to very low concentrations of LNG strongly bio-concentrate this substance in their blood plasma (Fick et al., 2010). Because it has been suggested that LNG is a potentially harmful substance (Fick et al., 2010; Christen et al., 2010), an increasing number of studies describing its effects on different aquatic organisms has been published recently (Zeilinger et al., 2009; Contardo-Jara et al., 2011; Kvarnryd et al., 2011; Lorenz et al., 2011a,b; Säfholm et al., 2012; Hoffmann and Kloas, 2012; Runnalls et al., 2013; Svensson et al., 2013). Given its use as a contraceptive in humans, it is not surprising that its main effects in aquatic vertebrates were exerted on reproduction, and LNG was also found to be a very potent endocrine disruptor in fish. For example, Zeilinger et al. (2009) showed that a water concentration of LNG as low as 0.8 ng/l, which is its lowest tested concentration, caused reduced fertility in the exposed adult fathead minnows (*Pimephales promelas*). Svensson et al. (2013) found androgenic effects at higher LNG concentrations (at ≥40 ng/l) in three-spined stickleback (*Gasterosteus aculeatus*). However, few data are available regarding the target sites of LNG in fish or the modes of action underlying its endocrine disrupting activity.

Therefore, the aim of the present study was to describe in detail the effects of LNG on the hypothalamus-pituitary-gonad (HPG) axis in a teleost fish, the roach (*Rutilus rutilus*). This widespread cyprinid fish lives in fresh and brackish waters of Europe and is a well-established sentinel for assessing endocrine disruption under both laboratory and field conditions (Tyler et al., 2007). Because progestins are not only important for final reproductive events but also known to be involved in the regulation of other steps during gametogenesis (Schulz et al., 2010; Lubzens et al., 2010), the present study focused on the effects of LNG on pubertal roach. Sub-chronic LNG-exposure was achieved using a flow-through system. The endpoints included gonad histology, plasma levels of sex steroids, and the expression of key genes (here and later the phrase gene expression is used as a synonym for gene transcription, although it is acknowledged that gene expression can also be regulated, e.g., at translation and protein stability level) involved in the regulation of reproduction. The mRNA expression levels of genes encoding gonadotropin β-subunits (*fshβ* and *lhβ*) in the pituitary and vitellogenin (*vtg*), sex steroid receptors (*ar, esr1, ers2a*, and *esr2b*), and steroid-hormone binding globulin (*shbg*) in the liver were studied. Furthermore, because steroid hormones, including LNG, have been demonstrated not to affect only reproductive physiology (Filby et al., 2006; Lorenz et al., 2011b), we included genes encoding the pituitary thyroid-stimulating hormone β-subunit (*tshβ*) and growth hormone (*gh*) in our gene expression analysis.

2. Materials and methods

2.1. Chemicals and dilution water

D(−)-norgestrel (=levonorgestrel, LNG; CAS number: 797-63-7, purity: 99%) obtained from Sigma–Aldrich (Steinheim, Germany) was used as the test substance and for the preparation of analytical standards for LC–MS/MS. To achieve the desired test concentrations, dimethylsulfoxide (DMSO, ≥99.8%, Roth, Karlsruhe, Germany) was used as a solvent. Milli-Q-grade water was used for the preparation of stock solutions. As a dilution water, the artificial tank water (ATW) was used. ATW was continuously filtered (0.45-mm filter), UV-sterilised, and temperature-conditioned Milli-Q-grade water containing 100 mg/l of Instant Ocean sea salt, 200 mg/l $CaCl_2$, and 103 mg/l $NaHCO_3$.

2.2. Experimental animals

Roach of one year of age (*R. rutilus*; mass: 9.3 ± 1.0 g) were obtained from a local fish farm, cultured in a pond (Vodnany, Czech Republic), and maintained for three months (over winter) in an indoor tank (300 l). Three weeks before the start of the experiment, the fish were transferred to the experimental flow-through system and acclimatised to this system until the start of the exposure. During acclimation period, the temperature was gradually increased to 21.0 ± 0.2 °C, the photoperiod was gradually changed from 12:12 h (light–dark) to 14:10 h, and the flow rate was 250 l of ATW per tank per day. At the beginning of the acclimation period there were several cases of fish death (up to 4 fish per tank) before the fish adapted to the experimental conditions. A subset of fish was subjected to a histological analysis of the gonads before the experiment to determine their maturational stage. Female and male roach displayed signs of puberty, as indicated by the presence of oocytes in the cortical alveolus stage in the ovaries and spermatogonia B in the testes, respectively (Levavi-Sivan et al., 2010; Schulz et al., 2010; Taranger et al., 2010).

2.3. Experimental design

The LNG exposure of roach lasted 28 days. A flow-through system with two parallel tanks (40 l) for each treatment was used to maintain constant conditions throughout the experiment. Fish at a density of 25 fish per tank (6 g/l) were exposed to the following nominal LNG concentrations: 3, 31, 312, and 3124 ng/l. Moreover, two control groups were included: (1) control group containing dilution water only (water control) and (2) solvent control (SC) containing 0.0005% DMSO (the same concentration used in the LNG groups to facilitate LNG dissolving). The flow-through system was operated at a flow rate of 250 l of exposure medium per tank per day, which is equivalent to a water exchange rate of approximately six tank volumes per day. One mixing chamber per tank received ATW and the corresponding concentrated stock solution. The exposure medium containing 0.0005% DMSO was supplied to the corresponding tanks. The actual test concentrations in each tank were verified weekly (five times in total) through LC–MS/MS. All of the exposure tanks were permanently aerated (oxygen saturation ≥80%). The temperature was set to 21.0 ± 0.2 °C, and the water pH was 8.1 ± 0.1. The photoperiod was maintained at 14:10 h. The light intensity at the tank level was 335 ± 58 lux. The fish were fed a commercial diet (Aller Futura; 64% protein, 12% fat) twice a day at an amount equal to 2% of the total body mass. The fish that died were removed from the tanks, and the mortality was recorded. The experiment was conducted in compliance with the local laws on animal welfare.

2.4. Sampling

At the end of the experiment, every experimental group was sampled randomly. The fish were anaesthetised with ethyl 3-aminobenzoate methansulfonate (MS222, Sigma). The standard length and body mass of each individual fish were measured to the nearest 1 mm and 100 mg, respectively. Blood was collected from

the caudal vein (10 samples/sex/group) using heparinised syringes (heparin concentration = 10 KU/ml), and the plasma was separated by centrifugation (10,000 × *g*, 5 min), immediately frozen in liquid nitrogen, and stored at −80 °C. After the blood sampling, the fish were killed by severing the spinal cord. The gonad and liver masses of the fish were measured to the nearest 1 mg using an analytical balance. The condition factor was calculated as CF = body mass [g] × 100/(standard length [cm])^3, the gonadosomatic index was calculated as GSI = gonad mass [g]/body mass [g] × 100, and the hepatosomatic index was calculated as HSI = liver mass [g]/body mass [g] × 100. The pituitary, liver, and gills were sampled for gene expression analysis (10 samples/sex/group), immediately frozen in liquid nitrogen, and stored at −80 °C. A piece of liver tissue (10 samples/sex/group) and samples from the brain and gills (3–6 samples/sex/group) were collected for chemical analysis and stored at −80 °C. For histological analysis, the gonad tissue was transferred to histology cassettes and fixed in Bouin's solution (Sigma) overnight.

2.5. LNG extraction and analysis

Water samples were extracted as described by Contardo-Jara et al. (2011). The LNG concentration in the water samples was determined using a HPLC Agilent 1200 Series instrument with MS/MS detection (Applied Biosystems 3200 Q Trap, Darmstadt, Germany). The analytical conditions are described in detail by Contardo-Jara et al. (2011). The homogenization and extraction of the tissue samples were performed using the method described by Ramirez et al. (2009) with some modifications. The sample extraction was performed using acetonitrile in a high-speed homogeniser with stainless-steel balls. The tissue samples were analysed using LC–MS/MS with APCI/APPI ionisation (Kvarnryd et al., 2011). These analyses were performed with a TSQ Ultra MS/MS instrument (Thermo Fisher Scientific, San Jose, CA, USA) coupled with an Accela 1250 instrument (Thermo Fisher Scientific, San Jose, CA, USA) and a HTS-XT autosampler (CTC Analytics AG, Zwingen, Switzerland). On average, the LOQs of LNG in the tissue and water samples were 2.5 ng/g and 0.5 ng/l, respectively. The average recoveries of LNG were 102% and 80% in the tissue and water samples, respectively.

The bioconcentration factor (BCF) was calculated according to OECD Guideline No. 305 (OECD, 1996) for the groups exposed to 3124 and 312 ng/l LNG. The concentrations of LNG in the tissues of fish exposed to the two lower LNG concentrations (31 and 3 ng/l) were below the limit of quantification (LOQ), and thus no BCF was derived. The BCF corresponds to the mean concentration of LNG in the fish tissue at the termination of the experiment divided by the mean concentration of LNG in all of the analysed water samples from the corresponding group.

2.6. Histology of gonads

The samples of gonad tissue (13–19 samples/sex/group) were preserved in Bouin's fixative for 12 h, dehydrated in a graded series of ethanol, and subsequently embedded in paraffin. Sections were cut at a thickness of 5 μm and stained with haematoxylin and eosin. The gonads were analysed with respect to the developmental stage of germ cells. In the case of the testes, the area occupied by cysts containing spermatogonia A or spermatogonia B (for 8 samples/group) was measured as described previously (Trubiroha et al., 2011), and the area occupied by spermatogonia B was expressed as a percentage of the total area investigated.

2.7. Plasma concentrations of sex steroids

Blood plasma (10 samples/sex/group) was extracted with diethyl ether (Penta), and the concentrations of E2 (oestradiol), T (testosterone), and 11-KT (11-ketotestosterone) were determined using specific enzyme-linked immunoassays (EIA; Cayman Chemicals) as described previously (Trubiroha et al., 2012). The absorbance at 412 nm was measured using a 96-well plate reader (Infinite M200, Tecan), and the hormone concentrations of the plasma samples were calculated according to the standard curve using logit/log plots and with respect to the dilution of the samples. The detection limits of the assays were 19 pg/ml for E2, 6 pg/ml for T, and 1.3 pg/ml for 11-KT (Cayman Chemicals).

2.8. RNA extraction and reverse transcription

Twenty samples per treatment group (10 males and 10 females) were used for the extraction of RNA from the corresponding tissues. The RNA in the gill and liver samples was extracted using Qiazol (Qiagen) and then treated with DNase I (AmpGrade; Life Technologies). The RNA in the pituitary samples was extracted using the RNeasy Mini kit (Qiagen), including on-column digestion with RNase-free DNase I (Qiagen), according to the manufacturer's instructions. The concentration and purity of total RNA was measured by UV light absorption using a NanoDrop ND-1000 spectrophotometer (Nano-Drop Products, Thermo Fisher Scientific). The high integrity of the RNA was verified with an Agilent 2100 Bioanalyser in a subset of 36 samples from each tissue. The total RNA was reverse transcribed using AMV reverse transcriptase (Finnzymes) for the gill and liver samples and MMLV reverse transcriptase (Affinity Script, Agilent Technologies) for the pituitary samples as described previously (Trubiroha et al., 2009, 2010).

2.9. PCR-based gene identification and analysis of gene expression by RT-qPCR

Primers directed to the conserved regions of the target genes were used for the qPCR-based identification of roach *shbg* and *tshβ*. The PCR products were identified by direct sequencing using a CEQ 8800 Genetic Analysis System (Beckman Coulter) according to the manufacturer's protocol. Their identity was confirmed by BLAST and multiple sequence alignments. New sequence information was submitted to the GenBank™ database (Supplementary Table S1).

Relative RT-qPCR assays [20-μl reaction volume: 2 μl of diluted cDNA, 7.5 pmol of each primer, and 1× FastStart Universal SYBR Green Master (Rox) (Roche)] were conducted with gene-specific primers for roach *ar*, *esr1*, *esr2a*, *esr2b*, *fshβ*, *gh*, *lhβ*, *rpl8*, *shbg*, *tshβ*, and *vtg* (Supplement Table S2) in a Mx3005p qPCR cycler (Agilent Technologies) as described previously (Trubiroha et al., 2009, 2010). The specificity of the amplicons was confirmed by direct sequencing. The PCR efficiencies ranged from 1.81 to 1.98 ($R^2 > 0.99$) (Supplementary Table S2). The relative values of the target transcript abundance in individual samples were determined by the comparative C_T method ($\Delta\Delta C_T$) according to Pfaffl (2001). The expression of the reference gene *rpl8* was used for normalisation because of its constant expression under the experimental conditions. The normalised expression of the target genes is presented as the means ± SD relative to the solvent control (SC).

2.10. Statistics

The statistical analyses were performed using the Statistica v.9 software (StatSoft, Czech Republic). The differences in the studied parameters between groups were analysed by analysis of variance (ANOVA) followed by post hoc Dunnett's test. When the conditions for using ANOVA were not satisfied (normal distribution and homoscedasticity), the data were log-transformed. The data are presented as the means ± SD. The different sexes were assessed separately. The significant differences compared with the solvent control are indicated by asterisks and were considered statistically significant at $p < 0.05$ (the data for the water control are shown

Table 1
Gonadosomatic index (GSI) of female and male roach, area covered by spermatogonia B (spgB) in the testes of male roach, and percentage of female roach with ovaries containing follicles in the cortical alveolus stage (ca) after exposure to LNG. The values are expressed as the means ± SD. Significant differences compared with the solvent control (SC) are indicated by asterisks (*$P < 0.05$; **$P < 0.01$).

Parameter/treatment (ng/l)		SC	3	31	312	3124
♀	GSI	1.6 ± 0.3	1.7 ± 0.4	1.7 ± 0.4	1.6 ± 0.3	1.4 ± 0.3
	(N)	(23)	(24)	(24)	(27)	(26)
	ca (%)	25	25	13	11	0
	(N)	(16)	(16)	(15)	(19)	(15)
♂	GSI	1.0 ± 0.3	1.0 ± 0.4	1.0 ± 0.4	1.2 ± 0.2	1.0 ± 0.3
	(N)	(22)	(19)	(22)	(17)	(20)
	spgB (%)	23 ± 21	35 ± 20	50 ± 17*	62 ± 9**	34 ± 18
	(N)	(8)	(8)	(8)	(8)	(8)

N = number of samples.

neither in graphs nor in tables because there were no significant differences in any of the studied endpoints between the solvent and water controls). The differences between treatment groups with respect to the presence/absence of follicles in the early cortical alveolus stage in the ovaries of the female fish were tested by Pearson's chi-square (χ^2) test.

3. Results

3.1. LNG concentration in water

The measured LNG concentrations, which are expressed as the means ± SD (min–max % of the nominal concentration), were 3 ± 1 ng/l (53–179%) for 3 ng/l LNG, 25 ± 19 ng/l (37–209%) for 31 ng/l LNG, 150 ± 46 ng/l (30–68%) for 312 ng/l LNG, and 2448 ± 1310 ng/l (37–167%) for 3124 ng/l LNG. The concentration of LNG in the control groups was below the LOQ.

3.2. LNG concentration in tissues and BCF

The LNG concentrations in the liver expressed as the means ± SD were the following: (1) at 3124 ng/l LNG – 62 ± 23 ng/g (females) and 50 ± 12 ng/g (males) and (2) at 312 ng/l LNG – 8 ± 1 ng/g (females) and 6 ± 4 ng/g (males). There were no statistically significant differences in the LNG concentrations in the liver between males and females. The BCF of LNG in the liver of roach ranged from 20 to 53.

In addition, a few brain and gill samples (3–6 samples per sex and group) were available for chemical analysis (results are shown in Supplementary Table S4). Based on these samples, the BCF of LNG in the brain and gills was estimated to be 17–33 and 20–21, respectively.

3.3. Mortality, morphological parameters, gonadosomatic index (GSI), and gonad histology

There was no difference in total mortality of fish among treatment groups and it did not exceed 10% in any group. There were no significant differences in body mass, standard length, condition factor (CF), and hepatosomatic index (HSI) among the groups (Supplementary Table S3).

The GSI was not affected by LNG exposure both in females and in males. The values ranged from 1.4 to 1.6 and 1.0 to 1.2 in females and males, respectively (Table 1). The ovaries of all of the females, irrespective of the treatment group, contained follicles mainly in the primary growth stage or sporadically in the early cortical alveolus stage. There was no significant difference in the number of females displaying follicles in the cortical alveoli stage among the treatment groups (Table 1). The testis of the males in the control group and the groups exposed to the lowest (3 ng/l) and highest (3124 ng/l) LNG concentrations comprised mainly spermatogonia A (Table 1). The testis of males exposed to 31 and 312 ng/l of LNG presented spermatogonia B at a significantly higher percentage (50% and 62% of the testis area, respectively) compared with the control (23%, Table 1).

3.4. Plasma concentrations of sex steroids

The females treated with 3124 ng/l LNG exhibited significantly lower plasma 11-KT (3-fold) and E2 (1.5-fold) whereas T (2-fold) was higher compared with the control (Fig. 1). The females exposed to 312 ng/l LNG presented significantly lower plasma E2 concentrations (1.5-fold; Fig. 1). Lower LNG concentrations (3 and 31 ng/l LNG) did not affect the levels of sex steroids in the plasma of exposed females. The males exposed to 31 ng/l and higher concentrations of LNG presented significantly lower 11-KT levels (8- to 17-fold) compared with the control (Fig. 1). The concentrations of T and E2 in males remained unaffected by LNG treatment.

3.5. Gene expression

3.5.1. Pituitary

The females treated with 3124 ng/l LNG exhibited significantly downregulated (7-fold) mRNA-expression of *fshβ* (Fig. 2), whereas the mRNA expression of *lhβ* was upregulated (3-fold) compared with the control (Fig. 2). Males exposed to 3124 and 312 ng/l LNG showed significantly lower levels of *fshβ* mRNA (12.5- and 5-fold, respectively) compared with the control. Furthermore, the highest LNG concentration (3124 ng/l LNG) caused significant upregulation (4-fold) of *lhβ* in males (Fig. 2). The mRNA expression levels of *tshβ* and *gh* were not affected by LNG exposure in both sexes.

3.5.2. Liver

Both males and females treated with 3124 ng/l LNG had significantly higher levels of *vtg* (2700- and 1500-fold, respectively) and *esr1* (9.5- and 6-fold, respectively) mRNA (Fig. 3). The expression of *esr2a*, *esr2b*, *ar*, and *shbg* mRNA remained unaffected by LNG exposure in both sexes (Fig. 3).

3.5.3. Gills

In the gills, the *shbg* mRNA expression level was very low ($C_T \geq 35$) and could not be quantified.

4. Discussion

In the present study, the highest tested concentration of LNG (3124 ng/l) exhibited marked oestrogenic activity in roach. In fact, LNG caused a significant upregulation of the mRNA expression levels of the genes encoding oestrogen receptor 1 (*esr1*) and vitellogenin (*vtg*) in the liver of exposed fish of both sexes. The induction of the expression/synthesis of VTG, the egg yolk precursor protein, which is a well-established biomarker of oestrogen exposure

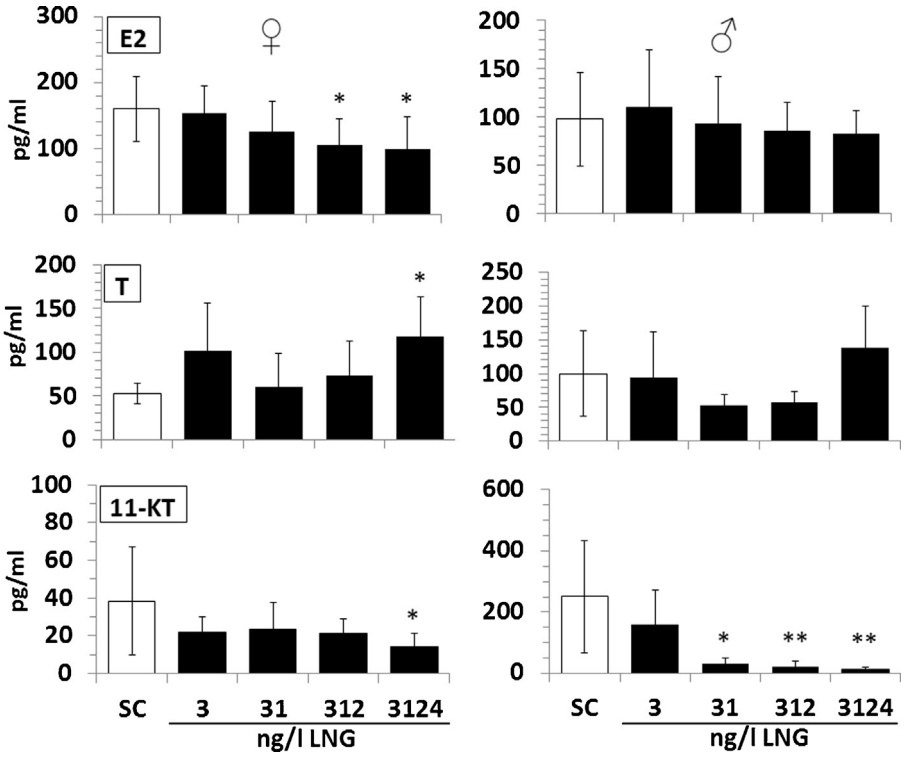

Fig. 1. Plasma levels of 17β-oestradiol (E2), testosterone (T), and 11-ketotestosterone (11-KT) in female and male roach after LNG exposure. The values are expressed as the means ± S.D. ($N = 10$). Significant differences compared with the solvent control (SC) are indicated by asterisks (*$P < 0.05$; **$P < 0.01$).

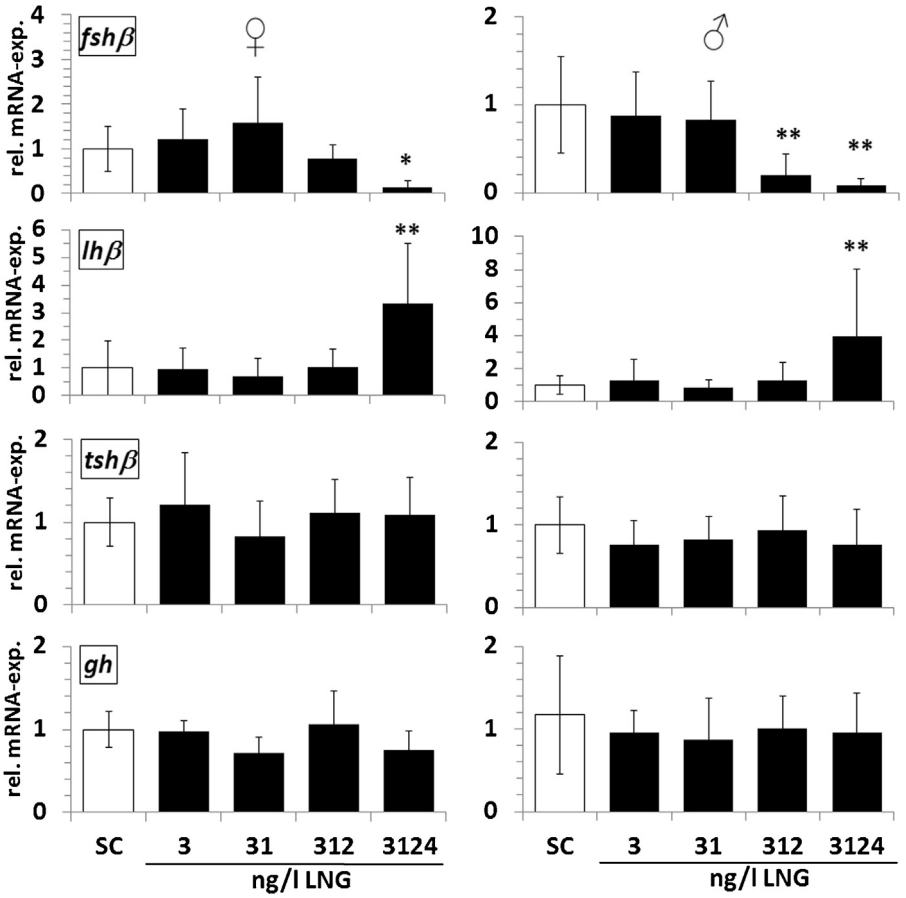

Fig. 2. mRNA expression levels of *fshβ*, *lhβ*, *tshβ*, and *gh* in the pituitary of female and male roach after LNG exposure. The values are expressed as the means ± S.D. ($N = 10$). Significant differences compared with the solvent control (SC) are indicated by asterisks (*$P < 0.05$; **$P < 0.01$).

