Umweltforschungsplan des
Bundesministeriums für Umwelt,
Naturschutz, Bau und Reaktorsicherheit

Forschungskennzahl 3713 65 412
UBA-FB-00 [trägt die UBA-Bibliothek ein]

Eignung des Rufverhaltens des Krallenfroschs als Endpunkt für die Erfassung der Effekte hormonell wirkender Stoffe auf aquatische Ökosysteme

von

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Im Auftrag des Umweltbundesamtes

Abschlussdatum April 2016

Kurzbeschreibung

Ziel dieses Projektes war es herauszufinden, ob der Endpunkt „Rufverhalten Südafrikanischer Krallenfrösche (*Xenopus laevis*)“ als valider Endpunkt für die Detektion und Beurteilung (anti)androgen und (anti)östrogen wirksamer endokriner Disruptoren (ED) im Rahmen der Umweltrisikobewertung herangezogen werden kann. In früheren Studien konnte gezeigt werden, dass männliche *X. laevis* ihr Rufverhalten während einer semi-statischen Exposition gegenüber ED verändern und zwar so spezifisch, dass alle Substanzen nach ihren Wirkmechanismen klassifiziert und in umweltrelevanten Konzentrationen nachgewiesen werden konnten. Um das Projektziel zu erreichen, wurde die semi-statische Testmethode an eine standardisierte Versuchsdurchführung in einem Durchflusssystem angepasst. Darüber hinaus wurden weitere Chemikalien mit (anti)androgener und (anti)östrogener Wirkungsweise getestet, um sicherzustellen, dass die spezifischen Veränderungen im Rufverhalten generell durch solche Substanzen ausgelöst werden. Ferner wurden die Auswirkungen einer Exposition mit sogenannten Negativsubstanzen untersucht, welche selbst keine (anti)androgene und (anti)östrogene Wirkungsweise innehaben, um gewährleisten zu können, dass die Veränderungen im Rufverhalten der Frösche tatsächlich ED-spezifisch sind. Die Ergebnisse dieser Studie zeigen, dass die neue, nicht-invasive Methode erfolgreich in einem Durchflusssystem durchgeführt werden und folglich als effektiver Biomarker für den Nachweis und die Beurteilung (anti)androgener und (anti)östrogener ED verwendet werden kann. Eine Exposition gegenüber Negativsubstanzen resultierte nicht in ED-spezifischen Effekten. Allerdings scheint die XENOCALL-Methode im Durchfluss weniger sensitiv zu sein als im semi-statischen System. Darüber hinaus konnten wir zeigen, dass eine Exposition gegenüber ED-Gemischen, aber auch gegenüber einzelnen ED zu starken Effekten im Ruf- und Umklammerungsverhalten der männlichen Frösche führen kann, welche mit einem reduzierten reproduktiven Erfolg exponierter Tiere einhergehen. Solche ED-Kontaminationen könnten somit sogar zum globalen Amphibiensterben beitragen.

Abstract

The aim of the present study was to resolve the question whether the calling behavior of male *X. laevis* is a suitable endpoint for the detection of endocrine disrupting chemicals (EDC) in the context of the environmental risk assessment. In recent studies we could show that male *X. laevis* alter their calling behavior during a semi-static exposure to ED so specifically, that all tested substances could be classified according to their mode of action (MOA) even at environmentally relevant concentrations. To achieve the aim of the project, the semi-static test system was transferred to a standardized experimental setup in a flow-through system. Further test chemicals with (anti)androgenic and (anti)estrogenic MOA were tested to ascertain that such EDC elicit the MOA-specific effects in the calling behavior of the frogs, in general. Furthermore, we tested the effects of so called negative substances, which do not elicit (anti)androgenic and (anti)estrogenic MOA, to ensure that these chemicals do not evoke the EDC-specific effects in the calling behavior of the males. The results of this project demonstrate, that the newly developed, non-invasive testing method can be successfully conducted in a flow-through system and, thus, be used as an effective biomarker for the detection and assessment of (anti)androgenic and (anti)estrogenic EDC. Moreover, the tested negative substances did not elicit the EDC-specific alteration in the calling behavior of the males. However, the new method seems to be less sensitive when performed in a flow-through system compared to the semi-static system. Hence, performing the experiments semi-statically might be more effective when testing for (anti)androgenic and (anti)estrogenic EDC using this non-invasive testing method. Furthermore, we could show that an exposure of frogs to a mixture of EDC with different MOA, but also to single EDC can lead to strong adverse effects on the calling and the clasping behavior, which can be accompanied by a reduced reproductive success. These effects might contribute to the global problem of amphibian decline.