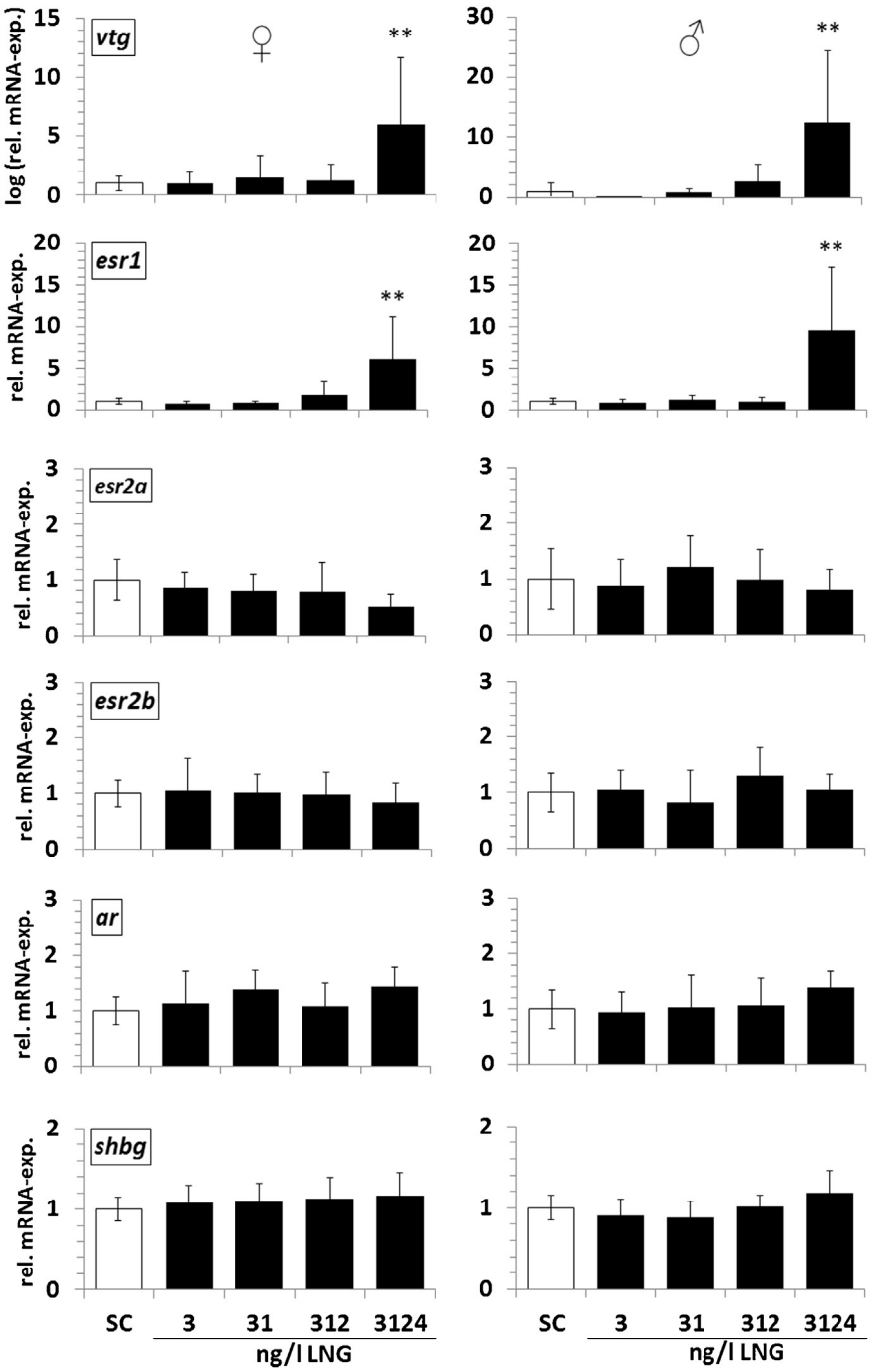

Fig. 3. mRNA expression levels of *vtg*, *esr1*, *esr2a*, *esr2b*, *ar*, and *shbg* in the liver of female and male roach after LNG exposure. The values are expressed as the means ± S.D. ($N = 10$). Significant differences compared with the solvent control (SC) are indicated by asterisks (*$P < 0.05$; **$P < 0.01$).

in fish, is known to be closely correlated with the upregulation of *esr1* (reviewed by Nelson and Habibi, 2013). This clear oestrogenic activity of LNG is contrary to the results of most of the previous studies in fish, which mainly reported the strong androgenic activity of LNG. Svensson et al. (2013) exposed adult female three-spined stickleback to LNG and observed a significant downregulation of *vtg* mRNA expression in the liver (at 40 and 358 ng/l LNG) that was paralleled by the induction of the mRNA expression of the gene encoding spiggin, a well-known biomarker for androgens, in the kidney. Decreased plasma VTG levels accompanied by the development of spawning tubercles, a secondary sexual characteristic typical of males during spawning season, was found in adult females of fathead minnow (*P. promelas*) exposed to 100 ng/l LNG (Runnalls et al., 2013). Spawning tubercles in adult females of the same species exposed to the same LNG concentration were also observed by Zeilinger et al. (2009). However, although male roach are known to display spawning tubercles during the breeding season (Kortet et al., 2003), this secondary sexual characteristic was not observed in any fish after LNG exposure in the present study. To the best of our knowledge, hormonal induction of spawning tubercles has not been demonstrated under experimental conditions in roach so far. Furthermore, the roach used in our study were in early puberty and it is not clear if the roach at this stage are even able to develop androgen-induced secondary sexual characteristics.

Regarding oestrogenic effects of progestins in fish, to date only one study using zebrafish (*Danio rerio*) embryos has reported a significant upregulation of *vtg* mRNA expression after exposure either to 200 ng/l LNG or to 20 ng/l norethindrone (a synthetic progestin, 19-nor-testosterone derivative) (Zucchi et al., 2012). Interestingly, LNG exposure was associated with the downregulation of the gene encoding androgen receptor (*ar*), but *esr1* was not affected (Zucchi et al., 2012). However, *vtg* induction was not observed in all of the developmental stages, and the results of the expression analysis were obtained using whole embryos, which hampers a comparison to our observations in the liver of roach. The human-oriented literature indicates that LNG itself does not bind to the mammalian oestrogen receptors (binding to fish ESRs has not been studied so far), but its oestrogen-like effects have been observed under both *in vitro* and *in vivo* conditions (Jeng et al., 1992; Kumar et al., 2000; García-Becerra et al., 2002). Its oestrogenic activity has been attributed to the A-ring-reduced metabolites of LNG. In a study by García-Becerra et al. (2002), the 3β,5α-tetrahydro derivative of LNG was found to cause a significant activation of oestrogen-dependent gene transcription in HeLa cells transfected with expression vectors for human ESR1 and ESR2 and an oestrogen-responsive reporter gene. This effect was selectively confined to the ESR1.

Assuming that LNG is converted in fish into similar metabolites as in humans, the oestrogenic effect of LNG exposure observed in the present study may also be attributed to its metabolites. The differences in VTG induction between roach in the present study and stickleback/fathead minnow (Runnalls et al., 2013; Svensson et al., 2013) may be due to a higher level of oestrogenic metabolites in roach because we used a LNG concentration that was at least one order of magnitude higher than that used in the experiments with stickleback/fathead minnow. However, different rates of the biotransformation reactions of LNG between the individuals of different ages and maturity stages used in the experiments may also contribute to the differences in the results. If the rate of metabolism is lower in adults, their circulating levels of the parent compound with androgenic and progestogenic properties may be higher compared with those observed in the juvenile/pubertal and early life stages. Similarly, in humans, metabolism and clearance of many pharmaceuticals is greater in children than in adults. Therefore, higher weight normalised or more frequent dosage of drugs has to be applied to children to reach the same therapeutic plasma levels (Kearns et al., 2003). Differences in metabolism have been also reported for different age classes of fish. For example, Wiegand et al. (2000) studied the activities of two detoxification enzyme systems during zebrafish development (from the 2- to 4-cell stage to adult fish) and found that juveniles showed the highest activities among all of the studied developmental stages. The activities of some enzymes were even higher in embryos or early larvae compared with adult fish. Moreover, Parks and LeBlanc (1998) exposed juvenile and adult fathead minnow to waterborne testosterone and studied its metabolic clearance. The adult and juvenile fathead minnow exhibited the same profile of testosterone metabolites. However, the juvenile fish eliminated testosterone and nearly all its metabolites at greater weight-normalised rates than the adults.

Recently, Miguel-Queralt and Hammond (2008) showed that LNG is rapidly taken up from water by zebrafish. This finding was explained by the fact that LNG has a high affinity for steroid hormone-binding globulin (SHBG), which serves as a portal for natural and synthetic steroids and controls their flux between the blood and aquatic environment (Miguel-Queralt and Hammond, 2008). The liver is the main site of *shbg* expression and a source of plasma SHBG in fish (Miguel-Queralt et al., 2007). We could not detect any effect of LNG exposure on the hepatic *shbg* mRNA in roach. Indeed, only a few studies have indicated the regulation of *shbg* expression by sex steroids in fish (Hoffmann et al., 2008), and a recent investigation suggested that plasma SHBG is most likely influenced by metabolic changes rather than by the sex steroid levels (Miguel-Queralt et al., 2007). The very low *shbg* expression we observed in the gills of roach ($C_T \geq 35$) is in agreement with the results of the study conducted by Miguel-Queralt and Hammond (2008) in zebrafish. Interestingly, these researchers demonstrated a strong accumulation of SHBG (protein) in the gills, and the binding of sex steroids, including LNG, to SHBG in the brachial filaments was suggested as a possible reason for the rapid sequestration of these hormones from water (Miguel-Queralt and Hammond, 2008). Assuming a similarly rapid uptake by roach and considering that juvenile rainbow trout (*Oncorhynchus mykiss*) exposed to very low concentrations of LNG strongly bioconcentrated this substance in their blood plasma (Fick et al., 2010), the relatively low bioconcentration factor of LNG in the liver (20–53) calculated in the present study indicates the rapid metabolism of LNG in roach. Unfortunately, the concentrations of LNG in the blood of roach could not be analysed due to an insufficient amount of plasma for both sex steroid hormone and LNG analyses.

Exposure to LNG at a concentration of 3124 ng/l caused a significant upregulation of *lhβ*, whereas the *fshβ* mRNA expression was suppressed in the pituitary of both male and female roach. The lower LNG concentration (312 ng/l) suppressed *fshβ* expression in males but not in females. The positive feedback of sex steroids on *lhβ* mRNA expression and the LH plasma levels has been frequently demonstrated in juveniles of several fish species (Levavi-Sivan et al., 2010; Zohar et al., 2010). This finding is particularly true for oestrogens and aromatisable androgens. Thus, the positive effect of the highest tested concentration of LNG on *lhβ* in the pituitary of roach may be associated with its oestrogenic activity, as demonstrated by the transcriptional induction of *vtg* and *esr1* in the liver. The effects of sex steroids on *fshβ* mRNA expression and FSH release are less documented. The available data are sometimes contradictory and may depend on the maturational stage of the investigated individuals (Zohar et al., 2010). A negative effect of T and E2 on the FSH plasma levels was reported during gametogenesis e.g., in coho salmon (*Oncorhynchus kisutch*; Larsen and Swanson, 1997; Dickey and Swanson, 1998) and Atlantic salmon (*Salmo salar*, Borg et al., 1998). Both T and E2 decreased *fshβ* mRNA expression in immature female Japanese eels (*Anguilla japonica*; Jeng et al., 2007; Levavi-Sivan et al., 2010). The present study provides the first analysis of the mRNA expression of genes encoding both gonadotropin β-subunits in the pituitary of fish exposed to a synthetic progestin; therefore, we can compare our data only with other data obtained in amphibians. Lorenz et al. (2011a), who chronically exposed African clawed frog (*Xenopus laevis*) tadpoles during the period of sex differentiation to the same concentrations of LNG as in the present study, reported that LNG represses the mRNA expression of *lhβ* in both sexes, whereas the expression of *fshβ* was regulated sex-specifically and was dependent on the developmental stage. Thus, although the effects of LNG on the expression of both *lhβ* and *fshβ* in *X. laevis* tadpoles are different of those found in roach, these data clearly show that LNG is able to exert feedback on the pituitary.

The gonadotropins FSH and LH are strong steroidogenic hormones in fish (Levavi-Sivan et al., 2010). Compared with LH, FSH is thought to be more important in regulating early gametogenesis in most fish species (Levavi-Sivan et al., 2010; Taranger et al., 2010). Consistently, a study by Trubiroha et al. (2012) showed that the onset of gametogenesis in male and female roach coincided with an upregulation of pituitary *fshβ* mRNA. Thus, the lowered plasma levels, particularly of 11-KT in male roach and E2 in female roach exposed to LNG, were likely caused by the strong suppression of *fshβ* in the pituitary. However, the plasma levels of FSH and LH were not determined in the present study, and the inhibition of sex steroid synthesis by LNG at the level of the gonads is similarly possible, as has been shown for androgen-treated testes explants

of African catfish (*Clarias gariepinus*) (Schulz et al., 2008). Independent of the mechanisms of action, a modulation of endogenous sex steroid levels seems to be a characteristic of progestin exposure in fish (Paulos et al., 2010; Runnalls et al., 2013).

The gonads of female roach were not affected by LNG exposure. In contrast, the testes of males exposed to 31 and 312 ng/l LNG presented a percentage of spermatogonia B that was significantly higher compared with that found for the control. Zeilinger et al. (2009) exposed adult fathead minnow to LNG and documented that LNG also causes advancement in testes development, namely an increase in the number of mature spermatids and testes size with an increase in LNG concentration. We can hypothesise that the stimulating effect of LNG on early spermatogenesis in roach found in the present study is due to the androgen- or progestin-like effects of LNG. Recently, a study by Chen et al. (2013) showed that treatment with a natural fish progestin, 17α,20β-dihydroxy-4-pregnen-3-one (DHP), stimulated the early stages of spermatogenesis in zebrafish. However, the reason for the inverted U-shaped concentration response with regard to spgB found in the testes of roach exposed to LNG is difficult to explain, although non-monotonic concentration responses are relatively common in the case of endocrine-disrupting chemicals (Vandenberg et al., 2012). A similar effect pattern on the testes was reported by Berglund et al. (1995) and Antonopoulou et al. (2009), who showed that the androgen T can exert both positive and negative effects on the growth of testes in immature male Atlantic salmon (the positive effects were more pronounced at the low concentrations, and the negative effects were more pronounced at the high concentrations). In the present study, it is likely that 31 and 312 ng/l LNG is able to offset the low levels of endogenous 11-KT and stimulates spermatogonial proliferation (by androgen- or progestin-like effects), whereas the strong oestrogenic activity of LNG at a concentration of 3124 ng/l may have counteracted these stimulatory effects because high levels of oestrogens are known to be detrimental for testis growth (e.g., Halm et al., 2002).

A recent study in fish showed that sex steroids such as E2 cannot only affect the reproductive physiology but also modulate the expression of genes important for growth and metabolism (Filby et al., 2006). However, at least at the level of *gh* expression, we were not able to demonstrate any effect of LNG.

Interestingly, Lorenz et al. (2011b) reported the adverse effects of LNG on the thyroid gland. Amongst others, the disruption of the thyroid system was indicated by increased *tshβ* mRNA expression levels in the brain/pituitary tissue of LNG-exposed *X. laevis* tadpoles. In the present study, using the pituitary *tshβ* mRNA level as a marker, we were not able to demonstrate any effect of LNG on the thyroid status of roach. Further investigations in fish, including thyroid histology, are necessary to determine whether disruption of the thyroid system by LNG is class-specific for amphibians.

In summary, the present study demonstrated a clear oestrogenic activity of LNG in both sexes of pubertal roach at the highest tested concentration (3124 ng/l). This finding is in contrast to the results of most studies on adult fish, which mainly demonstrated the androgenic activity of LNG. Furthermore, the present study provides the first demonstration that LNG differentially affects the expression of both gonadotropin β-subunits in the pituitary of fish. The *fshβ* expression was downregulated, and the expression of *lhβ* was stimulated, although the latter was only apparent with the highest LNG concentration. LNG exposure was also accompanied by decreased sex steroid levels (except for T), and the 11-KT levels in males decreased after exposure to an environmentally relevant concentration (Al-Odaini et al., 2010). Importantly, this study provides the first demonstration of the effects of progestin exposure during puberty in fish. Because of the importance of gonadotropins, particularly FSH and sex steroids for gametogenesis, LNG exposure during the juvenile or pubertal stages may have serious consequences for successful gonad maturation and spawning later in life.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.aquatox.2014.05.008.

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Synthetic progestin etonogestrel negatively affects mating behavior and reproduction in Endler's guppies (*Poecilia wingei*)

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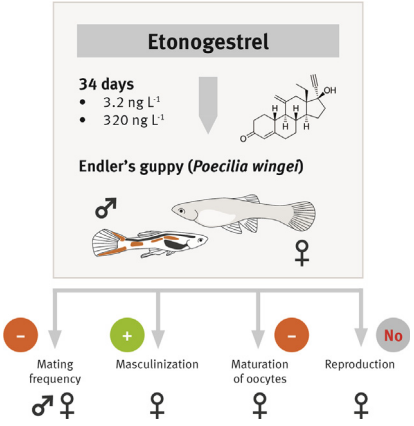
HIGHLIGHTS

- This is the first report on the effects in fish of etonogestrel exposure.
- Masculinization was seen in females exposed to the highest concentration.
- All exposed females were unable to reproduce.
- Exposure reduced males' mating activity but not their reproductive success.
- Alterations in reproductive behavior appeared to be sensitive endpoints.

GRAPHICAL ABSTRACT



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ABSTRACT

High rates of progestins consumption in the form of active ingredients in women's oral contraceptives and other hormonal preparations may lead to their increased concentrations in aquatic environments and subsequent harmful effect on fish reproduction. The objective of the present study was to assess the effect of etonogestrel, a third-generation synthetic progestin, on the reproductive behavior, fertility, gonads histology, and secondary sexual characteristics of male and female Endler's guppies (*Poecilia wingei*). Fish were subjected for 34 days to two concentrations of etonogestrel, including one possibly environmentally relevant (3.2 ng L^{-1}) and one sublethal (320 ng L^{-1}) concentration. A mating behavior study was subsequently conducted and revealed that the treatment with etonogestrel significantly reduced mating frequency in the exposed fish compared to controls. All the exposed females were unable to reproduce. In addition, female fish exposed to the highest level of etonogestrel were masculinized, as their anal fins and body coloration showed patterns similar to those of male fish. Etonogestrel-exposed females also had fewer developed oocytes. In conclusion, the low etonogestrel

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Sexual dimorphism
Steroid

concentration (3.2 ng L^{-1}) led to a reduction of mating activity in males without effect on their reproductive success, but it completely inhibited reproduction in females. Exposure to etonogestrel clearly has more severe consequences for females than males.

1. Introduction

Consumption of synthetic progestins as active ingredients in women's oral contraceptives and other hormonal preparations is relatively high (Kumar et al., 2015). Because these progestins are incompletely removed during wastewater treatment processes, their concentrations in aquatic environments may be as great as tens of nanograms per liter (Al-Odaini et al., 2010; Houtman et al., 2018). Such levels are of concern for inhabitants of aquatic ecosystems because they exceed concentrations that adversely affect fish and amphibians under laboratory conditions (Fent, 2015; Frankel et al., 2016a; Frankel et al., 2016b; Hoffmann and Kloas, 2012; Hou et al., 2018a; Kumar et al., 2015; Lorenz et al., 2011a; Lorenz et al., 2011b).

The synthetic progestin etonogestrel (13β-ethyl-17β-hydroxy-11-methylene-18, 19-dinor-17α-pregn-4-en-20-yn-3-one) is a testosterone derivative and the biologically active metabolite of desogestrel (Croxatto, 2002). Although etonogestrel and its precursor desogestrel have not been detected to date in waste and surface waters, there in fact have been only few attempts to analyze their levels (Golovko et al., 2018). Due to its increasing application, etonogestrel is suspected to be of environmental relevance. For instance, the use of single-rod, etonogestrel-releasing subdermal implants as a form of contraceptive increased by 50% in the U.S.A. between the years 2009 and 2012 (Kavanaugh et al., 2015; Odom et al., 2017). In the Czech Republic, the application of etonogestrel increased by approximately 45% between the years 2011 and 2015 and since that time has remained at almost the same level (State Institute for Drug Control, http://www.sukl.eu/ 2018) (Fig. S1). In addition, the predicted critical environmental concentration of etonogestrel is very low. A level of just 1.6 ng L^{-1} in water would result in a fish blood plasma concentration equal to the human therapeutic blood plasma level (Fick et al., 2010).

In mammals, progesterone receptor is the main target for progestins, and etonogestrel is characterized by very strong agonistic properties that exceed those of the natural ligand progesterone (Kumar et al., 2015; Sauer et al., 2018). In general, progesterone is involved in regulation of the hypothalamic–pituitary–gonadal axis and it regulates development, differentiation, and normal functioning of the female reproductive tract (Wagenfeld et al., 2016). It also triggers norepinephrine release from hypothalamus in order to mediate hormone-dependent sexual behavior in humans (Graham and Clarke, 1997). In addition to strong progestagenic activity, etonogestrel exerts androgenic, anti-estrogenic, and anti-gonadotropic activities in mammals (Kumar et al., 2015).

Several studies to date have documented adverse effects of progestins on the reproduction and development of fish and frogs (Hou et al., 2017; Kumar et al., 2015), but the exact mode of action is not fully understood (Hou et al., 2018a; Hou et al., 2018b). *In vitro* studies have demonstrated that progestins mostly do not bind to fish progesterone receptor, but some of them, mainly testosterone derivatives, including etonogestrel, possess high affinity to fish androgen receptors (Bain et al., 2015; Ellestad et al., 2014).

In ecotoxicological studies on aquatic organisms, behavioral endpoints are considered to be more sensitive than morphological, reproductive, and developmental parameters (Melvin and Wilson, 2013). Courtship and reproductive behaviors also have been identified as important endpoints for the study of endocrine disruption (Frankel et al., 2016a; Sebire et al., 2008; Zeilinger et al., 2009). Poeciliids are commonly used as model organisms in toxicological studies (OECD, 1992; Wester and Vos, 1994) and behavioral biological research (Schlupp, 2018a; Schlupp, 2018b). These viviparous fish have elaborate and well-defined courtship and mating behaviors (Pyke, 2005). A practical advantage is that they are able to breed year-round under laboratory conditions and with a short reproductive period (Baatrup and Junge, 2001). Moreover, morphological and histological responses and alterations in body color and mating behavior are readily observed in poeciliids exposed to endocrine-disrupting compounds (Angus et al., 2001; Hou et al., 2018b). In recent years, Endler's guppies (*Poecilia wingei*) increasingly have been used as an experimental animal for studying mate choice, social reproductive behavior, as well as activity and shoaling anxiety responses to new environments (Olsen et al., 2014; Sommer and Olsen, 2016). Only a few studies have thus far described the effects of synthetic progestins on the mating behavior of fish (Frankel et al., 2016b; Hou et al., 2018a; Kumar et al., 2015). Hou et al. (2018b) reported that long-term exposure of western mosquitofish (*Gambusia affinis*) to the testosterone derivative norgestrel at concentrations of 400 and 4000 ng L^{-1} led to reduced frequencies and durations of mating behavior in males and caused masculinization of females. Moreover, they observed impairment in females' reproduction. Similarly, Frankel et al. (2016b) showed reduced mating frequency in the eastern mosquitofish after eight-day exposure to 100 ng L^{-1} of levonorgestrel. Runnalls et al. (2013) reported that desogestrel, the precursor of etonogestrel, decreased egg production and caused masculinization of female fathead minnow (*Pimephales promelas*) after 21-day exposure at a high level (10,000 ng L^{-1}). Mating behavior was not evaluated in these studies, and, to the best of our knowledge, there is no report to date describing the effects of etonogestrel on fish.

The main objectives of the present study were to assess effects of sublethal etonogestrel concentrations on the different aspects of reproduction and morphology in Endler's guppies, namely (1) mate choice and mating behavior, (2) fertility, (3) secondary sexual characteristics, and (4) gonad histology.

2. Materials and methods

2.1. Chemicals and reagents

Methanol and acetonitrile (LiChrosolv® Hypergrade) were purchased from Merck (Darmstadt, Germany). Ultrapure water was produced using an Aqua-MAX-Ultra System (Younglin, Kyounggi-do, South Korea). Analytical standards were of high purity (mostly 98%). Etonogestrel [(13β-ethyl-17β-hydroxy-11-methylene-18, 19-dinor-17α-pregn-4-en-20-yn-3-one; CAS no.: 54048-10-1) was purchased from Sigma Aldrich (Steinheim, Germany). The internal standard drospirenone-13C6 was obtained from Toronto Research Chemicals, Inc. (Toronto Research Chemicals, ON, Canada). Absolute ethanol (100%) and xylene (mixture of isomers) were purchased from Penta (Czech Republic). Decalcification solution 1 (formic acid solution) was obtained from VWR (Czech Republic). Ten percent neutral buffered formalin, Mayer's hematoxylin, eosin, and histological ethanol (99.7–99.9%) were obtained from Diapath (Czech Republic).

Etonogestrel was dissolved in dimethyl sulfoxide (DMSO) to make a stock solution at a concentration of 320 mg L^{-1}.

2.2. Experimental design

Juvenile Endler's guppies were obtained from a local commercial breeder (Jindřichův Hradec, Czech Republic). Juvenile fish were kept and raised to maturity under conditions as described by Houde (1987). Briefly, the fish were acclimated to laboratory conditions for

3 months before beginning the exposure. During the acclimation period, males and females were raised separately to ensure virginity. The development of the fish inclusive of gender and virginity was checked regularly. Males were identified visually according to the developing gonopodium and body color. Pregnant females were identified based on the description of Norazmi-Lokman et al. (2016) and excluded from the experiment. Both genders were visually exposed to the opposite sex in the neighboring aquaria during the rearing. The fish were kept in dechlorinated tap water with added salt (NaCl, 1 g per 100 L) and calcium chloride (0.1 g per 100 L). These conditions were applied for all experiments. The temperature was kept constant at 25.0 ± 1.4 °C. Photoperiod (light:dark) was 14:10 h. Dissolved oxygen concentration and pH were 8.5 ± 0.5 mg L^{-1} and 7.9 ± 0.5, respectively. These parameters were measured every second day and at every experimental trial. During the acclimation and experimental periods, the fish were fed ad libitum with freshly hatched brine shrimp (*Artemia salina*) and a commercial preparation of freshly hatched brine shrimp (Artemia Sanders Premium, Sanders, USA) at 1% of their body weight per day. The fish were not fed on the sampling days.

Virgin, sexually mature fish with minimum length of 17 mm were selected (Herdman et al., 2004). Only colored males with fully developed gonopodium were used. The adult fish were randomly distributed into eighteen 100 L aquaria, each containing 22 fish. Males and females were kept separately. The fish were acclimated for 20 days to the experimental conditions. All sides of the aquaria were covered by polystyrene to prevent fish from being disturbed.

One hundred liters of the etonogestrel solution and of the water in control tanks were renewed daily. The fish were exposed to etonogestrel at environmentally relevant (E1: 3.2 ng L^{-1}) and sublethal (E2: 320 ng L^{-1}) concentrations under semi-static conditions for 34 days. DMSO with final concentration of 0.0005% was added to treatment groups to facilitate the dissolving of etonogestrel. Two control groups were included. One control group, designated C, was contained in dilution water only. The second, designated SC for solvent control, was contained in water with added dimethyl sulfoxide at the same concentration as in the treatment groups. All experimental and control trials were duplicated. The SC control was triplicated. Mortality was recorded during the acclimation and experimental periods. This study was performed in accordance with the principals of the EU-harmonized Animal Welfare Act of the Czech Republic.

2.3. Etonogestrel analysis

Individual stock solutions of the standards were prepared at 1 mg mL^{-1} concentration in methanol and stored at −20 °C. A spiking mixture of drospirenone-13C6 was prepared by diluting the stock solutions with methanol to a final concentration of 1 μg mL^{-1} for each compound. Working standard mixtures (0.01–10 μg mL^{-1}) of the native compound were prepared in methanol.

Water samples were collected from aquaria into 1 L amber glass bottles. The concentration of etonogestrel was measured immediately after water exchange (0 h) and 24 h post-exchange to check for its concentration and stability. The collected samples were stored at +4 °C in darkness until extraction, which was carried out within 24 h. Water samples were prepared according to a protocol developed for solid-phase extraction (Golovko et al., 2018).

An Accela 1250 LC pump (Thermo Fisher Scientific, San Jose, CA, USA) coupled with a hybrid quadrupole/orbital trap Q-Exactive mass spectrometer (Thermo Fisher Scientific) and an HTS XT-CTC autosampler (CTC Analytics AG, Zwingen, Switzerland) were used for analysis of water extracts. An analytical Hypersil Gold column (50 mm × 2.1 mm ID × 3 μm particles; Thermo Fisher Scientific) preceded by the same phase pre-column (10 mm × 2.1 mm ID × 3 μm particles) was used for the chromatographic separation of etonogestrel.

Ultrapure water and methanol were used as the mobile phases. The elution conditions were programmed as follows: 350 mL min^{-1} 30% methanol in water for 1 min, isocratically followed by a gradient change to 20/80 water/methanol at a flow of 400 mL min^{-1} for 8 min and a final gradient change to 100% of methanol at a flow of 400 mL min^{-1} for 10 min. These parameters were held for 2 min and then changed to the starting conditions and held for 1.5 min to equilibrate the column for the next run.

Atmospheric pressure photoionization (APPI) in positive mode was used to ionize target compounds. The instrument was calibrated daily (mass calibration) in positive modes using a standard procedure proposed by Thermo Scientific.

The atmospheric pressure chemical ionization/atmospheric pressure photoionization (APCI/APPI) parameters were set as follows: capillary temperature (300 °C), vaporizer temperature (300 °C), sheath gas pressure (40 arbitrary units), auxiliary gas (15 arbitrary units), and discharge current (4 μA in positive ionization mode). A UV krypton lamp (10 eV) was used in the source.

The analytical method for analyzing a wide range of progestins, including etonogestrel, in different water matrices using solid-phase extraction followed by liquid chromatography tandem atmospheric pressure chemical ionization/atmospheric pressure photoionization with hybrid quadrupole/orbital trap mass spectrometry operated in high resolution product scan mode (LC-APCI/APPI-HRPS) had previously been validated for linearity, repeatability, limit of quantification (LOQ), and trueness (Golovko et al., 2018).

The linearity of the calibration curve was tested in the range from 0.1 ng L^{-1} to 500 ng L^{-1}. Calibration curves were measured at the beginning and at the end of the sequence to check instrument stability. The calibration was prepared in water/MeOH (1/1).

Prior to extraction for each aquarium, a procedural blank (demineralized water) was extracted and analyzed to distinguish between positive detections and potential sample contamination. Each sample was analyzed simultaneously with matrix matching standards for the determination of matrix effects. Data analysis and calculation were performed using TraceFinder 3.3 software (Thermo Fisher Scientific).

2.4. Behavioral studies

Before and at the end of the exposure period, such activities of the fish as grouping, following, site preference, and swimming were studied (2.4.1). After 34 days of exposure, the behavioral tests, namely the choice (2.4.2) and mating tests, were conducted within 2 days. To determine fertility, females from the mating test (2.4.3) were kept in isolation for 90 days (Fig. 1).

2.4.1. Analysis of activity patterns

Behavioral parameters (grouping, following, site preference, and swimming activity) were recorded before and at the end of the exposure period. The behavior patterns were recorded over a period of 3 days with a monitoring system based on 3D cameras (Xbox One Kinect Sensor V2) placed above the aquaria. After 5 min of adaptation, the behavior was recorded for 40 min. Every aquarium was recorded 3 times and the experimental groups were recorded in parallel. The monitoring system detects the individual fish in 3D space and saves its position for later processing.

The recordings were later automatically quantified for following, grouping, and activity behaviors by software implemented in-house for fish 3D position processing and that had been developed by Saberioon and Cisar (2016). These parameters were defined based on the description of Kane et al. (2005) and with modifications as described below. Grouping of the fish (grouping behavior) was identified when >70% of the fish were present within a 40 cm radius. Following behavior was determined based on trajectories of movement, when the movement directions were not exceeding a 50° angle and fish were in a swarm-like pattern. Swimming activity is represented by the average velocity of the fish per second. This parameter was calculated frame by

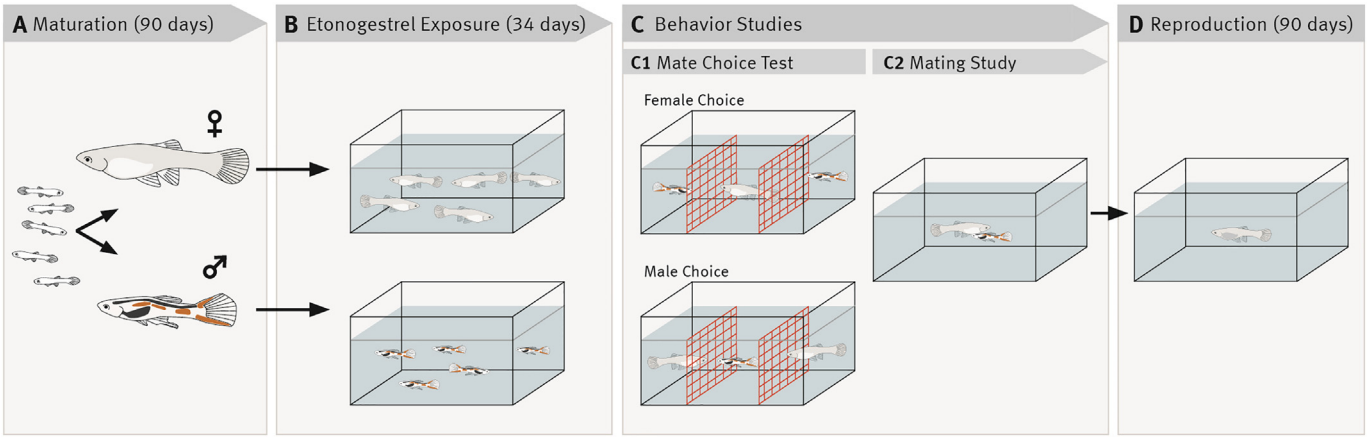

Fig. 1. Schematic workflow of different stages of the long-term reproductive study on Endler's guppies. A – Juvenile fish were raised for 3 months under laboratory conditions to mature stage. B – Exposure to etonogestrel for 34 days. C – Behavioral studies: C1, mate choice test; C2, mating study. D – Reproduction (mated females were kept in isolation for 90 days in order to determine fertility).

frame as the ratio between the length of the fish trajectory and the time of swimming, and it was then transformed into centimeters per second. The swimming activity was calculated for individual fish and averaged for the fish group. Site preference was defined as time spent in the center of the aquarium (50% of the surface) or the border area (50% of the surface). The site preference was calculated based on the time fish spent in each of the two regions.

2.4.2. Mate choice test

The mate choice test was conducted after the 34 days of exposure. This test was aimed at determining the effect of etonogestrel treatment on the preference for individuals of the opposite gender in both males and females. This binary mate choice experiment was conducted following Herdman et al. (2004) and Bierbach et al. (2011) and was carried out separately for males and females. In total, 112 naïve fish (56 males and 56 females) were tested. This test was carried out in 8 replicates for each experimental group. A 9 L aquarium (32 × 22 cm) was divided by fine mesh into three parts. To avoid disturbances, all sides of the aquaria were covered by black plastic sheeting. A naïve fish from the solvent control was placed into the middle part (16 × 22 cm), which was the test area. On the left and right sides, which were of dimensions 8 × 22 cm, exposed and unexposed fish (solvent control) of the opposite gender were placed. As an additional control, a naïve fish was placed into an aquarium with two unexposed fish from the opposite gender in the other parts. This procedure was conducted with fish from control and solvent controls (Fig. 1).

After an adaptation period of 5 min, the behavior of the fish in the middle section was recorded for 15 min. Nine aquaria were used for this experiment. The assignment of experimental trials to the aquaria was randomized. The behavior patterns were recorded with the monitoring system based on a 3D camera (Xbox One Kinect Sensor). One camera was placed above three aquaria to monitor each fish separately and save the 3D position for later processing. The experimental setup is illustrated in Fig. 1. The closeness was calculated from the recordings using software implemented in-house based on the program developed by Saberioon and Cisar (2016). Closeness was defined as spending time outside the midpoint area of the middle section located at one of the respective sides. The time of each behavior was automatically quantified by a mating processing program (Saberioon and Cisar, 2016). Attraction or avoidance was evaluated based on the presence on one of the sides. The water in the aquaria was changed after each experimental trial.

2.4.3. Mating experiment

Mating behavior was studied using an exposed male cohabitating with an unexposed female and vice versa. Moreover, male and female fish exposed to both concentrations of etonogestrel and unexposed couples were mated. In particular, combinations tested in the mating experiment were as follow: (1) male C × female C, (2) male SC × female SC, (3) male E1 × female SC, (4) male E2 × female SC, (5) male SC × female E1, (6) male SC × female E2, (7) male E1 × female E2, and (8) male E2 × female E2. A respective fish couple was transferred into a 15 L aquarium (32 × 22 cm) containing 5 L of water. The mating tests were conducted with nine replicates for each experimental group. After an adaptation period of 5 min, the mating was recorded by charge-coupled device camera (Sony, CCD-TR840E) for 15 min. The camera monitored four aquaria together from the top to record the complete trajectory and fish behavior. The videos were later analyzed for the presence of several behavior endpoints, including display of the S-shape (the S-shape is a sigmoid movement displayed by a male during the courtship), following, attending, and attempts by the male to copulate. The behavioral patterns were evaluated manually, blinded. Courtship and mating were determined as described by Baatrup and Junge (2001), Pyke (2005), Olsen et al. (2014), and Frankel et al. (2016b).

For determining fertility, female fish involved in the mating study were kept isolated in clean water for 90 days after mating. These fish were kept in isolated boxes (12 × 8 cm) under the conditions described above. The birth rate was recorded.

2.5. Secondary sexual characteristics

All fish involved were recorded for coloration analysis after the mating study. All females were photographed at the end of the mating test and 90 days post mating. During the recording, the fish were alive and placed in a special aquarium with dimensions 12 × 4 × 4 cm. A green background was selected for the recordings. The pictures were taken using an Olympus Alpha 501 camera. Blinded evaluation was made of the pictures, and alterations in body color were noted. These alterations were defined by the presence of orange or any other color different from the normal, metallic gray body color (Poeser et al., 2005).

Sixteen fish of each gender from each treatment and the water control as well as 24 fish from the solvent control were used for morphological analysis. Fish were stored in 10% neutral buffered formalin. Weight of fish was recorded after 4 months of fixation and stabilization of the relative post-fixation mass. The fish body, eye, caudal, dorsal, pelvic, pectoral fins, as well as the anal fin of the females and gonopodium of the males were photographed using an Olympus E600 camera mounted on an Olympus SZX7 stereomicroscope. All measurements were carried out using Quick Photo 2.3 software.

Measured were total (TL) and body (BL) lengths, horizontal eye diameter (ED), lengths of the first ray (DFL_{1st}) and last ray (DFL_{last}) of the dorsal fin, as well as total lengths of the caudal (CDFL), pelvic (PLFL), and pectoral (PECFL) fins. These parameters, with the exceptions

of TL and BL, were normalized to the BL. These parameters are described in detail in Wiecaszek et al. (2009).

In the cases of the anal fin and gonopodium, the thicknesses of the 3rd and 4th rays, lengths of the 4th and 6th rays, thicknesses and length ratios of 3rd:4th ray, relative length of 4th and 6th rays to BL, and the palp lengths of the gonopodium were measured as described by Angus et al. (2001). The rays were identified as described by Turner (1941). Abnormalities of the male gonopodium and the female anal fin were recorded.

2.6. Histological analysis

The in toto fixed fish (10% buffered formalin) were decalcified (for 4 h in decalcification solution 1). Male and female fish were oriented in longitudinal and sagittal directions in capsules. In the case of female fish, serial cuts were made of the area containing the ovarium. For 8 fish of every experimental group, the developmental stages of the gonads were identified (Edwards and Guillette, 2006; Golpour et al., 2016; Hou et al., 2018a). Ninety days post-exposure, possible pregnancy was checked histologically in females which had not given birth. Samples were dehydrated in an ascending series of ethanol concentrations, paraffin-embedded, cut by microtome (4 μm), then placed on slides. The sections were stained with hematoxylin and eosin (H&E), then examined by light microscopy at 10× to 1000× times magnification (Bancroft and Gamble, 2008).

2.7. Statistical analysis

The statistical analysis was performed using Statistica software version 13 (StatSoft, Czech Republic). Data were checked for normality and homoscedasticity by the Kolmogorov–Smirnov test, and by Cochran's, Hartley's and Bartlett's tests, respectively.

If these criteria were fulfilled, a one-way analysis of variance (ANOVA) was employed to detect significant differences among the experimental groups in the measured variables. Subsequently, Dunnett's multiple range test was applied to compare the control mean with the means of all treatment groups. If the conditions for ANOVA were not satisfied, nonparametric tests (Kruskal–Wallis test and Friedman ANOVA) were used. The significance level was set at $p < 0.05$.

The behavior during the experiment, namely time spent in the outer 50% of the aquarium, grouping, following, and swimming speed were analyzed by Kruskal–Wallis test and Friedman ANOVA. The mate preference of the fish was analyzed by Kruskal–Wallis test. Differences in coloration and the developmental stages of oocytes in the ovary were checked by chi-squared test. Fisher's exact test was applied for the birth rate and the presence of palp in female fish. The data are presented as mean ± standard deviation (SD).

Table 1
Concentration of etonogestrel in water in the sub-chronic toxicity test on Endler's Guppy (*Poecilia wingei*). Concentrations were measured immediately after water exchange (0 h) and 24 h post-exchange. The values are expressed as mean ± SD ($n = 4$). C = water control, SC = solvent control, LOQ = limit of quantification.

Group	Sample time (h)	Water concentration (ng L^{-1})	Stability during 24 h (%)	Min – Max
C	0	<LOQ		
	24	<LOQ		
SC	0	<LOQ		
	24	<LOQ		
3.2 ng L^{-1}	0	3.2 ± 1.8		1.6–5.1
	24	2.7 ± 1.4	88 ± 16	1.2–4.1
320 ng L^{-1}	0	483 ± 22		450–500
	24	435 ± 58	91 ± 16	360–490

3. Results

3.1. Analytical performance and water concentration of etonogestrel

Table 1 shows concentrations of etonogestrel measured in aquarium water for all treatment groups. Etonogestrel concentrations over 24 h were relatively stable (88–91%), with means corresponding to 3.0 ± 1.5 and 458 ± 48 ng L^{-1}, respectively, in treatments with nominal etonogestrel concentrations of 3.2 and 320 ng L^{-1}. In water samples from both controls, the concentrations of etonogestrel were below the LOQ (<0.35 ng L^{-1}).

3.2. Behavioral studies

3.2.1. Analysis of activity patterns

In male and female fish, the relative durations of grouping and following in all treatment groups were short, as these behaviors, respectively, were in the ranges of 1.0–1.9% and 0.3–2.2‰ of the total recorded time. Before and at the end of the exposure period, grouping and following of females and males did not differ significantly in the exposed fish compared to the controls (Friedman ANOVA, Kruskal–Wallis, and Kruskal–Wallis test applied on differences of grouping and following before and at the end of the exposure time, $p > 0.05$). Males and females exposed to nominal etonogestrel concentrations of 3.2 and 320 ng L^{-1} did not show significantly different swimming activity in comparison with the controls (ANOVA, $p > 0.05$). In all treatment groups, the fish avoided the central section and stayed preferentially in the corners of the aquaria. The site preference in fish of all treatment groups was not statistically different compared to that of the controls (Kruskal–Wallis, $p > 0.05$). The relative duration and frequency of grouping and following and the site preference are summarized in Supplementary Tables 1 and 2.

3.2.2. Mate choice test

No effect of etonogestrel on mate preference was found for treated male and female fish (Kruskal–Wallis, $p > 0.05$; Fig. S2). No site preference of the fish was found within the control groups (SC and C; Kruskal–Wallis, $p > 0.05$; Fig. S2).

3.2.3. Mating study

Both groups of exposed males (3.2 and 320 ng L^{-1}) showed significantly shorter duration and lower frequency of mating attempts compared to the controls (ANOVA, $p < 0.05$; Fig. 2), although the time for each attempt was not changed (Table S3). The courtship behaviors, namely duration and frequency of attending and following and frequency of display of the S-shape of the male fish, did not differ significantly in the etonogestrel-treated fish compared to the controls (Table S3).

3.3. Reproduction

All etonogestrel-exposed females at both concentration levels were unable to reproduce 90 days post mating (Fig. 3). In contrast, control females which mated with unexposed or 3.2 and 320 ng L^{-1} etonogestrel-exposed male fish were able to reproduce. Fisher's exact test confirms that the result is statistically significant both when we compare all control groups with all exposed groups as well as all SC groups with all exposed groups (Fisher's exact test, $p < 0.0002$).

3.4. Mortality

No mortality was recorded during the experimental period.

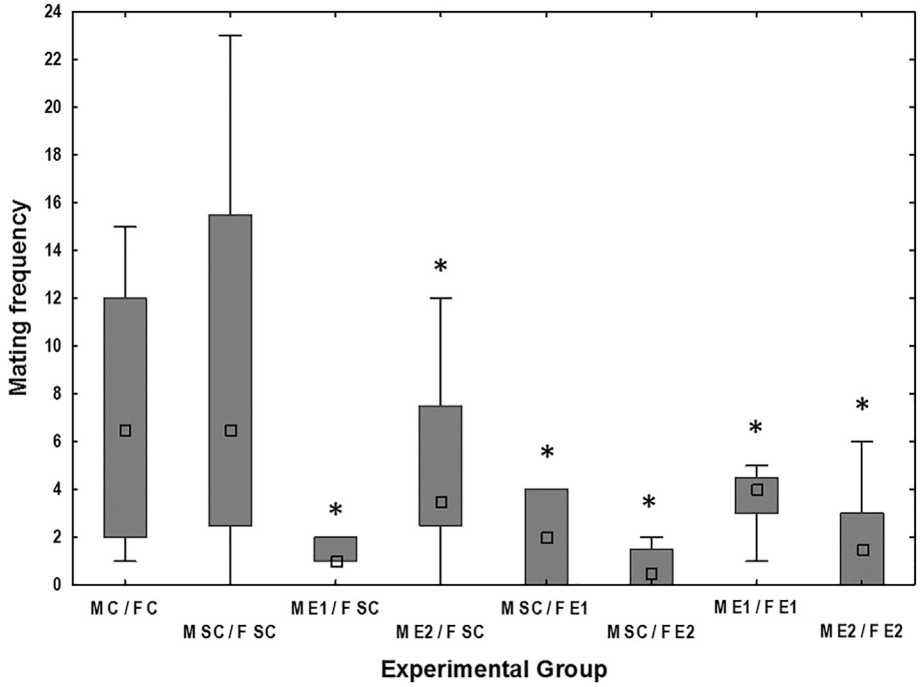

Fig. 2. Mating frequency during 15 min in Endler's guppies exposed to etonogestrel for 34 days. M = male, F = female, C = water control, SC = solvent control, E1 = 3.2 ng L^{-1} etonogestrel-exposed fish, E2 = 320 ng L^{-1} etonogestrel-exposed fish. Asterisks indicate significant difference from controls. *$p < 0.05$ (ANOVA, $n = 9$).

3.5. Secondary sexual characteristics

In female fish exposed to 320 ng L^{-1} etonogestrel, the relative length of the caudal fin (CFL/BL), absolute and relative width of the 3rd and 4th rays and length of the 4th and 6th rays of the anal fin, absolute length of the last ray of the dorsal fin (DFL_{last}), and relative length of the dorsal fin (DFL_{1st}/BL) were significantly increased compared to those of the controls (Table S5). All these parameters are close to those measured in male control fish (control and solvent control) and indicate masculinization of females exposed to the highest tested level of etonogestrel (Fig. 4). Ninety days post exposure, the length of the caudal fin and rays of anal fin remained altered (Table S6).

In males exposed to 320 ng L^{-1} etonogestrel, the ratio of DFL_{last}/BL and the absolute and relative length of the 6th ray of the gonopodium increased compared to the controls ($p < 0.05$). The ratio of the length of the 4th to the 6th fin ray in the gonopodium was significantly reduced in fish exposed to both 3.2 and 320 ng L^{-1} etonogestrel compared to the controls ($p < 0.05$, Table S4).

Other measured parameters (i.e., TL, BL, weight, FCF, ED, ED/BL, HL, HL/BL, PFL, and PFL/BL) were not significantly affected by the etonogestrel exposure either in males or females (Tables S4–6).

At the highest level of etonogestrel (320 ng L^{-1}), a significantly higher number of females displayed alterations in their body color (chi-squared test, $p < 0.05$; Fig. 5). These alterations were characterized by a yellow- to orange-colored caudal part of the body inclusive of the tail and dorsal fin. Typically, these changes started at the tip of the caudal fin. These male-like patterns indicated masculinization of fish in this experimental group (Figs. 4 and 5). This effect appeared to be reversible, as 90 days post mating only 8% of the females showed masculinization of the body color. These alterations were not present in any of the other groups.

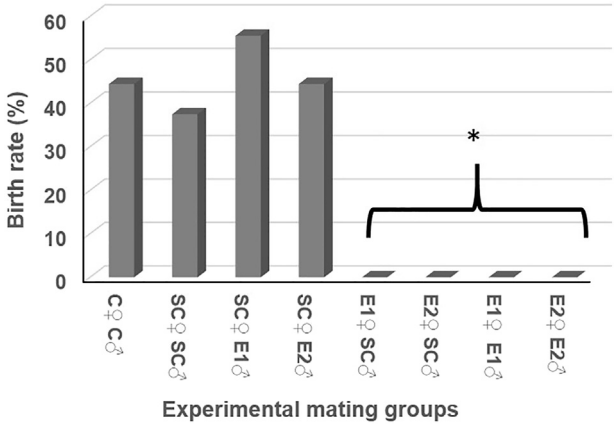

Fig. 3. Birth rate of Endler's guppies sub-chronically exposed to etonogestrel for 34 days. M = male, F = female, C = water control, SC = solvent control, E1 = 3.2 ng L^{-1} etonogestrel-exposed fish, E2 = 320 ng L^{-1} etonogestrel-exposed fish. Asterisks indicate significant differences from controls. *$p < 0.05$ (Fisher's exact test, n = 9).

3.6. Histology

Etonogestrel treatment affected maturation of oocytes in the exposed fish. In the control groups, mature oocytes were predominant, but the relative occurrence of mature oocytes was significantly lower in fish exposed to 3.2 and 320 ng L^{-1} etonogestrel compared to the controls (chi-squared test, $p < 0.05$; Fig. 6).

Etonogestrel did not affect the development of testes. Predominantly mature spermatocytes were present in all examined fish. Among the experimental groups, no statistically significant differences in frequency of the different developmental stages in the testes were found (chi-squared test, $p > 0.05$; Fig. S3). In none of the examined fish did the testes or ovaries show pathological alterations, and no signs of intersex were observed.

Among fish that did not give birth, no pregnancy was found 90 days post mating by the histological analysis in any of the exposed fish (3.2 and 320 ng L^{-1}) or controls.

4. Discussion

The main objectives of the present study were to assess effects of sublethal etonogestrel concentrations (3.2 and 320 ng L^{-1}) on Endler's

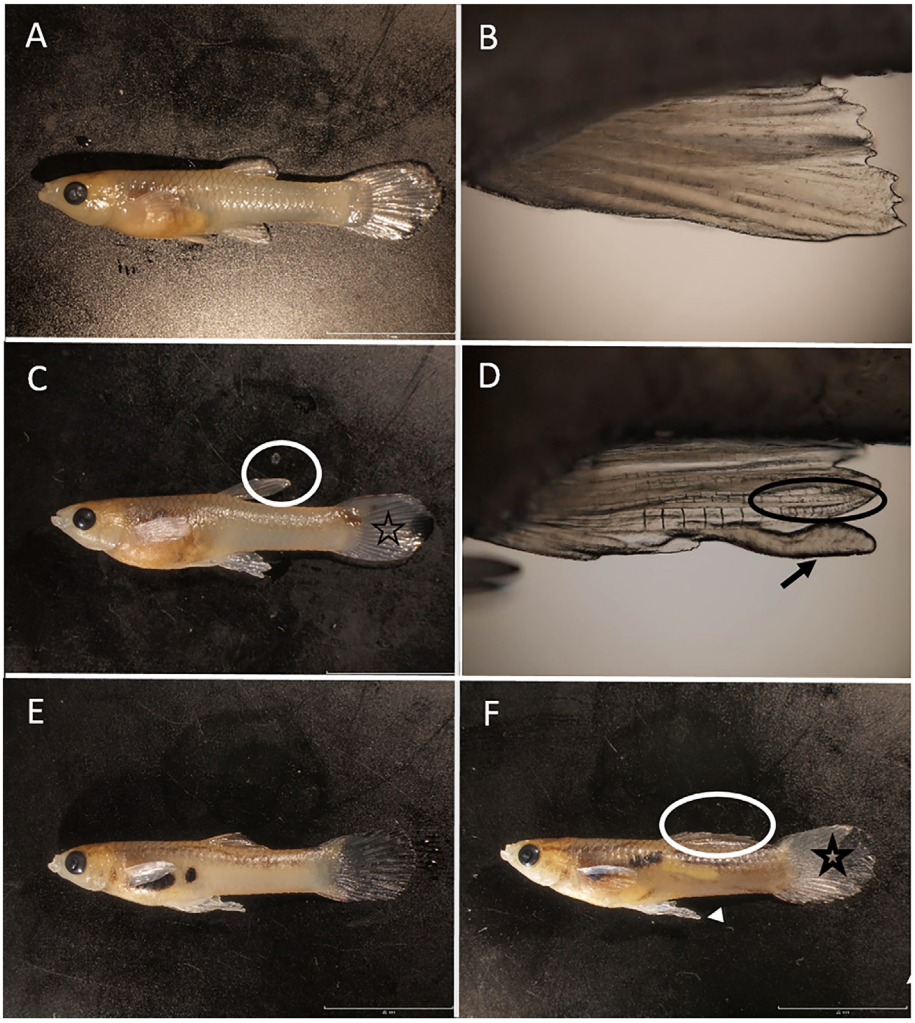

Fig. 4. Morphological changes in fins and gonopodium, of exposed Endler's guppies. (A) Whole body of an unexposed solvent control female (SC); the anal fin is shown in detail in image (B). (C) Whole body of a female exposed to 320 ng L^{-1} etonogestrel for 34 days; white circle and star indicate longer dorsal and caudal fins, respectively. (D) Detail of anal fin of etonogestrel-exposed female (from C); arrow and circle show palp and hooks, respectively. (E) Whole body of unexposed solvent control male (SC). (F) Whole body of male exposed to 320 ng L^{-1} etonogestrel for 34 days; circle and star show longer dorsal and caudal fins, respectively. Arrowhead indicates gonopodium with an altered shape.

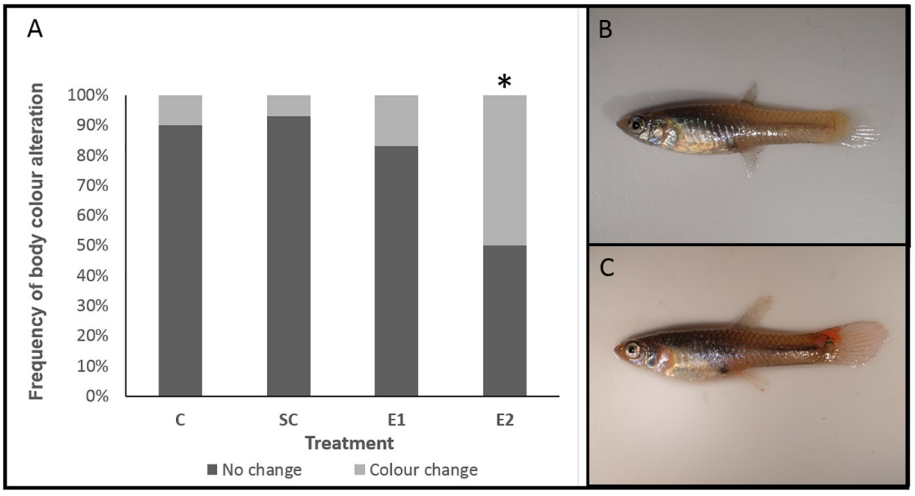

Fig. 5. (A) Percentage of color alterations in female Endler's guppies at the conclusion of 34 days of exposure to etonogestrel. C = water control, SC = solvent control, E1 = 3.2 ng L^{-1} etonogestrel-exposed fish, E2 = 320 ng L^{-1} etonogestrel-exposed fish. Asterisks indicate significant difference from controls. $^{*}p < 0.05$ (chi-squared test). $n = 9$. (B) Body color of a control female fish. (C) Altered body color of a female exposed to 320 ng L^{-1} of etonogestrel for 34 days.

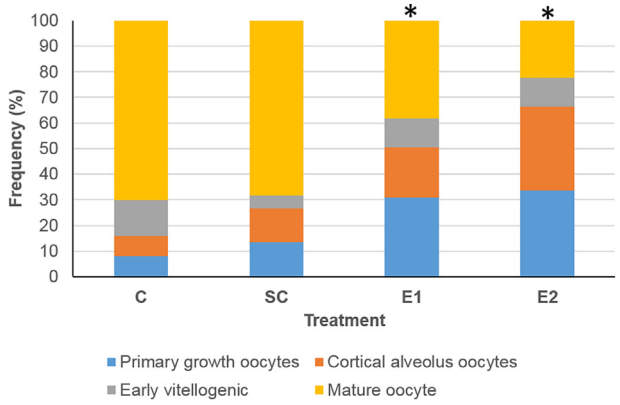

Fig. 6. Percentage of oocytes in different developmental stages within the ovaries of Endler's guppies at the conclusion of 34 days of exposure to etonogestrel. C = water control, SC = solvent control, E1 = 3.2 ng L^{-1} etonogestrel-exposed fish, E2 = 320 ng L^{-1} etonogestrel-exposed fish. $n = 8$. Asterisks indicate significant difference from controls. $^{*}p < 0.05$ (chi-squared test).

guppies. Changes in secondary sexual characteristics (morphology and coloration) are first discussed below (4.1), followed by behavioral alterations (activity patterns, mate choice and mating; 4.2), and then effects on reproduction and gonad development (4.3).

4.1. Secondary sexual characteristics

In the present study, female fish exposed to etonogestrel (320 ng L^{-1}) displayed a male-like coloration mainly at the caudal fin. In addition, the anal fin of females developed a gonopodium-like shape, including hooks and a palp, and the caudal fin was elongated. These fin alterations were still visible 90 days post exposure, thus indicating that they could be permanent. Taken together, all the changes in secondary sexual characteristics found in females exposed to etonogestrel (320 ng L^{-1}) are signs of masculinization of females and clearly show an androgenic effect of etonogestrel at high concentration. Our findings are well in line with the study by Bain et al. (2015), who reported that etonogestrel transactivated Murray-Darling rainbowfish (*Melanotaenia fluviatilis*) androgen receptors α and β with half maximal effective concentration (EC_{50}) values of 164 and 1469 ng L^{-1}, respectively.

Similar alteration of the anal fin and/or changes in color were described in female fish exposed to androgen-active pulp and paper mill wastewaters (Deaton and Cureton, 2011; Howell et al., 1980; Parks et al., 2001) or synthetic androgenic hormones (Larsson et al., 2002; Zamora et al., 2008).

Androgenic effect has been observed also in other testosterone-derived synthetic progestins, namely (levo)norgestrel, gestodene, and desogestrel. Hou et al. (2018a) and Frankel et al. (2016b) described masculinization of anal fin in adult females of western mosquitofish exposed to ≥35.8 ng L^{-1} of norgestrel for 42 days and in eastern mosquitofish exposed to ≥10 ng L^{-1} of levonorgestrel for 8 days, respectively. Female fathead minnow developed male secondary sexual characteristics after 21 days of exposure to gestodene (1 ng L^{-1}), levonorgestrel (29.6 ng L^{-1}), and desogestrel (10 μg L^{-1}) (Runnalls et al., 2013; Zeilinger et al., 2009).

In the present study, a longer dorsal fin was observed in males exposed to the highest level of etonogestrel (320 ng L^{-1}). Worthy of note is that *Poecilia latipinna* and *Poecilia mexicana* display a sexual dimorphism of dorsal fin, where females prefer males with longer dorsal fins (MacLaren et al., 2011). In addition, exposure to etonogestrel at both concentrations caused changes in the shape of the gonopodium, namely it reduced the ratio of the 4th to 6th ray. The gonopodium in Poeciliids functions as an intromittent organ but it is also important for pre-copulatory sexual selection (Kwan et al., 2013). Therefore, any alterations of its morphology may impact reproductive success (Evans et al., 2011; Kwan et al., 2013). Contrary to the present study, an increase of the 4th:6th ray ratio was observed in males of eastern mosquitofish after 8 days of exposure to 100 ng L^{-1} levonorgestrel (Frankel et al., 2016b). It is not clear, however, whether these particular changes increase or decrease attractiveness of the affected males.

4.2. Behavioral alterations

Etonogestrel did not cause any changes in activity, following, and grouping of fish in this study, indicating that the exposed fish did not suffer from anxiety, narcosis, or disorientation. Even such behavior changes, however, probably would not strongly interfere with mating. Olsen et al. (2014) showed, for example, that although Endler's guppies exposed to high levels of the psychoactive drug citalopram displayed signs of anxiety, mating and courtship were not affected. Contrary to the present study, Zhao et al. (2018) report that locomotor activities decreased in zebrafish embryos after 2 days of exposure to progesterone and synthetic progestins (levonorgestrel and gestodene) at the level of 16 ng L^{-1}. This might be explained by differences in the experimental setup, including the tested species, life stage, exposure time, and selection of progestins.

Reproductive behavior in guppies involves association, courtship, and mating (Poeser et al., 2005; Pyke, 2005). In this study, no difference in the association and courtship (i.e., preference or attending behavioral traits; S-shape display and following) were found in etonogestrel-treated fish compared to the controls. Nevertheless, mating frequencies (mating attempts) appeared to be the most sensitive behavioral endpoint in the present study. At both etonogestrel concentrations, the mating frequencies were significantly reduced. Exposed males appeared to be affected directly, as they made a lower number of attempts to pair with both control and exposed females. Moreover, they were affected indirectly, because reduced mating frequency was also observed in unexposed males paired with exposed females. This indicates a negative effect of etonogestrel on the attractiveness of the exposed females. At the high etonogestrel level, one of the reasons for low attractiveness of females was probably their strong masculinization. In females exposed to low etonogestrel concentration, however, there were no marked changes in secondary sexual characteristics. Therefore, additional issues had to have played a role, and pheromone signaling could be one of them. Mature females release sexual pheromones into the water and thus induce mating behavior in males (Kobayashi et al., 2002; Sorensen and Stacey, 2004). Inasmuch as etonogestrel exposure negatively affected maturation of the gonads in females within the present study, this could have interfered also with pheromone signaling in females and thus reduced males' interest in mating (i.e., decreased mating frequency). In agreement with this study, Frankel et al. (2016b) and Hou et al. (2018a) found that short-term (8 days) exposure to levonorgestrel (100 ng L^{-1}) and long-term exposure (42 days) to norgestrel (3.6 ng L^{-1}) resulted in changes of reproductive behavior in western and eastern mosquitofish, respectively, but the potential adverse outcomes of these changes on the birth rate have not been studied.

4.3. Reproduction and gonad development

Females exposed to both levels of etonogestrel were unable to produce offspring after mating with either control or exposed males. On the other hand, exposed males were able to reproduce with control females even if the mating frequency was reduced. The exposure to etonogestrel clearly has more serious consequences for females than males. At present, there is no information available regarding the effect of synthetic progestins on reproduction in Poeciliids. In agreement with the present study, reproduction in fathead minnow, Japanese medaka, and zebrafish have been shown to be affected adversely by low levels of synthetic progestins, which lead to inhibited or reduced egg production (Kumar et al., 2015). In fathead minnow, for example, egg

production was reduced after 21 days of exposure to low levels of levonorgestrel (0.8 ng L^{-1}) (Zeilinger et al., 2009) and gestodene (1 ng L^{-1}) (Runnalls et al., 2013), as well as higher levels of norethindrone (100 ng L^{-1}) (Paulos et al., 2010) and desogestrel (10 μg L^{-1}) (Runnalls et al., 2013).

Reproductive failure in females exposed to both levels of etonogestrel in the present study can be explained at least in part by their markedly lower percentage of mature oocytes (22–38%) in comparison to the controls (68–70%). It is noteworthy that Hou et al. (2018a) observed a higher percentage of atretic and post-ovulatory oocytes and higher incidence of intersex in western mosquitofish exposed to norgestrel at ≥35.8 ng L^{-1}.

We found no effect of etonogestrel exposure on testes. This fact corresponds with our findings of reproductive success among exposed males paired with control females. Likewise, Frankel et al. (2016a) observed no effect of levonorgestrel on the development of testes in fathead minnow exposed to concentrations of ≥10 ng L^{-1} for 8 days. In that study, they had not examined any possible effect on reproduction.

The results showing the effect of synthetic progestins on fish reproduction are not surprising inasmuch as these pharmaceuticals were primarily designed for contraception in human medicine (Baird and Glasier, 2000; Kroupova et al., 2014). Nevertheless, synthetic progestins have a low affinity to the fish progesterone receptors (Bain et al., 2015; Ellestad et al., 2014). Therefore, this effect is likely mediated through another pathway and further studies should be undertaken to elucidate synthetic progestins' mode of action in fish.

5. Conclusions

To the best of our knowledge, this study reports for the first time the effects of etonogestrel on fish. A concentration of etonogestrel as low as 3.2 ng L^{-1} reduced mating activity in males without affecting their reproductive success but caused complete failure of reproduction in females. This shows that etonogestrel exposure had more serious consequences for females. In addition, the high etonogestrel level (320 ng L^{-1}) caused marked masculinization of females. These findings strengthen current concerns about possible detrimental effects on fish populations of synthetic progestins reaching aquatic environments.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2019.01.276.

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Naturally-induced endocrine disruption by the parasite *Ligula intestinalis* (Cestoda) in roach (*Rutilus rutilus*)

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ABSTRACT

Fish represent the most frequently used vertebrate class for the investigation of endocrine disruption (ED) in wildlife. However, field studies are complicated by exposure scenarios involving a variety of anthropogenic and natural influences interfering with the endocrine system. One natural aspect rarely considered in ecotoxicological studies is how parasites modulate host physiology. Therefore, investigations were carried out to characterise the impacts of the parasitic tapeworm *Ligula intestinalis* on plasma sex steroid levels and expression of key genes associated with the reproduction in roach (*Rutilus rutilus*), a sentinel species for wildlife ED research. Parasitisation by *L. intestinalis* suppressed gonadal development in both genders of roach and analysis of plasma sex steroids revealed substantially lower levels of 17β-oestradiol (E2) and 11-ketotestosterone (11-KT) in infected females as well as E2, 11-KT, and testosterone in infected males. Consistently, in both, infected females and males, expression of the oestrogen dependent genes such as vitellogenin and brain-type aromatase in liver and brain was reduced. Furthermore, parasitisation differentially modulated mRNA expression of the oestrogen and androgen receptors in brain and liver. Most prominently, liver expression of oestrogen receptor 1 was reduced in infected females but not in males, whereas expression of oestrogen receptor 2a was up-regulated in both genders. Further, insulin-like growth factor 1 mRNA in the liver was increased in infected females but not in males. Despite severe impacts on plasma sex steroids and pituitary gonadotropin expression, brain mRNA levels of gonadotropin-releasing hormone (GnRH) precursors encoding GnRH2 and GnRH3 were not affected by *L. intestinalis*-infection. In summary, the present results provide basic knowledge of the endocrine system in *L. intestinalis*-infected roach and clearly demonstrate that parasites can cause ED in fish.

1. Introduction

Anthropogenic endocrine active compounds are widely present in surface waters and there is major concern about their potential health-effects exerted on the reproductive system in aquatic vertebrates (Kloas et al., 2009; Segner, 2005). Some of the best documented examples of endocrine disruption (ED)[1] in wildlife have been reported in fish, in particular roach (*Rutilus rutilus* Linnaeus, 1758), a species which has become a sentinel in ecotoxicological field-research (Jobling et al., 1998; Tyler et al., 2007). However under field conditions, correlations between pollutants and observed ED are difficult to prove and the interpretation of data is hampered by complex exposure scenarios involving a variety of anthropogenic as well as natural factors which modulate the physiology of the individuals investigated (Kloas et al., 2009). For example, increasing evidence suggests that changes of physico-chemical parameters of water such as pH (Ikuta and Kitamura, 1995; Zelennikov et al., 1999), temperature (Jin et al., 2009; Pankhurst et al., 1996; Watts et al., 2004), or dissolved oxygen (Thomas et al., 2007; Wu et al., 2003) can interfere with the reproductive function in fish. It is generally accepted that anthropogenic compounds in the aquatic environment are an important cause of ED in wildlife species but, nevertheless, the ecological relevance of ED is still discussed in a controversial manner (Mills and Chichester, 2005; Segner, 2005) and only a few studies link biomarker responses to actual effects on the population level (Ankley et al., 2008; Kidd et al., 2007; Miller

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[1] *Abbreviations used:* 11-KT, 11-ketotestosterone; AR, androgen receptor; Cyp19a, gonad-type aromatase; Cyp19b, brain-type aromatase; E2, 17β-oestradiol; EE2, ethinyloestradiol; ED, endocrine disruption; Esr, oestrogen receptor; FSH, follicle-stimulating hormone; GH, growth hormone; GnRH, gonadotropin-releasing hormone; IGF1, insulin-like growth factor 1; LH, luteinising hormone; T, testosterone; VTG, vitellogenin.

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and Ankley, 2004). One major challenge in ecotoxicology, therefore, is to place ED into context with other abiotic and biotic factors affecting wildlife populations.

An additional factor, mostly ignored in ecotoxicological studies, is the capability of certain parasites to modulate physiological processes of the host, resulting in a different sensitivity of infected individuals to environmental pollution (Sures, 2006, 2008). Moreover, pollution can affect the structure and abundance of parasite communities (Blanar et al., 2009). Given their ubiquity and pivotal role in ecosystems (Kuris et al., 2008), parasites have therefore to be considered in ecotoxicological studies as important parameters affecting host populations as well as the biomarker responses of hosts exposed to pollution. In the context of ED this becomes especially important when parasites affect the endocrine regulation of the host, in particular the reproductive system (Jobling and Tyler, 2003; Morley, 2006). Parasite-induced modulation of the host's reproductive system has been frequently documented in invertebrates and the physiological as well as ecological interactions are thoroughly investigated in some cases (Beckage, 1993; Hurd, 2001; Jong-Brink et al., 2001; Terry et al., 2004). In vertebrate hosts, similar observations have been studied rather occasionally (Arme, 1968; Azevedo et al., 2006; Fogelman et al., 2009; Morales-Montor et al., 2001; Sitja-Bobadilla, 2009).

In fish, the parasite *Ligula intestinalis* is known to cause reproductive dysfunction (Arme, 1968). This cestode is characterised by a complex life cycle involving three hosts with copepods as the first and fish as the second intermediate host. Final hosts are piscivorous birds (Dubinina, 1980). Plerocercoids (larval stages) of *L. intestinalis* are located in the body cavity of fish, most frequently cyprinids and, as a consequence, the gonads of infected individuals remain immature (Arme, 1968). Studies in bream (*Abramis brama*) along the river Elbe in Germany demonstrated significant impacts of *L. intestinalis* on common biomarkers for ED, including plasma levels of vitellogenin and sex steroids as well as brain aromatase activity (Hecker and Karbe, 2005; Hecker et al., 2007). Previous investigations on roach showed that arrested gametogenesis upon *L. intestinalis*-infection was accompanied by low mRNA expression of pituitary gonadotropin subunits (Carter et al., 2005; Trubiroha et al., 2009), indicating that gonadotropin-insufficiency is underlying depressed gametogenesis (Trubiroha et al., 2009). These results suggest that parasites can have serious implications for the significance of biomarkers associated with ED of the reproductive system. Thus, there is an urgent need to characterise the effects of parasitisation by *L. intestinalis* on endocrine parameters in fish.

The aim of the present study was to further our knowledge about the impacts of parasitisation by *L. intestinalis* on endocrine parameters and biomarkers of ED in roach, an important species for ecotoxicological field studies. We investigated the effects of infection on the sex steroids 17β-oestradiol (E2), testosterone (T), and 11-ketotestosterone (11-KT) in both genders and analysed the mRNA expression of key genes in liver and brain known to be involved in the regulation of reproduction and growth. Partial cDNA sequences of vitellogenin (VTG), oestrogen receptor 2a (Ers2a, formerly known as ERβ2), and insulin-like growth factor 1 (IGF1) were identified and real-time RT-PCR assays for these and further genes investigated were developed. Genes analysed in this study included receptors for sex steroids, namely androgen receptor (AR) and oestrogen receptors 1, 2a, 2b (Esr1, Esr2a, Esr2b; formerly known as ERα, ERβ2, ERβ1, respectively), the egg yolk protein precursor vitellogenin (VTG), and gonad-type (Cyp19a) as well as brain-type (Cyp19b) aromatase, the enzymes which catalyse the conversion of T to E2 (Payne and Hales, 2004). Furthermore, the expression of insulin-like growth factor 1 (IGF1) as an important factor mediating the interrelationship between growth and reproduction in fish (Davis et al., 2007) was analysed in liver. Previous investigation on the same individuals demonstrated that in both genders, infection prevented gonadal development accompanied by low pituitary expression of gonadotropin subunits (Trubiroha et al., 2009). To investigate whether gonadotropin-releasing hormones (GnRH) are involved in the impact on pituitary gonadotropins reported, the analysis of brain mRNAs encoding precursors for GnRH2 and GnRH3 (formerly known as chicken-type II GnRH and salmon-type GnRH, respectively) was included in the present study.

2. Materials and methods

2.1. Animals and sampling

Adult roach (3–6 years old) were caught by electro-fishing from Lake Mueggelsee (Berlin) in October 2007 when uninfected females and males underwent vitellogenesis and spermatogenesis, respectively. Investigated individuals (respectively, 12 uninfected and 12 infected roach of each sex) showed comparable size parameters as given in detail by Trubiroha et al. (2009). Blood and tissue samples were taken on the day of catchment. Blood was collected by puncture of the caudal vein using heparinised syringes and was subsequently centrifuged to obtain cell-free plasma. Tissue and plasma samples were stored at −80 °C until further processing.

2.2. Hormone assays

Plasma (100 μl) was extracted with 1 ml diethyl ether (Roth) in glass vials. After freezing of the aqueous phase at −80 °C, the organic phase was transferred to a new vial. The remaining aqueous phase was extracted once again. Obtained ether fractions were pooled and allowed to evaporate over night at room temperature. For steroid analysis, residuals were reconstituted in assay buffer (Cayman Chemicals) and concentrations of E2, T, and 11-KT were determined by specific enzyme-linked immunosorbent assays (ELISA) (Cayman Chemicals) according to the manufacturer's instructions. Absorption at 412 nm was measured in a 96-well plate reader (Infinite M200, Tecan).

2.3. RNA extraction and reverse transcription

RNA extraction and reverse transcription was conducted as described in Opitz et al. (2006) with some modifications. Briefly, total RNA from brain and liver was extracted by Trizol (Invitrogen). Content of total RNA was measured by UV absorption using a NanoDrop ND-1000 spectrophotometer (NanoDrop Products, Thermo Fisher Scientific) and subsequently 1 μg RNA was treated with 1 U DNAse I (Ampgrade, Invitrogen). For a subset of RNA samples, integrity was checked on a RNA 6000 Nano LabChip (Agilent Technologies) using the Agilent 2100 bioanalyser. RNA integrity numbers of these samples were >9 confirming high integrity of RNA (Fleige and Pfaffl, 2006). Total RNA (1 μg) was reverse transcribed using AMV reverse transcriptase (Finnzymes) as described in Opitz et al. (2006).

2.4. Gene expression analysis by real-time PCR

By applying a PCR approach based on primers directed against conserved regions of the respective genes, partial cDNA sequences of roach VTG, Esr2a, and IGF1 were amplified and directly sequenced using a CEQ 8800 Genetic Analysis System (Beckman Coulter) according to the manufacturer's protocol. Identity was confirmed by BLAST and multiple sequence alignments. Using gene specific primers (Table 1), real-time PCR assays were carried out in a Mx3005P qPCR Cycler (Stratagene) as described in Trubiroha

Table 1
Primers for real-time PCR.

Assay	NCBI Accession No.	Primer	5′–3′ Sequence	Amplicon size (bp)
AR	FJ769371	Sense Antisense	GAGCCCTCACTTGTGGAAGC TGAACGGAGCCGACCTCATC	226
Cyp19a	AB190291	Sense Antisense	AGGGCTACAGAGTGAAGAAAGG ACACGGAGAAACGAGACAAC	231
Cyp19b	AB190292	Sense Antisense	TGGCAGGCAGTTCTCATAC TTGCGAAGTCCGTCTCATC	166
Esr1	AB190289	Sense Antisense	TTAGGCGGCGAATCCAGCAG ACGAGGTGTCCAGGTAGTAG	124
Esr2a	GQ303561	Sense Antisense	AAAGAGGAGCACAGCAGAAG GATGCGAGCGATTAGTTC	155
Esr2b	AB190290	Sense Antisense	CATCTTCTCACCCGACCTC CGACTCTCCACACCTTCAG	210
GnRH2	U60668	Sense Antisense	GTCTAAGTGCCCAGTTTG GCATCCAGCAGTATTGTC	184
GnRH3	U60667	Sense Antisense	GGCGGAAAGAGAAGTGTTG CCTCGTCTGTTGGGAAATC	179
IGF1	GQ303562	Sense Antisense	CGAATGCTGCTTTCAGAG AGGAAGAGTGGCTATGTC	155
rpL8	FJ769335	Sense Antisense	ATCCCGAGACCAAGAAATCCAGAG CCAGCAACAACACCAACAACAG	94
VTG	EU930850	Sense Antisense	CCACCATTACACCTCCTCTC CACTCCCATCACAGCAAAG	131

AR, androgen receptor; Cyp19a, gonad-type aromatase; Cyp19b, brain-type aromatase; Esr1, oestrogen receptor 1; Esr2a, oestrogen receptor 2a; Esr2b, oestrogen receptor 2b; GnRH2, gonadotropin-releasing hormone 2; GnRH3, gonadotropin-releasing hormone 3; IGF1, insulin-like growth factor 1; rpL8, ribosomal protein L8; VTG; vitellogenin.

et al. (2009). Amplification efficiencies were determined by a dilution series of pooled liver or brain cDNA and ranged between 1.83 and 2.06 ($R^2 > 0.99$). Relative target transcript abundance was calculated by the comparative C_T method (Pfaffl, 2001). Expression of target genes was normalised to the level of ribosomal protein L8 mRNA, since expression of this gene was constant between genders and remained unchanged by infection in liver and brain, respectively. The expression of target genes is presented as mean ± SD relative to the value of uninfected females.

2.5. Statistical analysis

Data were log-transformed, if necessary, to meet the criteria of normal distribution and homogeneity of variances, and were analysed by ANOVA and post hoc Tukey–Kramer's multiple comparison test. All data are presented as means ± SD The level of statistical significance was set at $p < 0.05$. Statistical analyses were performed using the software package GraphPad Prism 4.03.

3. Results

3.1. Plasma sex steroid levels

Uninfected roach showed clear sex-specific differences in plasma levels of E2 and 11-KT. E2 levels were significantly higher in females compared to males, whereas 11-KT levels were higher in males than in females (Fig. 1). In uninfected roach, no significant differences in levels of T could be detected between genders. Infected roach of both genders had significantly lower levels of plasma E2 and 11-KT (Fig. 1) compared to uninfected conspecifics. In infected females, plasma levels of E2 and 11-KT reached only 8.1% and 27.3% of the levels of uninfected conspecifics. In infected males, E2 and 11-KT were only 30.3% and 7.0% of levels in uninfected conspecifics, respectively. Upon infection by *L. intestinalis*, plasma T was significantly decreased only in males (23.3% of levels of uninfected males).

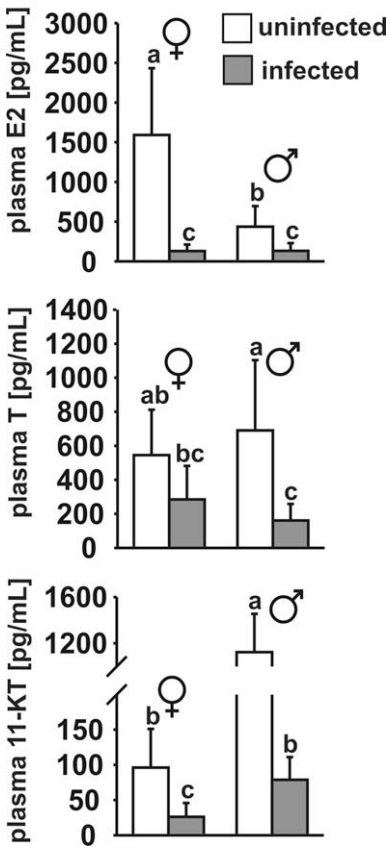

Fig. 1. Plasma levels of the sex steroids 17β-oestradiol (E2), testosterone (T), and 11-ketotestosterone (11-KT) in uninfected (white bars) and *Ligula intestinalis*-infected (grey bars) female and male roach. Bars represent means ± SD of 12 individual samples (10 samples for uninfected males). Different letters indicate significant differences ($p < 0.05$, Tukey's multiple comparison test).

3.2. Gene expression in liver

Partial cDNA sequences of roach VTG, Esr2a, and IGF1 were identified and submitted to GenBank™ (Table 1). Predicted amino-acid sequences showed highest similarities to the respective genes of *Pimephales promelas* (AF130354; 92.3%), *Oncorhynchus mykiss* (NM_001129985.1; 97.4%), and *Megalobrama terminalis* (AY247412.1; 97.8%), respectively.

Sex-specific differences in gene expression were detected in uninfected fish. Expression of VTG, Esr1, and AR mRNAs in the liver was significantly higher in uninfected females compared to uninfected males (Fig 2). Infection by *L. intestinalis* resulted in a significant decrease of liver expression of VTG and Esr1 in female roach. Similarly, expression of VTG was significantly lower in infected males. No significant difference could be detected concerning Esr1 mRNA between infected and uninfected males. In both genders, infection resulted in higher expression of Esr2a. A similar pattern was recorded for Esr2b although this was not significant in females. AR mRNA was significantly lower only in females infected by *L. intestinalis*. Analysis of liver IGF1 mRNA revealed significantly higher levels in infected females compared to uninfected individuals, but no significant effect of infection was detected in males.

3.3. Gene expression in brain

Analysis of GnRH2 and GnRH3 mRNAs in the brain did not reveal any impact of infection by *L. intestinalis* in females or in males. Still, infection had a significant impact on brain mRNA expression of Cyp19b in both genders whereas expression of Cyp19a mRNA was not changed in infected fish (Fig 3). In uninfected fish, expression of Cyp19a was significantly higher in males than in females. Expression of Ers1 was significantly reduced in infected males but not in females. Infection by *L. intestinalis* had no effect on mRNA expression of Esr2a, Esr2b, and AR in the brain of both genders.

4. Discussion

Parasites can induce a variety of physiological changes in their hosts. In particular the host's reproductive capacity is very commonly targeted (Hurd, 2001). However, this phenomenon has been rarely considered in field studies concerning endocrine disruption (ED) in fish and the endocrine status of infected individuals is only poorly understood (Hecker et al., 2007; Hecker and Karbe, 2005; Jobling and Tyler, 2003). Therefore, in the present study, several endocrine parameters associated with reproduction were analysed in wild-caught roach parasitised by the tapeworm *L. intestinalis*. As shown recently for these individuals, gonad development in infected roach was suppressed accompanied by considerably lower pituitary expression of FSHβ and LHβ in females and males, suggesting that decreased expression of gonadotropins causes the stagnation of gametogenesis (Trubiroha et al., 2009). In fish, gonadotropins regulate gametogenesis mainly via the induction of gonadal E2 and 11-KT which subsequently stimulate vitellogenesis in females and spermatogenesis in males (Lubzens et al., 2010; Schulz et al., 2010). Consistent with the low expression of gonadotropins and the arrested development of the gonads, in the present study, substantially reduced levels of E2 and 11-KT were detected in infected roach of both genders. The effect of infection on T was less pronounced, revealing a significant decrease only in males. Similarly in bream infected by *L. intestinalis*, T in both genders was less affected compared to E2 in females and 11-KT in males (Hecker and Karbe, 2005). As a consequence, it has to be expected that decreased expression of gonadotropins during infection leads more selectively to a suppression of the last step in gonadal steroidogenesis, namely the synthesis of E2 and 11-KT. It is now well accepted that oestrogens are indispensable hormones for testes development in male vertebrates, including fish (Schulz et al., 2010). Similarly in female fish, 11-KT has been reported to play a role in oocyte development in Atlantic cod (Kortner et al., 2009) and thus, the significant impact of parasitisation on E2 and 11-KT in both genders may contribute to impaired gametogenesis during parasitisation.

Consistent with lower plasma E2, expression of VTG mRNA in the liver was severely reduced in infected female roach. These findings are in agreement with investigations in bream, showing reduced plasma VTG in females infected by *L. intestinalis* (Hecker and Karbe, 2005). Furthermore, liver VTG mRNA was clearly quantifiable by real-time PCR ($C_T \leqslant 30$) also in male roach and became, concomitant with lowered plasma E2, significantly reduced during infection. Induction of plasma VTG as well as mRNA expression in

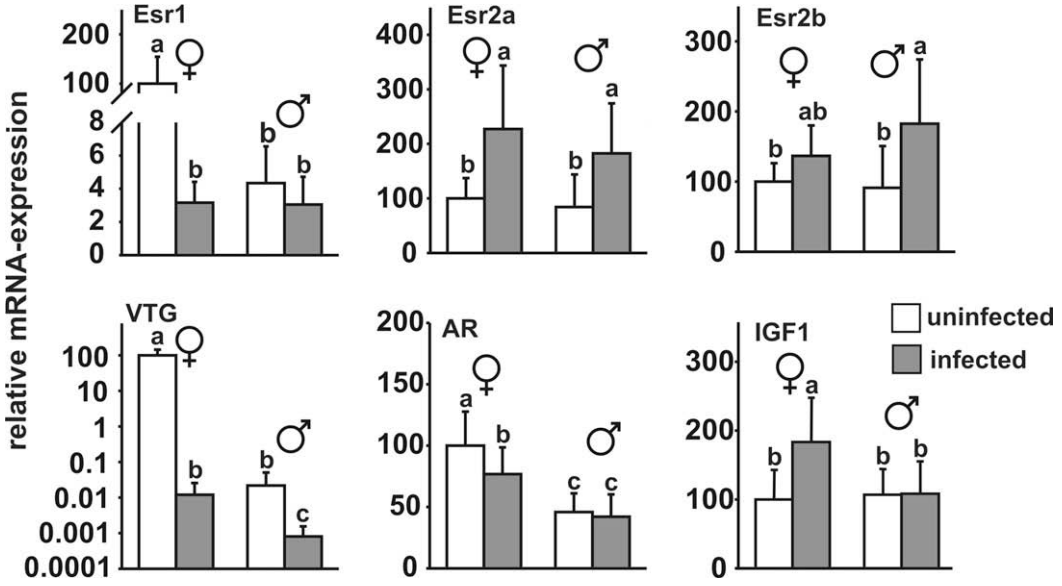

Fig. 2. Liver mRNA expression of vitellogenin (VTG), oestrogen receptors (Esr1, Esr2a, Esr2b), androgen receptor (AR), and insulin-like growth factor 1 (IGF1) in uninfected (white bars) and *Ligula intestinalis*-infected (grey bars) female and male roach. Bars represent means ± SD of 12 individual samples. Different letters indicate significant differences ($p < 0.05$, Tukey's multiple comparison test).

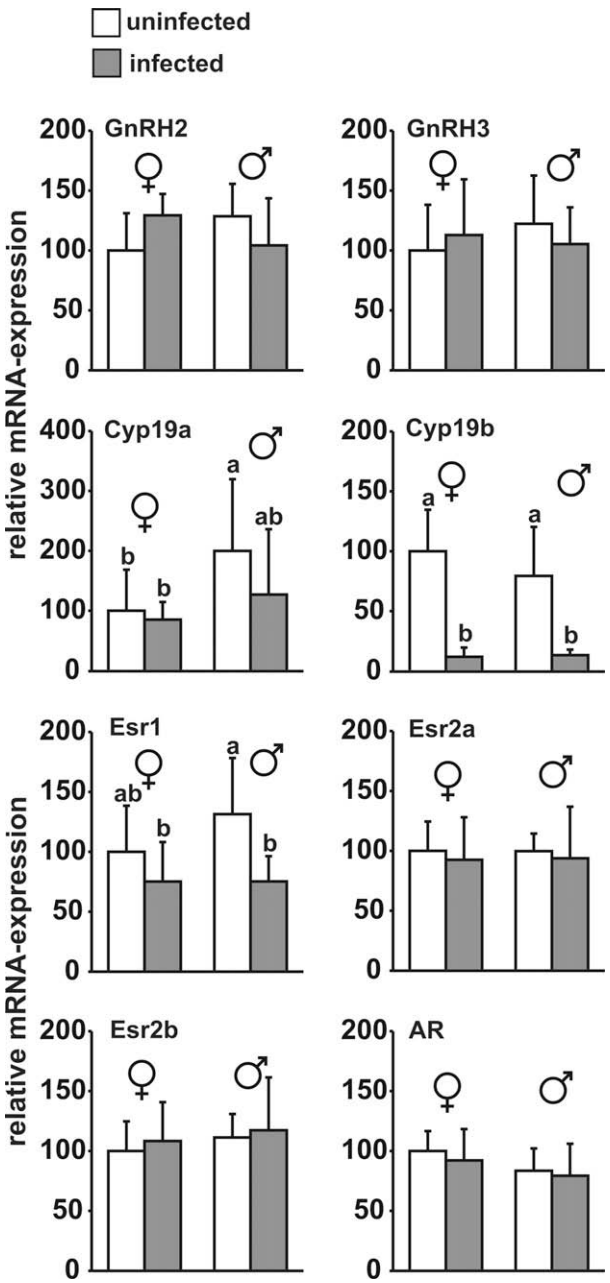

Fig. 3. Brain mRNA expression of gonadotropin-releasing hormones (GnRH2, GnRH3), aromatases (Cyp19a, Cyp19b), oestrogen receptors (Esr1, Esr2a, Esr2b), and androgen receptor (AR) in uninfected (white bars) and *Ligula intestinalis*-infected (grey bars) female and male roach. Bars represent means ± SD of 12 individual samples. Different letters indicate significant differences ($p < 0.05$, Tukey's multiple comparison test).

male fish is a widely used biomarker indicating exposure to oestrogenic compounds (Biales et al., 2007; Sumpter and Jobling, 1995). VTG has generally been claimed to be a female-specific protein but, nevertheless, an increasing amount of data show that VTG or its mRNA is also present at low levels in male fish which were not exposed to oestrogenic compounds (Bowman et al., 2000; Rodgers-Gray et al., 2001). As Lake Mueggelsee is located upstream of sewage discharging areas (Massmann et al., 2004), it seems very unlikely that the levels of VTG mRNA detected in male roach are caused by anthropogenic pollution. In addition, samplings of several hundreds of roach from Lake Mueggelsee (2006–2009) revealed only a negligible incidence of testicular oocytes (unpublished data). Since in roach, the occurrence of intersex phenomena has been demonstrated to be the best predictor of exposure to oestrogenic compounds (Jobling et al., 2006), this further confirms that low levels of hepatic VTG mRNA are constitutively expressed in male roach even at unpolluted sites. Recent findings show that in fish, VTG functions as a pattern recognition receptor and exhibits opsonic activity, suggesting that VTG plays a role in innate immunity of oviparous vertebrates independent of gender (Li et al., 2008; Liu et al., 2009). The strong reduction of liver VTG mRNA upon *L. intestinalis*-infection in male roach raises interesting questions about the possible physiological significance of decreased plasma VTG during parasitisation in male or juvenile fish.

Auto-induction of Esr1 mRNA has been reported in several fish species and this process is thought to be part of the oestrogen signalling pathway leading to hepatic vitellogenin-synthesis in oviparous vertebrates (Filby et al., 2006; Menuet et al., 2004; Sabo-Attwood et al., 2004). The hormonal regulation of oestrogen receptor mRNAs specifically in liver of roach has not been investigated so far but exposure of roach fry to ethinyloestradiol (EE2) similarly demonstrated the high sensitivity of Esr1 to oestrogenic compounds in this species (Katsu et al., 2007; Lange et al., 2008). Thus, the decreased expression of Esr1 in the liver of *L. intestinalis*-infected female roach concomitant with severely depressed VTG mRNA expression is most likely a result of the differences in E2 levels observed between infected and uninfected individuals. However, despite infected males also displayed significantly lower plasma E2 and liver VTG mRNA, no reduction of Esr1 expression in liver was observed. It is possible that different E2 threshold concentrations induce VTG and Esr1 mRNA expression in roach. In contrast to Esr1, differential effects of oestrogens on mRNA expression of Esr2a and Esr2b have been reported in fish (Filby et al., 2006; Menuet et al., 2004). In roach fry, the regulation of Esr2b mRNA by EE2 in body trunks was dependent on the developmental stage of exposed individuals (Lange et al., 2008). In the present study, liver expression of Esr2a was significantly higher in infected fish. Furthermore, Ers2b mRNA increased in males when infected by *L. intestinalis* and showed a similar trend in females. To our knowledge, expression of Esr2a has not been investigated in roach yet and it cannot be concluded whether liver Esr2a (and Esr2b in males) are regulated in response to changes in sex steroid levels or alternatively, as a consequence of parasitism. Similarly, the slight but significant suppressive effect of *L. intestinalis* on AR expression in liver of females is difficult to interpret but might also be a consequence of reduced E2 levels.

In addition to liver VTG, also the levels of Cyp19b mRNA in brain were strongly decreased in roach of both sexes when parasitised by *L. intestinalis*. Cyp19b is well known as oestrogen-inducible in fish due to the presence of oestrogen responsive elements (EREs) in the promoter region of this gene (Callard et al., 2001). Therefore, Cyp19b expression has been widely used as an oestrogenic marker in ecotoxicological studies using fish (Cheshenko et al., 2008). In contrast, the Cyp19a gene which is predominantly expressed in the gonads but also in the brain of teleosts does not contain EREs in the promoter region (Callard et al., 2001). Congruently, no effect of infection by *L. intestinalis* on Cyp19a mRNA in the brain of roach was detected in the present study. Despite major changes of sex steroid levels and in contrast to Cyp19b, brain expression of oestrogen and androgen receptors was not markedly affected by infection. Only in males, Esr1 mRNA was slightly reduced by *L. intestinalis*-infection. However, Esr1 mRNA tended to be lower also in infected females and differences in local expression were possibly masked due to the use of whole brains for expression analyses.

In vertebrates, growth and reproduction are tightly coupled via the endocrine system and the growth hormone/insulin-like growth factor (GH/IGF) axis in fish is thought to play a key role in co-ordinating energy allocation from somatic growth to reproduction

(Davis et al., 2007; Shved et al., 2007) and for the initiation of puberty (Campbell et al., 2003; Huang et al., 1998; Wuertz et al., 2007a,b). In fish, it is well established that starvation leads to a desensitisation of the liver towards GH, resulting in down-regulation of IGF1 mRNA expression and decreased plasma IGF1 concentrations (Duan, 1998; Fukada et al., 2004). Surprisingly, analysis of IGF1 mRNA expression in liver revealed no effect of *L. intestinalis*-infection in males and a slight but significant up-regulation in females. Albeit decreased glycogen and lipid stores have been reported in different species of parasitised cyprinids (Dubinina, 1980), the present results do not indicate that *L. intestinalis* leads to severe energy depletion of the host. This is supported by the fact that the infected individuals investigated in the present study showed only slightly reduced condition indices and no growth retardation (Trubiroha et al., 2009). Interestingly, down-regulation of IGF1 in liver by oestrogens has been demonstrated in fish and is thought to be part of the endocrine signalling pathway redirecting energy from somatic to reproductive tissues (Davis et al., 2007; Shved et al., 2007). Given the substantial differences in E2 and androgens between infected and uninfected fish as well as between genders, more pronounced effects on IGF1 mRNA would be expected. Obviously, further studies are needed to investigate the regulation of IGF1 by sex steroids in roach.

In accordance with low expression of gonadotropin subunits (Trubiroha et al., 2009), infected roach showed reduced levels of sex steroids and depressed gonadal development concomitant with down-regulation of oestrogen dependent genes. The mechanisms that underlie reduced gonadotropin expression, however, are still unknown. Thus, we compared the brain mRNA expression of gonadotropin-releasing hormone precursors (GnRH2 and GnRH3) between infected and uninfected roach. Based on its higher expression in the preoptic area and hypothalamus, GnRH3 is supposed to be the predominant hypophysiotropic form in roach (Penlington et al., 1997). Interestingly, neither for GnRH2 nor for GnRH3, any impact of infection was observed. Since GnRHs are widely present throughout the nervous system, effects of infection on neurons projecting to the pituitary were possibly masked due to the use of whole brains for expression analyses. Nevertheless, these results are rather surprising, given that significant changes in GnRH mRNAs as well as peptide levels were detected in other fish species by analysing whole brains of individuals throughout puberty, seasonal reproductive cycles, or after treatment with xeno-oestrogens (Filby et al., 2008; Gray et al., 2002; Vetillard and Bailhache, 2006). Notably, also immuno-histochemical studies on the brain GnRH-system could not demonstrate any difference between infected and uninfected roach (Williams et al., 1998). These findings may suggest an inhibitory mechanism of *L. intestinalis* at the pituitary level but further studies on hypophysiotropic neurons in distinct brain areas as well as on the pituitary GnRH content and responsiveness toward GnRH stimulation are clearly needed to elucidate the mechanism underlying reduced gonadotropin expression in infected roach.

In summary, we identified partial sequences of Esr2a, IGF1, and VTG in roach and developed real-time PCR assays for several key genes associated with reproduction and growth. This provides valuable molecular tools for ecotoxicological studies based on roach, a sentinel species for ED research. Most important, we documented that these biomarkers are highly impacted by parasitisation. The results presented here demonstrate severe effects of infection on levels of sex steroids, mRNA expression of their respective receptors (AR, Esr1, Esr2a, Esr2b), and on oestrogenic biomarkers such as VTG and Cyp19b in liver and brain, respectively. These recent data provide basic knowledge on the endocrine system of *L. intestinalis*-infected roach and demonstrate clearly that parasites can induce ED in fish. Accordingly, during field surveys the presence of the tapeworm *L. intestinalis* has to be considered as an important factor affecting gonadal development in fish. In this context, it should be noted that all endocrinological studies available so far were restricted to adult fish harbouring plerocercoids of considerable size, enabling a clear identification of parasitised individuals. New infections seem to occur particularly in prepubertal roach (Arme, 1968) and effects on biomarkers indicative for ED during the early development of *L. intestinalis* have not been characterised in any fish species. Given its widespread distribution (Stefka et al., 2009) and the considerable impact of *L. intestinalis* on fish populations (Kennedy et al., 2001), it remains to be investigated to which extent this parasite contributes as a natural source to ED in fish.

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Inhibition of gametogenesis by the cestode *Ligula intestinalis* in roach (*Rutilus rutilus*) is attenuated under laboratory conditions

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SUMMARY

Reproductive parameters of *Ligula intestinalis*-infected roach (*Rutilus rutilus*) which were held under long-tem laboratory conditions with unlimited food supply were investigated. Although uninfected and infected roach showed no difference in condition factor and both groups deposited perivisceral fat, the gonadosomatic-index was significantly lower in infected female and male roach. Quantitative histological analysis revealed that gonad development was retarded upon parasitization in both genders. In contrast to the phenotype described in the field, infected females were able to recruit follicles into secondary growth, but a high percentage of secondary growth follicles underwent atresia. In both genders, the histological data corresponded well with reduced expression of pituitary gonadotropins and lowered plasma concentrations of sex steroids, as revealed by real-time RT-PCR and ELISA, respectively. Furthermore, a reduction of vitellogenin mRNA and modulated expression of sex steroid receptors in the liver was demonstrated. Like in the field, there was a significant adverse impact of *L. intestinalis* on host reproductive physiology which could not be related to parasite burden. Our results show, for the first time, that maintenance under laboratory conditions can not abolish the deleterious effect of *L. intestinalis* on gametogenesis in roach, and indicate a specific inhibition of host reproduction by endocrine disruption.

Key words: parasite, tapeworm, host fecundity, parasitic castration, endocrine disruption, gonadotropin, sex steroids, oestrogen, androgen, vitellogenin.

INTRODUCTION

Reduction of the host's reproductive capacity is a common outcome of parasitic infections (Poulin, 2007). This phenotypic change may reflect non-adaptive side-effects of infection, defensive host adaptations to mitigate the impact of parasitism, or adaptive manipulations of the parasite to promote its own propagation (Ewald, 1980; Heins and Baker, 2003; Hurd, 2001, 2009; Lafferty and Kuris, 2009). In vertebrates, examples of reduced fecundity are best documented in fishes parasitized by larval cestodes (plerocercoids), namely in three-spined sticklebacks (*Gasterosteus aculeatus*) and in roach (*Rutilus rutilus*) infected by *Schistocephalus solidus* and *Ligula intestinalis*, respectively (Arme, 1997; Heins and Baker, 2008; Heins *et al*. 2010; Hoole *et al*. 2010). Both parasites are diphyllobothridean cestodes characterized by complex life cycles, involving a free-swimming coracidium, a procercoid in a copepod (first intermediate host), a plerocercoid in fish (second intermediate host), and an adult worm in piscivorous birds (final host) (Dubinina, 1980). Most of the parasite's growth takes place as a plerocercoid in the body cavity of a fish, and plerocercoids can attain a considerable size causing an energetic drain on the host (Dubinina, 1980; Barber *et al*. 2008). Despite striking similarities between *S. solidus* and *L. intestinalis* with regard to life cycle and host involvement, their effects on host reproduction differ. Both are generally considered as parasitic castrators which selectively target host reproductive energy and severely suppress host reproduction (Lafferty and Kuris, 2009). Impacts of *S. solidus* infection on stickleback reproduction involve delayed gametogenesis (Tierney *et al*. 1996; Heins and Brown-Peterson, 2010), reduced egg size (Heins and Baker, 2003), or decreased expression of secondary sexual characteristics and reduced courtship behaviour (MacNab *et al*.

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2009). There is increasing evidence that the effects of *S. solidus* infection on host reproduction are variable and correlated with the parasite load (Tierney *et al.* 1996; Bagamian *et al.* 2004; Heins and Baker, 2003, 2008; Heins *et al.* 2010; Heins and Brown-Peterson, 2010). These data are consistent with the hypothesis that simple nutrient depletion is the cause of the parasite's impact on host reproduction (Ewald, 1980; Hurd, 2001, 2009). In fact, Schultz *et al.* (2006) demonstrated no change in the reproductive effort (the proportion of energy devoted to reproduction) in response to *S. solidus*-infection in female sticklebacks and consequently concluded that reproductive curtailment is a side-effect of reduced host energy reserves caused by the demands of the parasite. In roach on the other hand, infection by *L. intestinalis* always seems to prevent host reproduction by inhibiting gonad development at an early stage of gametogenesis (Arme, 1997; Hoole *et al.* 2010), and it has been shown that parasitization disrupts the endocrine system controlling reproductive function (Kloas *et al.* 2009; Geraudie *et al.* 2010; Trubiroha *et al.* 2010). In particular, both genders of *L. intestinalis*-infected roach are characterized by a low expression of gonadotropins in the pituitary (Carter *et al.* 2005; Trubiroha *et al.* 2009). The gonadotropins, follicle-stimulating hormone (FSH) and luteinizing hormone (LH), are heterodimeric glycoprotein hormones that consist of a common glycoprotein-hormone α-subunit (αGSU) non-covalently linked to a specific β-subunit (FSHβ or LHβ) (Levavi-Shivan *et al.* 2010). Both are the key-hormones for reproduction in all vertebrates, regulating gonad development and the synthesis of sex steroids. FSH is considered to play a major stimulatory role during ovarian follicle growth and testicular spermatogenesis while LH is mainly involved in final gamete maturation (Lubzens *et al.* 2010; Schulz *et al.* 2010). Congruently, recent studies under laboratory conditions suggest the involvement of FSH rather than LH in mediating effects of parasitization by *L. intestinalis* early during gonad development in roach (Trubiroha *et al.* 2009). Further impacts of infection on reproductive physiology in roach comprise, for example, low plasma concentrations of the sex steroids 17β-oestradiol (E2), testosterone (T), and 11-ketotestosterone (11-KT) as well as decreased expression and plasma levels of vitellogenin (VTG), the hepatic precursor of yolk proteins (Geraudie *et al.* 2010; Trubiroha *et al.* 2010). The described endocrine disruption appears to become manifested in a density-independent manner (Arme, 1997; Hoole *et al.* 2010) which indicates adaptive host manipulation by *L. intestinalis*. Irrespective of these studies on host endocrinology, nothing is known about the role played by environmental factors in the curtailment of roach reproduction following *L. intestinalis*-infection. Scientists are becoming increasingly aware of the profound effects nutrition can have on the outcome of parasitic infections (Smith, 2007). In this context, laboratory studies allow controlling for variables such as food supply and can provide insights into the mechanisms and the evolutionary significance that underlie host phenotypes observed in the field.

The aim of the present study was to investigate whether long-term maintenance of roach under laboratory conditions with unlimited food supply can modulate or abolish the effects of parasitization by *L. intestinalis* on the reproductive physiology of its host. We investigated morphological features and reproductive parameters of *ad libitum*-fed infected and uninfected roach that were held in the laboratory for 2 years. The reproductive parameters studied comprise quantitative histological data on gonad development, plasma levels of the sex steroids E2, T, and 11-KT, as well as the expression of gonadotropin subunits (αGSU, FSHβ, LHβ) in the pituitary. Furthermore, the expression of hepatic VTG and of sex steroid receptors was investigated. The results are compared to field data from roach parasitized by *L. intestinalis*.

MATERIALS AND METHODS

Animals and sampling

In November/December 2006, roach were collected by electrofishing from Lake Grosser Mueggelsee (52° 260′ N, 13° 390′ E) (Berlin, Germany). Visual inspection revealed that several fish were parasitized by plerocercoids of *L. intestinalis*. Immediately after catching, mass and length of a subsample of 1+ and 2+ fish were recorded. The following morphological parameters (females and males pooled; mean ± S.D) were used as a basis for comparison of growth after 2 years of maintenance: uninfected $n = 12$; total length = 10·5 ± 1·0 cm; somatic mass = 8·78 ± 3·0 g; infected $n = 11$; total length = 11·2 ± 1·2 cm; somatic mass = 10·9 ± 4·0 g; parasitization index = 7·5 ± 4·1% (range 1·0–12·7%). Gonad histology showed that all fish were immature, except for 2 uninfected males that had entered spermatogenesis. Approximately 80 roach collected during this survey, including infected individuals, were transferred to the laboratory for investigation of the effects of *L. intestinalis* in fish receiving unrestricted food supply, in order to distinguish between direct effects of the tapeworm on gonad maturation and indirect effects caused by limited possibilities of food uptake. Roach were maintained in an aerated 1000 L tank under natural photoperiod and at a constant flow of tap water at 15 ± 2 °C temperature. Fish were fed daily *ad libitum* with commercial trout pellets (DAN-Ex 1750, Dana Feed) and living *Chaoberus* spp. larvae. Roach are classified as single-spawners, and gametogenesis is initiated in summer and proceeds throughout winter (Rinchard and Kestemont, 1996) with spawning occurring in late April/early May at Lake Mueggelsee (personal observation). Investigations of roach held in the laboratory

under the same conditions as described above showed that gametogenesis progressed in parallel to the situation in the field (personal observation). Spontaneous spawning in captivity, however, has not been observed.

After 2 years of maintenance, 59 remaining roach were sacrificed in November 2008. Blood was collected by puncture of the caudal vein using heparinized syringes and was centrifuged to obtain plasma. Liver tissue and pituitaries were removed for gene expression analyses. Tissue and plasma samples were stored at −80 °C until further processing. Upon sampling, fish total length, fish total mass as well as fish somatic mass (eviscerated bodies excluding parasite mass) were measured to the nearest 1 mm and 0·1 g, respectively. Roach gonads and parasites when present were weighed to the nearest 0·01 g using precision scales. The number of parasites per fish was also recorded. Fish condition factor was calculated as CF = fish somatic mass × 100/(fish total length)^3, and the gonadosomatic index as GSI = fish gonad mass/fish somatic mass × 100. Parasitization index was calculated as PI = parasite mass/fish somatic mass × 100. For histological analysis, samples of roach gonads were preserved in Bouin's fixative (Sigma) for 12 h, dehydrated in a graded series of ethanol and subsequently embedded in paraffin. Samples were sectioned at a thickness of 5 μm and stained with haematoxylin-eosin (H&E) or Goldner's modification of the Masson trichrome stain. All experimental procedures were conducted in compliance with the institutional guidelines for the care and use of animals.

Histological analysis of gonads

Gonads were analysed under an Axiovert 200 microscope (Zeiss) equipped with a Show View II digital camera (Olympus). Image analysis was performed using AnalySIS software (Soft Imaging Systems). Staging of roach gonads was conducted as described previously (Rinchard and Kestemont, 1996; Nolan *et al*. 2001). In females, the following stages of ovarian follicles were distinguished: primary growth (vacuole-free cytoplasm), early cortical alveolus (cortical alveoli occupy 1–3 rings in the cytoplasm), late cortical alveolus (cortical alveoli occupy more than 3 rings in the cytoplasm), vitellogenic (appearance of yolk globuli) and atretic. Ovaries were analysed based on the relative frequencies of follicles in the respective stage. Relative frequencies were calculated by dividing the number of follicles in a certain stage by the total number of follicles counted (a minimum of 100 follicles was counted per female). Early and late cortical alveolus, vitellogenic, as well as atretic follicles were summarized to be recruited into secondary growth and the percentage of follicles in secondary growth was calculated by dividing the number of secondary growth follicles by the total number of follicles. Furthermore, the ratio of atretic follicles to secondary growth follicles was calculated. For analysis of testes, pictures of 2 randomly selected fields of vision (200x magnification; area: 23,600 μm^2) were taken and the area occupied by cysts containing spermatogonia A or spermatogonia B was measured (no other germ cell stages were present). The area occupied by spermatogonia B is expressed as a percentage of the total area investigated.

Hormone assays

Blood plasma was extracted twice with diethyl ether (Roth) as described previously (Trubiroha *et al*. 2010) and concentrations of E2, T, and 11-KT were determined by specific enzyme-linked immunoassays (Cayman Chemicals) according to the manufacturer's instructions. Absorption at 412 nm was measured in a 96-well plate reader (Infinite M200, Tecan).

RNA extraction and reverse transcription

RNA extraction and reverse transcription was conducted as described previously (Trubiroha *et al*. 2009, 2010) with minor modifications. Briefly, total RNA from individual pituitary glands was extracted using the RNeasy Mini-Kit (Qiagen) including on-column treatment with DNase I (Qiagen). RNA from liver samples was extracted using Trizol (Invitrogen) followed by treatment with DNase I (AmpGrade; Invitrogen). The concentration of total RNA samples was measured by UV absorption using a NanoDrop ND-1000 spectrophotometer (NanoDrop Products, Thermo Fisher Scientific), and high integrity of the RNA was verified with an Agilent 2100 Bioanalyzer. Total RNA of pituitary (0·3 μg) and liver samples (1 μg) was reversely transcribed as reported previously (Trubiroha *et al*. 2009; 2010) using Affinity Script (Agilent) and AMV reverse transcriptase (Finnzymes), respectively.

Gene expression analysis by real-time PCR

Real-time PCR assays were carried out in an Mx3005P qPCR Cycler (Stratagene) using gene-specific primers as described previously (Trubiroha *et al*. 2009, 2010). Relative target transcript abundance was calculated by the comparative C_T method (Pfaffl, 2001) and was normalized to the expression of ribosomal protein L8 (rpL8). Expression of target genes is presented as mean ± S.D. relative to the expression value of uninfected females.

Statistical analysis

Normally distributed parameters (log transformed) of uninfected and infected roach of the same sex

Table 1. Morphological and parasitological features of the sampled roach

(Data are given as mean ± S.D. Range is shown in parentheses in the case of parasite abundance and PI. Significant differences between infected and uninfected roach of the same sex are marked by asterisks.)

		n	Total length [cm]	Somatic mass [g]	[a]CF	Parasite abundance	[b]PI
♀	Uninfected	26	16·3 ± 1·0	34·3 ± 6·6	0·79 ± 0·06	—	—
	Infected	9	18·5 ± 1·6*	53·0 ± 18·7*	0·81 ± 0·07	1·6 ± 1·1 (1–4)	10·1 ± 3·3 (6·1–15·6)
♂	Uninfected	12	15·4 ± 1·2	29·6 ± 7·8	0·79 ± 0·06	—	—
	Infected	12	17·7 ± 1·8*	46·5 ± 17·4*	0·80 ± 0·10	1·8 ± 0·8 (1–3)	12·1 ± 3·5 (6·0–19·0)

[a] CF, condition factor; [b] PI, parasitization index.

were analysed by Student's *t*-test, whereas not normally distributed data were evaluated using Mann-Whitney *U*-test. The level of statistical significance was set at $P < 0{\cdot}05$. Statistical analyses were performed using the software package SPSS 14.0.

RESULTS

Morphological and parasitological parameters

Summarized data on fish and parasites are given in Table 1. Length and somatic mass of infected female and male roach was significantly higher compared to their uninfected conspecifics (Table 1). No difference in CF was detected between uninfected and infected roach (Table 1) and both groups deposited perivisceral fat. PI of infected roach and number of parasites per fish was not significantly different between male and female roach. GSI was significantly lower in infected roach of both genders (Fig. 1).

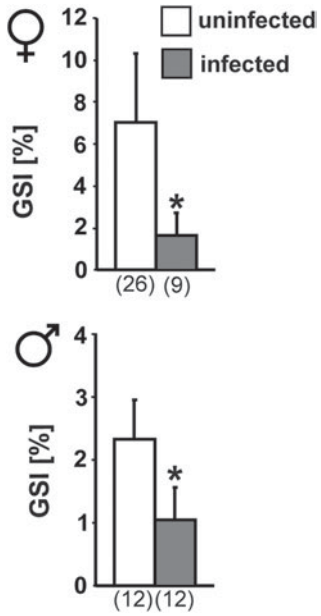

Fig. 1. Gonadosomatic index (GSI) of female and male roach, uninfected and infected by *Ligula intestinalis*. The number of individual samples is given in parentheses. Data are presented as mean ± S.D. Asterisks indicate significant differences between uninfected and infected roach of the same gender ($P < 0{\cdot}05$).

Gonad histology

Ovaries of uninfected female roach contained follicles in the primary growth stage and developing follicles in secondary growth. Vitellogenic follicles were the most advanced stages found in the ovaries of uninfected females (Fig. 2). Infected females were also able to recruit follicles into secondary growth with the most advanced follicles observed being in the late cortical alveolus stage. Ovaries of infected females contained a significantly higher percentage of follicles in the primary growth and in the early cortical alveolus stage compared to uninfected conspecifics, whereas no vitellogenic follicles were present (Figs 2 and 4). The relative frequency of follicles which were recruited into secondary growth was significantly higher in uninfected females (uninfected 23·2%, infected 13·2%) (Fig. 5). Both uninfected and infected females contained atretic follicles in their ovaries but the percentage of atretic follicles in relation to follicles in secondary growth was significantly higher in infected females (uninfected 13·6%, infected 33·7%) (Fig. 5). In males, testes of uninfected individuals comprised mainly spermatogonia B (90·8% of testes area) (Figs 3 and 4). In testes of infected males, spermatogonia B were also present but at a significantly lower percentage (56·4% of testes area) than in uninfected conspecifics (Figs 3 and 4).

Expression of gonadotropin subunits in the pituitary of roach

Analysis by real-time RT-PCR revealed significantly lower levels of FSHβ mRNA in *L. intestinalis*-infected roach of both genders (Fig. 6). In infected females and males, mRNA expression of FSHβ reached only 68·1% and 51·9% of levels in uninfected conspecifics, respectively. Parasitization also had a significant negative impact on the levels of LHβ and αGSU mRNA in females (60·8% and 72·0%, respectively, of levels in uninfected females), whereas in males no significant difference was detected in the expression of these two genes. Nevertheless, LHβ mRNA expression tended to be lower (54·0%; $P < 0{\cdot}06$) in infected male roach (Fig. 6).

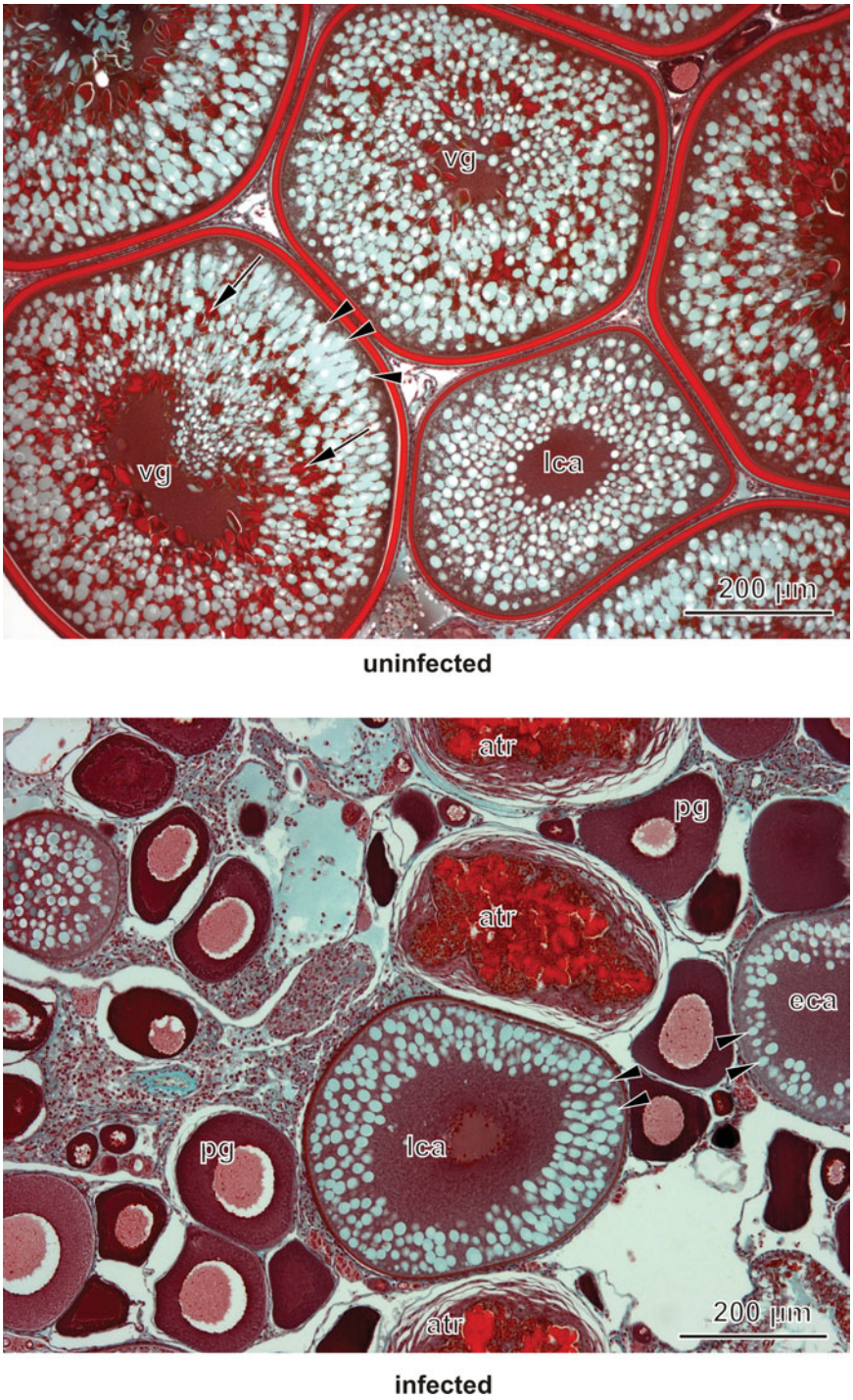

Fig. 2. Histological sections of ovaries of female roach uninfected and infected by *Ligula intestinalis*. Sections were cut at 5 μm thickness and stained with Goldners modification of Masson's trichrome stain to facilitate distinguishing between the carbohydrate-containing cortical alveoli (blue/green, arrowheads) from the yolk globules (red, arrows). atr, atretic follicle; eca, early cortical alveolus follicle; lca, late cortical alveolus follicle; pg, primary growth follicle; vg, vitellogenic follicle.

Plasma sex steroid levels

Infected females had significantly lower plasma concentrations of E2, T, and 11-KT, reaching only 16·9%, 18·4%, and 35·2% of the levels in uninfected conspecifics, respectively (Fig. 7). In males, only 11-KT was significantly reduced in infected fish (32·9% compared to uninfected males) but there was a trend toward lower levels of E2 (67·8%; $P<0·06$) and T (43·1%; $P<0·06$).

Expression of VTG and receptors for sex steroids in roach liver

The expression of VTG mRNA in the liver was significantly lower upon parasitization in both genders

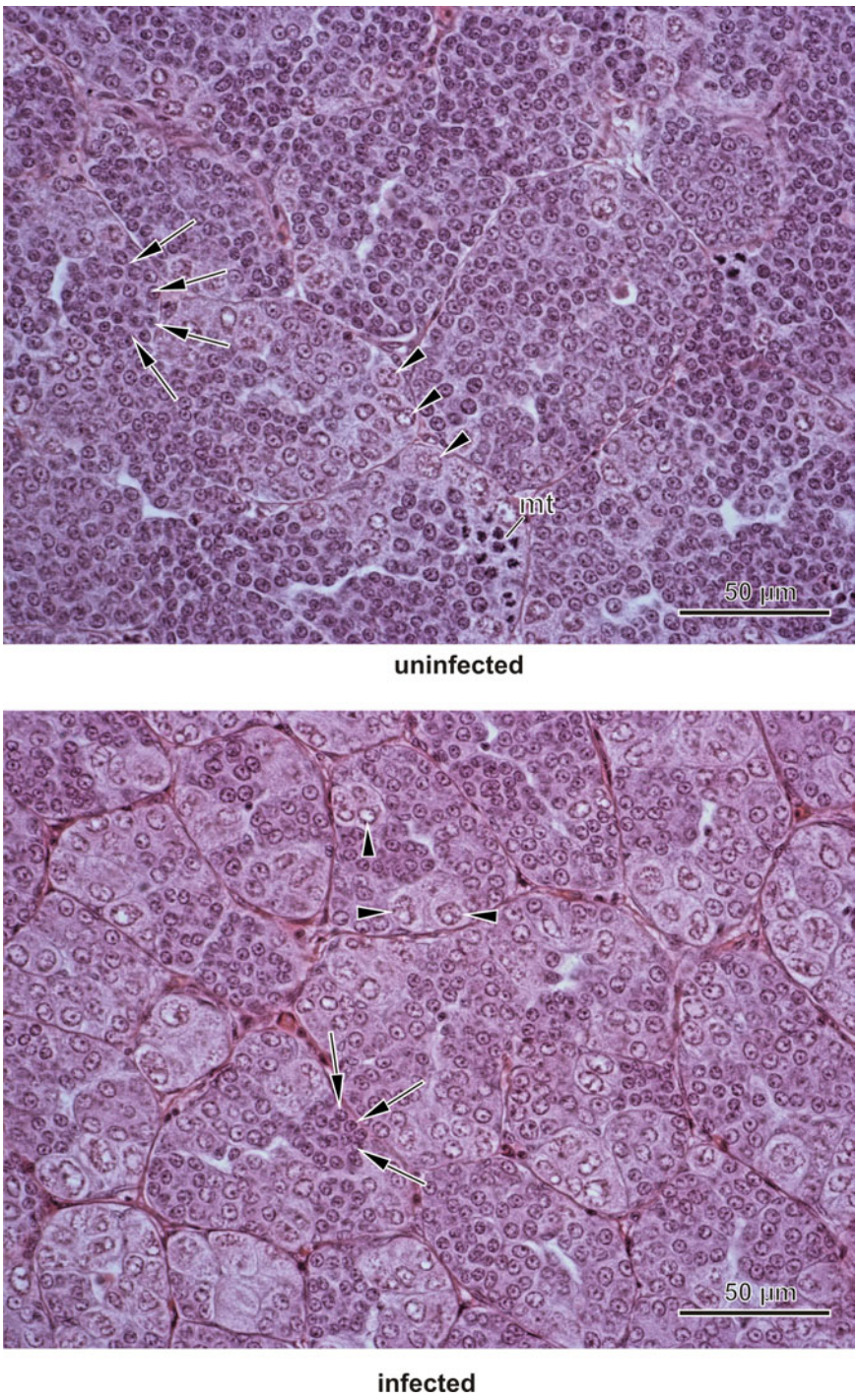

Fig. 3. Histological sections of testes of male roach uninfected and infected by *Ligula intestinalis*. Sections were cut at 5 μm thickness and stained by haematoxylin and eosin. mt, mitotic spermatogonia; arrows, spermatogonia B; arrow heads, spermatogonia A.

and reached 5·3% and 2·8% of levels in uninfected females and males, respectively (Fig. 8). Expression of sex steroid receptors was affected in a gender-specific manner by *L. intestinalis* infection. Compared to uninfected conspecifics, oestrogen receptor 1 (Esr1, synonymous to ERα) mRNA was significantly reduced in infected females (reaching 9·0% of levels in uninfected females) but not in males. Oestrogen receptor 2a (Esr2a, synonymous to ERβ2) mRNA expression was significantly higher (167%) in infected males but not in infected females compared to uninfected conspecifics. The liver expression of oestrogen receptor 2b (Esr2b, synonymous to ERβ1) and androgen receptor (AR) mRNA was not significantly different between uninfected and infected roach of both genders.

DISCUSSION

After long-term maintenance under laboratory conditions with unlimited food supply, infected roach of

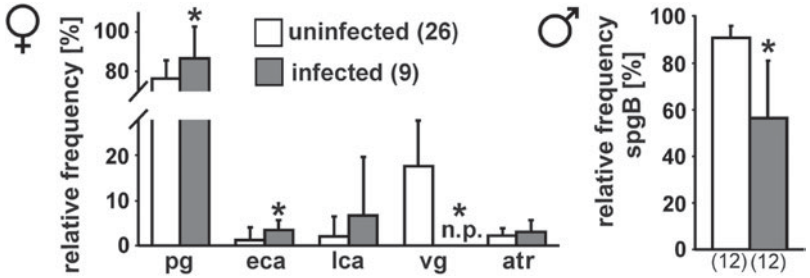

Fig. 4. Relative frequency of germ cell types in female and male roach uninfected and infected by *Ligula intestinalis*. The number of individual samples is given in parentheses. Data are presented as mean ± S.D. Asterisks indicate significant differences between uninfected and infected roach regarding the relative frequency of a certain cell type ($P < 0{\cdot}05$). Note that vitellogenic follicles were not present (n.p.) in infected females. atr, atretic follicles; eca, early cortical alveolus follicles; lca, late cortical alveolus follicles; pg, primary growth follicles; spgB, spermatogonia B; vg, vitellogenic follicles.

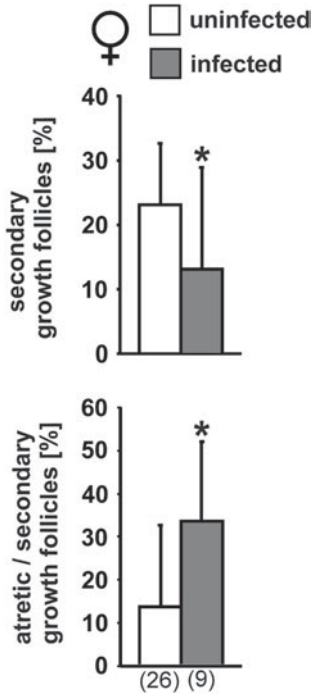

Fig. 5. Effects of parasitization by *Ligula intestinalis* on follicle recruitment into secondary growth and atresia of secondary follicles in female roach. Data are presented as mean ± S.D. The number of individual samples is given in parentheses. Asterisks indicate significant differences between uninfected and infected individuals ($P < 0{\cdot}05$).

both genders had lower GSI than their uninfected conspecifics. In females, this was a consequence of inhibited gonad development, reduced follicle recruitment into secondary growth and increased follicular atresia, whereas in males, the lower GSI values reflected inhibited spermatogonial proliferation. Although the negative effects of *L. intestinalis*-infection on gonadal growth and development in roach were significant, the present histological data are in contrast to observations under field conditions. Usually oogenesis is arrested in the primary growth stage in infected females (Arme, 1968; Arme and Owen, 1968; Trubiroha *et al*. 2009; Geraudie *et al*. 2010), and in infected males, spermatogonia B are more sparsely observed (occupying only about 20% of the testes area – unpublished data). Under field conditions, the gonads of roach parasitized by *L. intestinalis* resemble those of hypophysectomized fish. Based on molecular biological investigations, depressed gonadal growth and development in infected fish is thought to be a result of inhibited gonadotropin expression in the pituitary (Carter *et al*. 2005; Trubiroha *et al*. 2009). Similarly in the present study, the expression of pituitary gonadotropins was negatively affected by *L. intestinalis*. Nevertheless, the differences between uninfected and infected individuals in the present study were not as pronounced as under field conditions when mRNA expression of FSHβ and LHβ in infected roach did not exceed 10% and 30%, respectively, of the levels in uninfected conspecifics (Trubiroha *et al*. 2009). In teleosts, gonadotropins regulate important aspects of gametogenesis via the induction of gonadal E2 and 11-KT, in particular, vitellogenesis in females and spermatogenesis in males (Lubzens *et al*. 2010; Schulz *et al*. 2010). Consistent with the low expression of gonadotropins, significantly reduced plasma concentrations of sex steroids, namely E2 in females and 11-KT in males, were detected in infected roach in the present study. Similar observations have been made in bream (*Abramis brama*) (Hecker and Karbe, 2005) and roach (Geraudie *et al*. 2010; Trubiroha *et al*. 2010) under field conditions. Again, the negative effect of parasitization on sex steroids was less severe in the present study compared to previous field observations, where plasma concentrations of E2 in females and 11-KT in males reached only 7–8% of levels in uninfected conspecifics (Trubiroha *et al*. 2010).

In general, the expression of gonadotropin subunits in the pituitary and concentrations of sex steroids in plasma correlated well with the histological data, which showed a more progressed stage of gametogenesis in infected roach kept under long-term laboratory conditions compared to field observations.

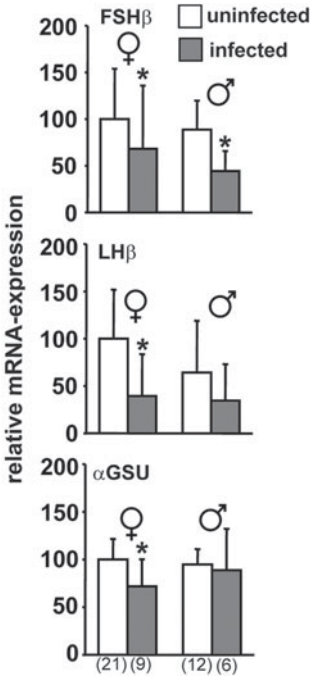

Fig. 6. Pituitary mRNA expression of follicle-stimulating hormone β-subunit (FSHβ), luteinizing hormone β-subunit (LHβ), and glycoprotein-hormone α-subunit (αGSU) in uninfected and *Ligula intestinalis*-infected female and male roach. Data are presented as mean ± S.D. The number of individual samples is given in parentheses. Asterisks indicate significant differences between uninfected and infected roach of the same gender ($P < 0{\cdot}05$).

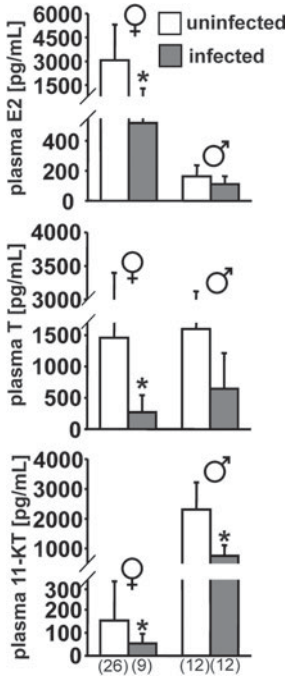

Fig. 7. Plasma levels of 17β-oestradiol (E2), testosterone (T), and 11-ketotestosterone (11-KT) in uninfected and *Ligula intestinalis*-infected female and male roach. Data are presented as mean ± S.D. The number of individual samples is given in parentheses. Asterisks indicate significant differences between uninfected and infected roach of the same gender ($P < 0{\cdot}05$).

Interestingly, the recruitment as well as the maintenance of secondary growth follicles was impaired in females infected by *L. intestinalis* in the present study. This might be a consequence of the lower expression of FSHβ and LHβ in these individuals, since FSH plays a key role in follicle recruitment, and gonadotropins in general constitute important anti-apoptotic/anti-atretic factors in the ovary (Wood and Van Der Kraak, 2002; Lubzens *et al*. 2010). It should be noted here that mature oocytes and sperm have been reported recently in a small number of roach infected by *L. intestinalis* during the spawning season, even under field conditions (Geraudie *et al*. 2010). The reason for this exceptional observation remains unknown but these data together with the present results show that the phenotype of *L. intestinalis*-infected roach with regard to reproductive physiology can vary under certain circumstances.

Investigations on gene expression in the liver demonstrated significantly lower mRNA levels of VTG in parasitized roach of both genders compared to uninfected conspecifics. In infected females, this is most probably a result of their lower E2 plasma concentrations, since in oviparous vertebrates hepatic VTG synthesis is induced by oestrogens (Wahli, 1988). Our findings are in agreement with field data for female bream (Hecker and Karbe, 2005) and roach (Geraudie *et al*. 2010; Trubiroha *et al*. 2010). Similar to previous findings in the field (Trubiroha *et al*. 2010), we measured considerably lower levels of liver VTG mRNA in infected male roach in the present study. Interestingly, plasma E2 concentrations were not significantly lower in infected compared to uninfected males after long-term maintenance under laboratory conditions. This indicates an effect of *L. intestinalis* on liver VTG mRNA expression in males which is probably not related to oestrogen levels. Lower VTG mRNA in infected males could, nevertheless, also be a consequence of their reduced plasma concentrations of androgens, since VTG induction by exposure to low concentrations of methyltestosterone has been demonstrated in male zebrafish (*Danio rerio*) (Andersen *et al*. 2006). Furthermore, VTG has been shown to be involved in immune function in fish (Li *et al*. 2008) and it would be interesting to address whether reduced VTG expression in infected roach is potentially associated with immune interactions between *L. intestinalis* and its host.

Oestrogen-induced synthesis of hepatic VTG in oviparous vertebrates is mediated via nuclear oestrogen receptors. Recent studies in rainbow trout (*Oncorhynchus mykiss*) (Leanos-Castaneda and Van Der Kraak, 2007) and goldfish (*Carassius auratus auratus*) (Nelson and Habibi, 2010) showed that the Esr2 subtypes are crucial for vitellogenesis in fishes,

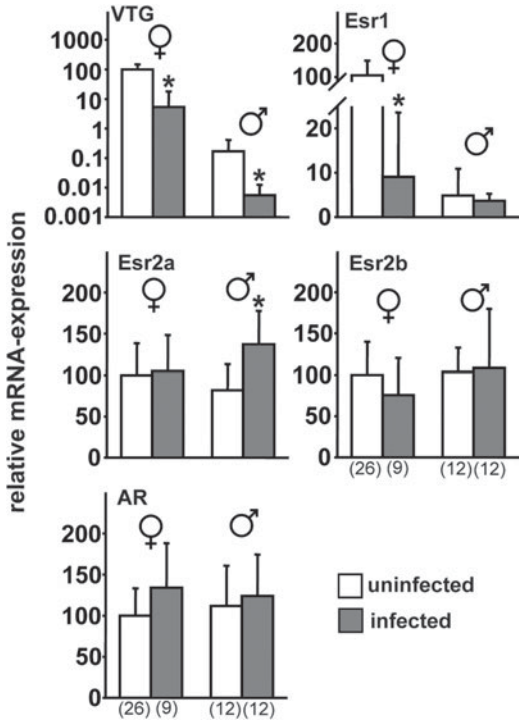

Fig. 8. Liver mRNA expression of oestrogen receptors (Esr1, Esr2a, and Esr2b), androgen receptor (AR), and vitellogenin (VTG) in uninfected and *Ligula intestinalis*-infected female and male roach. Data are presented as mean ± S.D. The number of individual samples is given in parentheses. Asterisks indicate significant differences between uninfected and infected roach of the same gender ($P < 0{\cdot}05$).

with Esr1 being auto-induced via Esr2 to sensitize the liver to further stimulation of VTG synthesis by oestrogens. Auto-induction of Esr1 mRNA has been consistently documented in teleosts, including roach, whereas the regulation of Esr2 subtypes by oestrogens is more complex and may depend on species, sex, and reproductive stage (Menuet *et al*. 2004; Filby and Tyler, 2005; Katsu *et al*. 2007; Lange *et al*. 2008). Thus, in accordance with a previous field investigation (Trubiroha *et al*. 2010), the lower mRNA expression of hepatic Esr1 in infected female roach in the present study is likely to be a result of their lower plasma E2 compared to uninfected conspecifics. In contrast to Esr1 mRNA, the expression of Esr2a, Esr2b, and AR differed in some cases from the situation described in the field where hepatic Esr2a mRNA in both genders (and Esr2b mRNA in males) was higher in infected individuals and AR mRNA was down-regulated in response to parasitism in females (Trubiroha *et al*. 2010). Nevertheless, we detected a higher expression of Esr2a in infected males also in the present study. Whether this is a result of changes in plasma sex steroids upon infection or a response to other parasite factors remains yet unknown.

Differences in the outcome of parasitism on host reproduction between field and laboratory studies have been reported in several host-parasite systems, including the three-spined stickleback infected by plerocercoids of *S. solidus* (Candolin and Voigt, 2001; Barber and Svensson, 2003). Under favourable laboratory conditions, no effect of *S. solidus* on reproduction in male sticklebacks was observed (Candolin and Voigt, 2001). In experimentally infected females, Barber and Svensson (2003) even reported an increase in GSI compared to uninfected conspecifics. On the other hand, we demonstrate here for the first time that long-term maintenance under laboratory conditions with unlimited food access can attenuate but not abolish the negative impact of parasitization by *L. intestinalis* on reproductive physiology in roach. In the present study, CF did not differ significantly between infected and uninfected individuals and perivisceral fat was abundant in both groups, showing that long-term energy reserves were deposited. As described for the three-spined stickleback infected by *S. solidus*, reproductive parameters of the host should be negatively related to parasite burden if inhibition of gametogenesis is a simple side effect of nutrient deprivation (Hurd, 2001; Hall *et al*. 2007; Heins and Baker, 2008). In roach parasitized by *L. intestinalis* however, even small plerocercoids that are unlikely to pose significant energetic demands cause an arrest of host gametogenesis (Arme, 1968; Arme and Owen, 1968). Consistent with previous investigations (Arme, 1968; Arme and Owen, 1968; Trubiroha *et al*. 2009), no negative correlation was detected between PI and any of the reproductive parameters in roach after long-term maintenance under laboratory conditions (data not shown). Therefore, it is concluded that the observed impact of *L. intestinalis* on roach gametogenesis is not due to energetic drains upon parasitization but reflects a specific strategy of the parasite, which is manipulating the allocation pattern of host energy away from reproduction (Hurd, 2001, 2009; Lafferty and Kuris, 2009). Such a strategy seems advantageous for the parasite because the high energy costs required for reproduction of roach might then be available for the growing plerocercoid. This would concomitantly minimize the harm exerted on host somatic tissues due to general nutritional drains, thereby avoiding a decrease in host viability (Hurd, 2001; Ebert *et al*. 2004; Lafferty and Kuris, 2009). In addition, preventing host reproduction may provide the temporal storage of energy reallocated from reproduction already during early infection before the parasite can fully exploit these resources. If the energy liberated from reproduction is higher than the demands of the parasite plus the energetic costs for the host in mounting an immune defence, this in turn can lead to enhanced growth (gigantism) of the infected host under certain circumstances (Ebert *et al*. 2004; Hall *et al*. 2007). In the present study, data relevant in this context were not collected and therefore no conclusions about the influence of *L. intestinalis* on the growth of the investigated roach can be drawn.

Gametogenesis in fish is triggered by body energy stores, and the pituitary gonadotropins as well as gonadal steroidogenesis are influenced by metabolic hormones (e.g. Campbell *et al*. 2006). Given the large biomass *L. intestinalis* can attain relative to the host, it seems possible that the reproductive dysfunction of infected roach under field conditions is in part mediated by energetic drains, acting in concert with the specific endocrine-disrupting effects of the parasite. Thus, unlimited food supply was possibly a predominant factor for the more advanced gonadal development of infected roach in the laboratory as compared to the field. Notwithstanding the experimental feeding regime, changes in additional confounding variables under laboratory conditions are potentially involved. For example, roach are known to release steroid hormones into the water (Lower *et al*. 2004) and the action of pheromones in fishes is not restricted only to the final reproductive events such as synchronization of spawning (Van Weerd *et al*. 1991; Huertas *et al*. 2006). Fishes infected with *L. intestinalis* do not exhibit normal shoaling patterns (Orr, 1966; Loot *et al*. 2001) and thus, pheromonal stimuli that would normally be received within the group-shoaling situation are probably absent from parasitized individuals. Under the present conditions, i.e. simultaneous maintenance of infected and uninfected fish in one tank, infected roach could also inevitably receive pheromonal stimuli from their uninfected conspecifics, which in turn might stimulate gametogenesis in parasitized individuals to some degree. However, nothing is known about the responsiveness of infected roach towards pheromonal/hormonal stimuli and further studies involving exposure experiments would help to get a better picture of the state of the endocrine system in roach parasitized by *L. intestinalis*.

In summary, we characterized for the first time the influence of *L. intestinalis* on reproductive physiology of roach kept for a long time under laboratory conditions with unlimited food supply. Effects of parasitism involved inhibited gametogenesis in both genders of roach, accompanied by reduced expression of pituitary gonadotropin subunits and lowered plasma concentrations of sex steroids. Furthermore, a strong negative impact of parasitization in both genders on hepatic VTG mRNA was observed. In general, the effect of *L. intestinalis* on reproductive physiology appeared attenuated under laboratory conditions compared to the phenotype found in the wild. Still, there was a clear deleterious impact of parasitization on host gametogenesis despite the fact that infected individuals were apparently in a good condition as indicated by CF and the deposition of perivisceral fat. Our observation is in contrast to studies on the related stickleback-*S. solidus* host-parasite system, where the negative impact of parasitism on host reproduction has been documented to be a side-effect of nutritional drains and can become abolished under favourable conditions. It is concluded here that arrested gametogenesis in roach upon infection by *L. intestinalis* in the wild is mediated only in part by environmental factors or nutritional drains of parasitism. The present results support the hypothesis that the cestode *L. intestinalis* selectively inhibits roach reproduction via endocrine disruption.

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Nutritional status and gene expression along the somatotropic axis in roach (*Rutilus rutilus*) infected with the tapeworm *Ligula intestinalis*

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ABSTRACT

The tapeworm *Ligula intestinalis* inhibits gametogenesis of its fish host, the roach (*Rutilus rutilus*). We investigated whether *L. intestinalis* infection makes significant demands on nutritional resources and consequently manipulates the endocrine somatotropic axis of roach. Two groups of naturally infected and uninfected roach were studied: a field group (natural feeding) and a laboratory group (*ad libitum* food supply). In females, no significant impact of parasitization on storage substrates (glycogen, lipids, and protein) was detected, whereas in males, either lipid content of the liver (field group) or lipid of the muscle and glycogen of the liver (laboratory group) were slightly decreased. Except for the females of the field group, higher mRNA expression of growth hormone (*gh*) in the pituitary of infected fish was observed. Furthermore, the expression of hypophyseal somatolactin α and β (*slα*, *slβ*) was up-regulated in infected females of the field and laboratory group, respectively. In liver and muscle, mRNA expression of insulin-like growth factors (*igf1*, *igf2*) and *igf* receptor (*igfr*) remained either unchanged or were up-regulated with infection. Parasitization showed inconsistent effects on *gh* receptor 1 (*ghr1*) expression in liver and muscle, whereas *ghr2* mRNA was mostly not influenced by infection. In general, the expression profile of genes involved in the somatotropic axis as well as the content of storage substances in infected roach did not resemble that of food-deprived fish either under natural or *ad libitum* feeding. In conclusion, the present study does not indicate starvation of *L. intestinalis* infected roach, and it is suggested that the inhibition of reproduction attenuated the nutritional demand of parasitization.

1. Introduction

Ligula intestinalis is a common and widespread diphyllobothridean cestode with a life cycle comprising three hosts. Definitive hosts are fish-eating birds in which the parasite reaches sexual maturity. Eggs released into the water with the bird's feces hatch to produce free-swimming coracidia which are eaten by copepods, the first intermediate host. The parasite develops into a procercoid that is trophically transmitted to fish, mainly cyprinids [13,28]. In the fish, the second intermediate host, *L. intestinalis* develops into a plerocercoid which grows rapidly within the body cavity, usually reaching 10–20% of the host mass. Upon consumption by a piscivorous bird the life cycle is completed [13]. The most remarkable outcome of *L. intestinalis* infection in fish is inhibition of host reproduction [2,72]. In both sexes of infected fish, gonads remain in an immature state, irrespective of season and fish age [1,2]. This effect of parasitism is reported to be independent of the number or size of parasites, and even small plerocercoids, which are unlikely to pose a significant energy demand on the host, are reported to impair gametogenesis [1,28]. Thus, *L. intestinalis* represents a parasitic castrator that gains energy by eliminating host reproduction by shifting energy allocation in its host away from reproduction [21,35,71]. This strategy minimizes harm to its host caused by general nutritional drain due to parasitism. In this way, impairment of the host's and parasite's survival can be avoided [35]. Recent studies have demonstrated that inhibition of gametogenesis in the *L. intestinalis*-infected roach (*Rutilus rutilus*) was accompanied by a pronounced disruption of the hypothalamus–pituitary–gonad (HPG) axis, the prime endocrine system regulating reproduction. In particular, the expression of gonadotropins in the pituitary, as well as plasma concentrations of sex-steroids, were lower in infected fish than in uninfected [9,72,73].

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The HPG-axis is interlinked with other endocrine systems, jointly synchronizing reproduction and determining the reproductive outcome. It is well known that activation of the HPG-axis, and hence reproductive capacity, is gated by metabolic and nutritional factors [16,61]. In fish the somatotropic axis, basically comprising growth hormone (GH), insulin-like growth factors (IGF1, 2, and 3), and their corresponding receptors and binding proteins [57,58], plays a key role in integrating body growth and condition with reproductive events [79]. For example, in salmonids [3,20,39] and European eel (*Anguilla anguilla*) [29], IGF1 has been shown to increase gonadotropin expression and release from the pituitary. Additionally, the paracrine IGF-system is suggested to play an important role in fish gonad development [58,64,80].

Growth hormone, a pituitary protein hormone, is fundamental to development and differentiation processes, normal somatic growth, and reproduction of vertebrates, and also modulates important metabolic processes [8]. The effects of GH are mediated via endocrine (liver-derived) or paracrine (locally produced) IGF1, though GH also displays IGF1-independent action [7,8,46,61]. In most bony fish, there is evidence that IGF2 is also controlled by GH [51,66], but its role has not been fully elucidated [59]. Furthermore, GH synthesis and secretion is modulated via a negative feedback loop involving endocrine IGF1, i.e. high blood levels of IGF1 lead to decreased synthesis and secretion of GH in the pituitary [57]. In addition, somatolactin (SL), a fish-specific member of the GH/prolactin peptide hormone family [42], regulates metabolic processes along with GH [6,75]. In fish, two distinct forms of SL have been described: SLα, which is present in all fish species studied so far and SLβ, which has been found in some species, including cyprinids such as roach [6]. Although their physiological role is not fully understood, several potential functions have been suggested, including involvement in gametogenesis, calcium regulation, stress response, control of lipolysis and energy mobilization, acid–base regulation, and background adaptation [6,12,19]. The physiological actions of GH and SL are mediated by corresponding receptors in the target tissues. Two distinct phylogenetic clades of fish growth hormone receptors (GHR), GHR1 and GHR2, have been described, involving differential biological functions during fasting and starvation [32,54,63]. Somatolactin receptors (SLRs) have not been identified, but it has been suggested that GHR1 is the SLR, whereas GHR2 has been attributed to GHR [18]. However, this hypothesis remains controversial, since functional studies e.g. by Chen et al. [11] have demonstrated that the two SLs of zebrafish (*Danio rerio*) show only negligible affinity to both GHR1 and GHR2 and do not activate these receptors, indicating that GHR1 is unlikely to be the SLR in this species. Similar results were also reported in Japanese eel (*Anguilla japonica*) [48] and rainbow trout (*Oncorhynchus mykiss*) [56].

Given that *L. intestinalis* affects the allocation of host resources between reproductive and somatic tissues, and since reproduction, growth, and metabolism are coordinated by the endocrine system, it is hypothesized here that infection by *L. intestinalis* also modifies the somatotropic axis of infected fish. The aim of the present study was to characterize the nutritional status of *L. intestinalis*-infected roach and to test whether parasitization is associated with changes in the endocrine somatotropic axis. We compared the main nutritional resources (glycogen, lipid, and protein) in liver and muscle and the mRNA expression of *gh*, *sl*α, and *sl*β in the pituitary as well as the expression of *ghr1*, *ghr2*, *igf1*, *igf2*, and *igfr* in liver and muscle between groups of uninfected and infected roach that were expected to differ in nutritional status. The first investigated group was sampled directly from the field, and the second was maintained under long-term laboratory conditions, receiving *ad libitum* feeding.

2. Materials and methods

2.1. Experimental system and sampling

Two groups including both infected and uninfected roach were examined: a field group with fish sampled immediately upon capture from a lake, and a group of fish that were transferred to the laboratory after capture and reared for 2 years under ideal nutritional conditions (*ad libitum* feeding). A detailed description of the experimental conditions is given in [71,73]. In brief, roach were caught in Lake Grosser Mueggelsee (Berlin, Germany) in October 2007 (field group) and in November/December 2006 (laboratory group). Field group consisted of 12 uninfected and 12 *L. intestinalis* infected adult fish of each sex (3–6 years of age). In infected fish, gonad development was retarded in both sexes [73]. The laboratory group comprised roach caught as juveniles (1+/2+ years of age) and subsequently reared under laboratory conditions [71]. Fish received *ad libitum* feeding and were kept under a natural photoperiod at water temperature of 15 ± 2 °C. Sampling was conducted after 2 years (12 infected and 12 uninfected males, and 26 uninfected and 9 infected females). Gonad development was retarded in both sexes of infected fish, although this was not as pronounced as in the field group [71].

Upon sampling, the following biometric and parasitological parameters were measured: fish total length (to the nearest mm); total and somatic (=carcass) wet mass of the fish; and parasite wet mass (to the nearest 0.1 g). Condition factor of the fish was calculated as CF = fish carcass (=somatic) mass × 100/(fish total length)^3 and parasitization index was calculated as PI = (parasite mass/fish somatic mass) × 100. Both parameters refer to somatic mass instead of total weight with regard to the focus of the study on somatic growth. Pituitary, liver, and muscle tissue were sampled for gene expression and biochemical analyzes and stored at −80 °C. In the laboratory group, blood was collected from the caudal vein with heparinised syringes, and plasma was separated by centrifugation (10,000*g*, 5 min) and stored at −80 °C.

2.2. Biochemical analysis of liver and muscle

Determination of glycogen content in liver tissue was carried out as described in [33] with slight modifications according to [26]. Briefly, liver tissue was homogenized under liquid nitrogen, extracted by 0.2 M perchloric acid (PCA), and neutralized by $KHCO_3$. An aliquot of 100 µL was incubated with glucoamylase at 40 °C for 2 h. After addition of 0.6 M PCA and neutralization with 5 M KOH, 20 µL aliquots of samples were mixed with 200 µL glucose-6-phosphate dehydrogenase solution containing 20 mM TRA buffer [1 mM ATP, 0.5 mM NADP, 5 mM $MgCl_2$] in microtitre plates. Absorbance was measured at 340 nm before and after addition of 1 unit hexokinase per well with a microplate reader (Infinite M200, Tecan, Männedorf, Switzerland) and corrected for background glucose.

Protein was extracted from liver and muscle samples as described by Munro and Fleck [43] and concentrations were determined according to the Bradford method with a commercial protein assay (Bio-Rad) using bovine serum albumin (BSA) as a standard.

Total lipids were determined by the sulphophospho-vanillin method according to Zoellner and Kirsch [81] with modifications according to Saborowski and Buchholz [62]. Briefly, upon homogenization with a mortar under liquid nitrogen, 1 mL of a chloroform:methanol solution (1:2 v/v) was added to 20–50 mg liver or 100 mg muscle. The tubes were sonicated in a water bath, incubated for 30 min (16 h for muscle) at room temperature and centrifuged for 10 min at 15,000*g*. The supernatant (50 µL for liver,

Table 1
Primers used for RT-qPCR. *rpl8*, ribosomal protein L8; *igf1*, insulin-like growth factor 1; *igf2*, insulin-like growth factor 2; *igfr*, igf receptor; *gh*, growth hormone; *ghr1*, growth hormone receptor 1; *ghr2*, growth hormone receptor 2; slα, somatolactin alpha; slβ, somatolactin beta; S, sense; As, antisense; bp, base pairs.

Target gene	NCBI Accession	Primer	5′–3′ Sequence	Amplicon size (bp)
rpl8	FJ769335	S	ATCCCGAGACCAAGAAATCCAGAG	94
		As	CCAGCAACAACACCAACAACAG	
igf1	GQ303562	S	CGAATGCTGCTTTCAGAG	155
		As	AGGAAGAGTGGCTATGTC	
igf2	JN656973	S	TTCAGCCACATCCCTACAG	171
		As	TGCCGCCTAAACTTCTTG	
igfr	JN656976	S	GCGACTTCTGGGTGTGGTTTC	186
		As	TATGCCATCCCGTCCGCTATC	
gh	JN656972	S	AGCGGAGCCGTCTCAAACAG	152
		As	AAAGGCAGCGGTAGGGAGTC	
ghr1	JN656974	S	CTGCTCACTGACGAAGAGTATG	205
		As	TCTGCTGCTGGGAGAAGATG	
ghr2	JN656975	S	CTGGAGAATGGCAAAGAC	189
		As	ACCAACAGCAGAGAAGAC	
slα	JN656977	S	TGTTTGTCCCGTATCCTC	125
		As	GTGAAGCAGCCATTTGTC	
slβ	JN656978	S	CCTTCACTCTGTCCTGATTC	96
		As	AGGGCACTTGAGGTGTTC	

and 500 μL for muscle) was transferred to glass reaction tubes and evaporated until dry. Two milliliters of concentrated sulfuric acid were added to the reaction tubes and incubated for 10 min at 100 °C in a water bath. After cooling to room temperature, 20 μL of this solution was transferred to microplates, 300 μL of phospho-vanillin reagent (11.9 M phosphoric acid, 8 mM vanillin) were added, and the absorbance was read at 530 nm in a microplate reader (Infinite M200, Tecan).

2.3. Biochemical analysis of plasma

Plasma glucose and triglycerides were determined by the GPO–PAP method using an enzymatic colorimetric kit (Greiner, Frickenhausen, Germany) and calculated with a standard dilution series. Plasma protein was quantified according to Bradford with a Roti-Quant kit and a BSA standard dilution series. All colorimetric assays were measured in duplicate with a microplate reader (Infinite M200, Tecan). Recoveries were determined by spike experiments and were >90% for all plasma parameters analyzed [74].

2.4. RNA extraction and reverse transcription

RNA extraction and reverse transcription (RT) of liver, muscle, and pituitary samples were conducted according to Trubiroha et al. [72,73]. In brief, muscle and liver samples were extracted using Trizol (Invitrogen) followed by treatment with DNase I (AmpGrade; Invitrogen). Pituitary samples were extracted with a RNeasy Mini kit (Qiagen) including on-column digestion with RNase-free DNase I (Qiagen) according to the manufacturer's instructions.

The concentration of total RNA samples was measured by UV absorption using a NanoDrop ND-1000 spectrophotometer (NanoDrop Products, Thermo Fisher Scientific) and by Ribogreen RNA-quantitation kit (Invitrogen), and high integrity of the RNA was verified with an Agilent 2100 Bioanalyzer in a subset of samples. Total RNA was reverse transcribed using AMV reverse transcriptase (Finnzymes) for liver and muscle samples and MMLV transcriptase (Affinity Script, Invitrogen) for pituitary samples as described previously [72,73].

2.5. PCR-based gene identification and analysis of gene expression by RT-qPCR

Primers directed to conserved regions of the target genes were used for the qPCR based identification of roach *igf2*, *igfr*, *gh*, *ghr1*, *ghr2*, *slα*, and *slβ*. PCR products were identified by direct sequencing using a CEQ 8800 Genetic Analysis System (Beckman Coulter) according to the manufacturer's protocol. Their identity was confirmed by BLAST and multiple sequence alignments. New sequence information was submitted to the GenBank™ database (see supplement).

Gene-specific primers (Table 1) for roach *rpl8, gh, ghr1, ghr2, igf1, igf2, ifgr*, *slα*, and *slβ* were used for relative RT-qPCR quantification in a Stratagene Mx3005p qPCR cycler. Specificity of amplicons was confirmed by direct sequencing. PCR efficiencies ranged from 1.81 to 1.98 ($R^2 > 0.99$) (Table 1). Relative values of target transcript abundance in individual samples were determined by the comparative C_t method [52]. Expression of target genes was normalized to *rpl8* mRNA and is presented as mean ± SD relative to the expression value of uninfected females.

2.6. Statistical analysis

Differences in studied parameters between uninfected and infected roach were analyzed by analysis of covariance (categorical variable: infection, covariate: somatic mass) followed either by post hoc Tukey HSD (honest significant difference) test or by Unequal N HSD test when the number of samples differed among groups. When the conditions for using ANCOVA were not satisfied (normal distribution and homoscedasticity) the data were log transformed. Data are presented as mean ± standard deviation (SD). Sexes were assessed separately and differences were considered statistically significant at $p < 0.05$. Statistical analyzes were performed using the software Statistica v. 9 (StatSoft, Czech Republic).

3. Results

3.1. Morphological parameters

In the field group, infected and uninfected individuals exhibited comparable size parameters, but condition factor was significantly lower in infected fish (Table 2). In contrast, in the laboratory group, infected individuals of both sexes showed higher somatic mass and total length compared to their uninfected counterparts. Furthermore, no difference in condition factor was observed between uninfected and infected individuals (Table 2). Parasitization index of infected roach was comparable ($p > 0.05$) in male and female fish of all groups (Table 2).

3.2. Storage substrates in the liver and muscle

The results of biochemical analysis of glycogen, lipid, and protein of liver and muscle in the field and laboratory groups were inconsistent. In the field group, lower lipid content and higher protein content were detected in the liver of infected males compared to uninfected conspecifics (Table 3). In the laboratory group, infected males showed lower glycogen content in the liver as well as lower lipid content in the muscle compared to uninfected males (Table 3). No effect of parasitization on energy stores was observed in females in either group.

3.3. Glucose, triglycerides, and total protein content in the blood plasma

No significant effect of infection on plasma glucose, triglycerides, and total protein was detected in the laboratory group (Table 4). Individual variation was relatively high as indicated by high standard deviation.

Table 2
Morphological features and parasitization index of infected and uninfected roach of both sexes. Values are expressed as mean ± SD. Asterisks indicate significant differences between uninfected and infected fish of the same sex.

		n	TL (cm)	*w* (g)	CF	PI
Field group[a]						
♀	Uninfected	12	15.0 ± 0.9	29.8 ± 6.2	0.88 ± 0.10	–
	Infected	12	15.3 ± 1.8	28.6 ± 10.1	0.78 ± 0.08*	9.7 ± 3.1
♂	Uninfected	12	15.3 ± 2.6	32.3 ± 17.1	0.82 ± 0.05	–
	Infected	12	13.9 ± 1.0	20.2 ± 4.9	0.74 ± 0.05*	11.3 ± 3.4
Laboratory group[b]						
♀	Uninfected	26	16.3 ± 1.0	34.3 ± 6.6	0.79 ± 0.06	–
	Infected	9	18.5 ± 1.6*	53.0 ± 18.7*	0.81 ± 0.07	10.1 ± 3.3
♂	Uninfected	12	15.4 ± 1.2	29.6 ± 7.8	0.79 ± 0.06	–
	Infected	12	17.7 ± 1.8*	46.5 ± 17.4*	0.80 ± 0.10	12.1 ± 3.5

n, number of fish; TL, total length; w, somatic mass; CF, condition factor; PI, parasitization index.
[a] Trubiroha et al. [73].
[b] Trubiroha et al. [71].

3.4. Gene expression

3.4.1. Pituitary

In the laboratory group, mRNA expression of *gh* was significantly higher in infected fish of both sexes compared to uninfected. In the field group *gh* mRNA was slightly, though significantly, increased in infected males, but not in females (Fig. 1). mRNA expression of *slα* was significantly up-regulated only in infected females of the field group, and expression of *slß* was increased only in females in the laboratory group (Fig. 1). However, *slα* and *slβ* mRNA tended to be higher, although not significantly, in infected individuals in general. *Slα* and *slβ* mRNA expression exhibited high individual variation.

3.4.2. Liver

Infected females of the field group had significantly higher mRNA expression of *igf1*, *igf2*, *igfr*, and *ghr1* in the liver compared to uninfected counterparts, but no changes in hepatic gene expression with parasitization were observed in males from the field (Fig. 2).

In females of the laboratory group, no effect of *L. intestinalis* parasitization was observed on the hepatic expression of any of the investigated genes. In males, only the expression of *ghr1* mRNA was significantly down-regulated compared to uninfected counterparts (Fig. 2).

3.4.3. Muscle

No changes in gene expression in the muscle were observed in parasitized males with the exception of an up-regulation of *ghr2* in infected males in the laboratory group (Fig. 3). Females in the field group exhibited significantly higher mRNA expression of *ifg1* compared to uninfected counterparts (Fig. 3).

4. Discussion

Although the roach investigated in the present study harbored plerocercoids of considerable size, the impact of *L. intestinalis*-infection on the main storage substrates of its host was slight and restricted to males. In females, no differences in any of the

Table 3
Content of storage substrates (glycogen, lipid, and protein) in the liver and muscle tissue of infected and uninfected roach of both sexes. Values are expressed as mean ± SD (*n* = 8 samples in every case). Asterisks indicate significant differences between uninfected and infected fish of the same sex.

Sex/infection status		Liver			Muscle	
		Glycogen (mg g^{-1})	Lipid (mg g^{-1})	Protein (mg g^{-1})	Lipid (mg g^{-1})	Protein (mg g^{-1})
Field group						
♀	Uninfected	45.0 ± 25.2	41.2 ± 13.8	123.3 ± 26.0	9.6 ± 2.0	148.8 ± 9.2
	Infected	59.1 ± 27.6	33.3 ± 8.4	113.7 ± 15.1	10.6 ± 2.7	141.2 ± 17.6
♂	Uninfected	62.3 ± 30.5	45.9 ± 9.8	104.8 ± 20.4	10.7 ± 2.0	151.5 ± 18.5
	Infected	55.2 ± 30.2	28.2 ± 2.0*	133.7 ± 14.4*	8.8 ± 1.7	156.6 ± 11.2
Laboratory group						
♀	Uninfected	67.7 ± 37.3	52.3 ± 15.4	139.6 ± 24.0	32.4 ± 0.1	166.2 ± 18.8
	Infected	62.4 ± 21.1	41.1 ± 9.0	139.0 ± 24.3	26.8 ± 4.4	176.3 ± 9.0
♂	Uninfected	110.3 ± 12.6	43.5 ± 5.7	110.6 ± 26.4	28.8 ± 4.0	172.6 ± 11.1
	Infected	65.8 ± 35.1*	43.6 ± 9.5	128.8 ± 30.8	21.8 ± 4.2*	165.8 ± 25.5

Table 4
Content of glucose, triglycerides, and protein in the blood plasma of infected and uninfected roach of both sexes from the laboratory group. Values are expressed as mean ± SD. *n*, number of samples.

Sex/infection status		*n*	Glucose (mmol L^{-1})	Triglycerides (g L^{-1})	Total protein (g L^{-1})
♀	Uninfected	26	3.8 ± 0.9	7.4 ± 2.1	28.4 ± 8.0
	Infected	9	4.2 ± 0.7	7.4 ± 3.2	21.0 ± 7.9
♂	Uninfected	12	3.9 ± 0.6	8.4 ± 2.3	19.1 ± 6.9
	Infected	11	3.8 ± 1.0	7.6 ± 3.8	19.6 ± 9.3

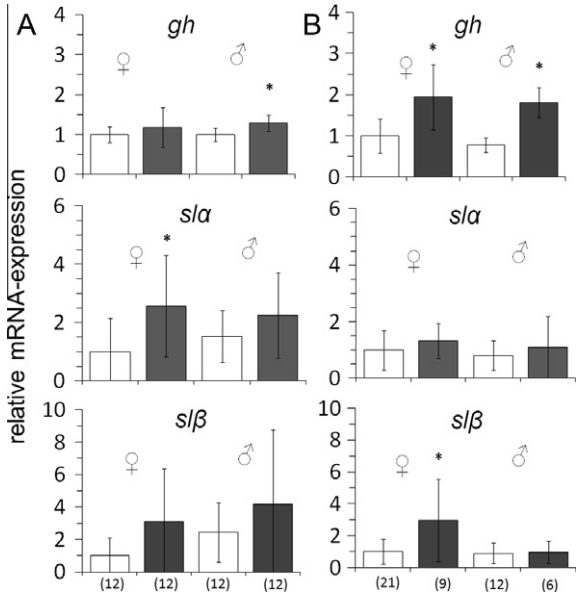

Fig. 1. mRNA expression of growth hormone (*gh*), somatolactin α and β (*slα* and *slβ*) in the pituitary of infected (black bars) and uninfected (white bars) roach in the field group (A) and the laboratory group (B). Number of samples is given in brackets. Asterisks indicate significant differences between uninfected and infected roach of the same sex.

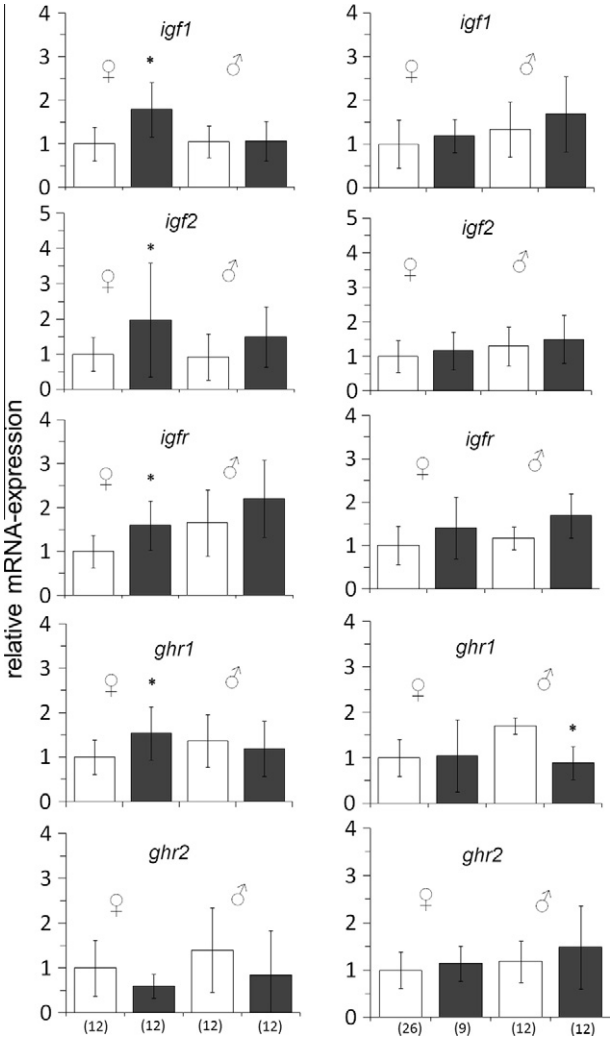

Fig. 2. mRNA expression of insulin-like growth factors (*igf1*, *igf2*), *igf* receptor (*igfr*), growth hormone receptor 1, and 2 (*ghr1*, *ghr2*) in the liver of infected (black bars) and uninfected (white bars) roach in the field (A) and the laboratory group (B). Number of samples is given in brackets. Asterisks indicate significant differences between uninfected and infected roach of the same sex.

storage substrates were detected between infected and uninfected fish. Infected males of the field group showed lower hepatic content of lipids, and males of the laboratory group had lower glycogen and lipid content in liver and muscle, respectively, compared to uninfected counterparts. Although the content of liver glycogen in infected males of the laboratory group was lower than in uninfected counterparts, it was comparable to that of uninfected males in the field, and the value fell well into the commonly observed range of 1–6% (w/w) [44]. Furthermore, under laboratory conditions, the muscle lipid content of infected roach of both sexes was higher than that of uninfected fish in the field, most likely due to the *ad libitum* feeding regime. Therefore, the present data do not reflect starvation in *L. intestinalis*-infected roach under laboratory conditions by any means and suggest minor changes in the nutritional status of infected males from the field. This is also in agreement with the absence of any impact of parasitization on the plasma parameters assessed (glucose, triglycerides, and total protein) in infected fish of the laboratory group. To the best of our knowledge, no data on storage substrates in *L. intestinalis*-infected fish under laboratory conditions are available, and there are only a few fragmentary studies of fish caught in the field. For instance, Kosareva [34] reported 34–73% decreased liver glycogen in infected bream (*Abramis brama* L.) compared to uninfected individuals, but lipid content was not determined. Shpolyanskaya [68] detected a reduction of 34% and 42% in the body fat of sunbleak (*Leucaspius delineatus*) and roach (*R. rutilus*), respectively, but glycogen content was not determined. In both studies only low numbers of infected individuals were available. Harris and Wheeler [23] also commented on a decrease of fat reserves in *L. intestinalis*-infected bleak (*Alburnus alburnus*) without giving any details. None of the studies cited above distinguished between sexes, and only one focused on roach [68]. In this case whole body fat was analyzed, while we differentiated between liver and muscle. Thus, future studies should characterize the nutritional impact of *L. intestinalis* on various host tissues and with consideration to fish sex.

Based on biochemical parameters, the effects of *L. intestinalis* on the nutritional status of roach appeared mild. Given that parasitization severely inhibited gametogenesis in the roach investigated, it is suggested that the nutritional demands of *L. intestinalis* were predominantly satisfied by resources which normally would have been incorporated into the host gonads. If we accept this hypothesis, the sex-specific effects of *L. intestinalis* on the content of the main storage substrates found under field as well as laboratory conditions could be explained by the fact that inhibition of gametogenesis releases more energy/resources in females than in males. Sex-specific effects of *L. intestinalis*-infection were also reported by Szalai et al. [70] in spottail shiner (*Notropis hudsonius*). Male spottail shiner appeared to be more sensitive than females towards the nutritional stress of parasitization, as indicated by reduced growth rates. It is known that females of several salmonid species mobilize more carbohydrates from the muscle and viscera during gonad maturation than males [60]. Moreover, Idler and Bitners [30] found that the lipid reserves transferred to the gonads of sockeye salmon (*Oncorhynchus nerka*) are approximately 16 times higher in females than in males. In the present study, the inhibition of gametogenesis in both sexes seemed to release enough energy to overcome (in females) or at least to minimize (in males) the nutritional drains exerted by parasitization. Otherwise, parasitization should have resulted in a much more pronounced depletion of

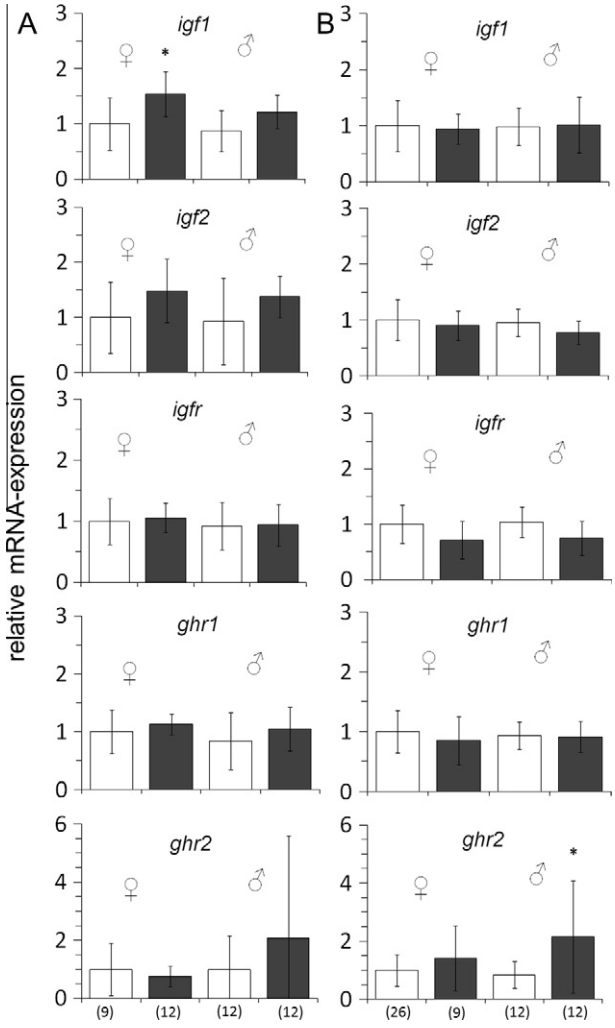

Fig. 3. mRNA expression of insulin-like growth factors (*igf1*, *igf2*), *igf* receptor (*igfr*), growth hormone receptor 1, and 2 (*ghr1*, *ghr2*) in the muscle of infected (black bars) and uninfected (white bars) roach in the field (A) and the laboratory group (B). Number of samples is given in brackets. Asterisks indicate significant differences between uninfected and infected roach of the same sex.

the main storage substrates, as has been demonstrated in the three-spined stickleback (*Gasterosteus aculeatus*) infected by plerocercoids of *Schistocephalus solidus*, a cestode related to *L. intestinalis* that seems not to specifically interfere with host reproduction [24,25,65]. Nonetheless, we cannot rule out the possibility that infected roach additionally compensated for parasite-induced loss of resources by, for example, increased food intake, increased food conversion efficiency, or reduced activity. For technical reasons, food intake and activity of roach was not measured in the present study. To investigate the effects of *L. intestinalis* on energetics of roach, laboratory infections and experiments such as those reported for the three-spined stickleback parasitized by *S. solidus* are necessary [4].

Due to their important role in the regulation of somatic growth and nutrient metabolism in fish [41,57,58,77], the expression of key genes associated with the somatotropic axis was assessed in the pituitary, liver, and muscle in the laboratory and field study. During food-deprivation, pituitary *gh* mRNA usually increases, but the expression of *igf1*, both at hepatic and extra-hepatic sites, declines [57]. This seems to be caused by GH resistance developed during food restriction [17,40,50,57], i.e. mRNA expression of hepatic *ghrs* is down-regulated and/or capacity of GH binding is reduced [45,54,57]. In the present study, except for infected females from the field group, pituitary mRNA expression of *gh* was higher in infected roach compared to their uninfected conspecifics, but contrary to what is expected for starved fish, infected roach displayed either unchanged or significantly higher levels of *igf1* and *igf2* mRNA in liver and muscle. Furthermore, hepatic expression of *ghr2* mRNA was not affected by *L. intestinalis*, and occasionally, *ghr2* mRNA was slightly elevated in the muscle of infected males of the laboratory group. Congruently, the data on pituitary *sl*(*s*) mRNA did not indicate starvation of roach infected by *L. intestinalis*. The expression of *sl*(*s*) mRNA in the pituitary is usually down-regulated during food-deprivation [36,75]; whereas, in the present study, mRNA levels of both *slα* and *slβ* were either increased or remained unchanged in infected individuals. The pattern of *ghr1* (putative *slr*) mRNA expression in the liver was inconsistent among the groups tested here, and contradictory information on the effect of fasting can be also found in the literature [17,45,54]. Nonetheless, as a general conclusion, the pattern of gene expression along the somatotropic axis did not indicate starvation of roach infected by *L. intestinalis*, which corresponds well with the results on storage substrates.

Parasitic castration is frequently associated with gigantism (enhanced growth) of the host, because when castration frees more host resources than the parasite currently requires, the host channels some of the excess energy and resources into its somatic growth [5,15,21,35,69]. As both GH and IGF1 have growth promoting effects [46], and SL is supposed to be involved in energy mobilization and in helping to expedite growth [12], it could be assumed that the *L. intestinalis*-infected roach investigated in the present study might have had a higher growth rate than their uninfected conspecifics at the time of sampling. Notably in the laboratory group, the significantly higher length and somatic mass of infected female and male roach compared to their uninfected conspecifics indicate that these infected fish had grown faster. However, plasma levels of the above-mentioned hormones are not necessarily correlated with their mRNA expression, and data on the growth rate of the investigated fish were not collected. So far, the phenomenon of gigantism due to parasitism by *L. intestinalis* has been reported in only a single roach population [38], and there are many studies reporting opposite effects of *L. intestinalis* in fish [22,31,55]. All of these studies rely on scale analysis and back-calculated growth rates. Particularly in older fish, results obtained in this way can be inaccurate, since the age at which the host became infected is difficult to assess. To resolve the question of under what circumstances *L. intestinalis* promotes growth of infected fish, laboratory experiments with individually tagged fish would have to be carried out.

Direct manipulation of the somatotropic axis has been reported by plerocercoids of the diphyllobothridean tapeworm *Spirometra mansonoides*. This parasite stimulates the growth of its second intermediary host (which could be any vertebrate except fish) by releasing a so-called plerocercoid growth factor, a cysteine proteinase with GH agonistic activity [53]. However, it seems unlikely that *L. intestinalis* secretes a growth factor similar to that of *S. mansonoides*. If this were the case in roach, a decrease in pituitary *gh* and an increase in hepatic *igf1* mRNA expression would be expected [53]. Most importantly, an obvious increase in the growth rate of infected fish should be apparent in more than just one roach population [37]. Nonetheless, the mechanisms and proximate clues leading to alterations along the somatotropic axis in *L. intestinalis*-infected roach reported for the first time in the present study are an interesting area for further investigation. For example, inhibited reproduction in parasitized fish and observed effects on key genes involved in growth regulation could be based on similar mechanisms. In this context, Cezilly and Perrot-Minnot [10] proposed that alterations of several phenotypic traits in infected hosts might result from dysregulation of one or few key-neuromodula-

tors, particularly biogenic monoamines such as serotonin or dopamine (DA). It is important to note that DA inhibits gonadotropin release and mRNA expression in several fish species thereby regulating final gamete maturation as well as puberty initiation [14]. Concurrently, DA has been reported to stimulate GH release and/or *gh* mRNA expression in fish [27,49,78]. Stimulation of the brain DA system (or DA receptor synthesis or interaction with DA receptors) would not only explain the severe depression of pituitary gonadotropins in *L. intestinalis*-infected roach [9,28,71,73] but also the increase of pituitary *gh* mRNA reported in the present study. Alterations of brain biogenic monoamines due to parasitic infections have also been associated with behavioral changes leading to enhanced transmission to the final host. Examples in fish include three-spined sticklebacks and the killifish (*Fundulus parvipinnis*) infected by the tapeworm *S. solidus* or the trematode *Euhaplorchis californiensis*, respectively [47,67]. Similarly, *L. intestinalis* induces behavioral changes in roach that increase the risk of predation by birds [37,76]. Thus, studies on the brain monoaminergic system would be an important step to elucidate the mechanisms underlying the multiple phenotypic alterations observed in fish infected by *L. intestinalis*.

In conclusion, neither the content of storage substrates nor the gene expression pattern along the somatotropic axis indicated starvation of *L. intestinalis* infected roach under field or laboratory conditions. In accordance with the concept of parasitic castration, it is suggested that inhibition of host gametogenesis compensated or at least attenuated the nutritional demands induced by parasitization. The observed changes in gene expression with infection, most prominently increased *gh* expression in the pituitary, raises interesting questions for further studies on growth and metabolism of *L. intestinalis* infected roach and the role played by the neuroendocrine system in mediating phenotypic alterations during parasitization.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ygcen.2012. 04.007.

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