Publications

Published:

Garmshausen J, Kloas W, Hoffmann F (2015). 17alpha-Ethinylestradiol can disrupt hemoglobin catabolism in amphibians. Comparative Biochemistry and Physiology, Part C 171, 34 – 40.

Submitted:

Efosa NJ, Kloas W, Kleiner W, Hoffmann F. Diclofenac exhibits direct estrogenic modes of action in male Xenopus laevis, and causes further side effects disturbing the hypothalamus-pituitary-gonad axis and mating vocalizations. (Environmental Science & Technology, eingereicht)

Garmshausen J, Siegel J, Castle D, Kloas W, Hoffmann F. 17alpha-Ethinylestradiol can impact amphibian mating success. (Hormones and Behavior)

Hoffmann F, Kloas W. Co-exposure to the estrogen ethinylestradiol and the androgen methyldihydrotestosterone causes antagonistic, independent and synergistic impacts on calling behavior of male Xenopus laevis, vitellogenin induction and heme metabolism, respectively. (Proceedings of the Royal Society)

In preparation:

Hoffmann F. P,p‘-dichlordiphenyldichloroethylene (p,p’-DDE) can elicit antiandrogenic and estrogenic modes of action in an amphibian (*Xenopus laevis*).

Planned:

Brüning A, Kloas W, Hoffmann F. Exposure to bisphenol A results in androgen-like effects on the calling behavior of male *Xenopus laevis.*

Hoffmann F, Kloas W. Dose-dependent differences in persistence of effects resulting from repeated exposures to the estrogen 17alpha-ethinylestradiol.

Hoffmann F, Kloas W. The thyroid hormone T3 can affect the heme catabolism of *Xenopus laevis*.

Contents

Figures

Tables

Abbreviations

APOA	Anterior preopitc area
AR	Androgenrezeptor; androgene receptor
ARO	Aromatase; aromatase
BLVRA	Biliverdinreduktase A; biliverdin reductase A
BPA	Bisphenol A
cDNA	Komplementäre DNA; complementary DNA
CF	Conceptual Framework
CTRL	Kontrolle; control
DDE	Dichlordiphenyldichlorethen; dichlorodiphenyldichloroethylene
DDT	Dichlordiphenyltrichlorethan; dichlorodiphenyltrichloroethane
DHT	Dihydrotestosteron, dihydrotestosterone
DCF	Diclofenac
DMSO	Dimethylsulfoxid; dimethyl sulfoxide
DTAM	Dorsal tegmental area of the torus semicircularis
E2	Östradiol, estradiol
ED	Endokriner Disruptor
EDC	Endocrine disrupting chemical
EE2	Ethinylestradiol, ethinylestradiol
EF	Elongationsfaktor 1 alpha; elongation factor 1 alpha
ELISA	Enzyme-linked immunosorbent assay
EPA	United States Environmental Protection Agency
ER	Östrogenrezeptor; estrogen receptor
EtOH	Ethanol
FSH	Follikelstimulierendes Hormon; follicle stimulating hormone
GC	Gaschromatographie; gas chromatography
GLMM	General linear mixed model
GnRH	Gonadotropin Releasing-Hormon; gonadotropine-releasing hormone
hCG	Humanes Choriongonadotropin; human chorionic gonadotropin
HHG-Achse	Hypothalamus-Hypophysen-Gonaden-Achse
HO	Hämoxigenase; heme oxigenasis
HPG-axis	Hypothalamus-pituitary-gonad-axis
ICI	Fulvestrant
IGB	Leibniz-Institut für Gewässerökologie und Binnenfischerei;

	Leibniz-Institute of Freshwater Ecology and Inland Fisheries
LAGeSo	Landesamt für Gesundheit und Soziales
LH	Luteinisierendes Hormon; luteinizing hormone
LTOR	Laminar nucleus of the torus semicircularis
MDHT	Methyldihydrotestosteron
METO	Metoprolol
MOA	Mode of action
OECD	Organisation for Economic Co-operation and Development
qPCR	Quantitative Polymerasen-Kettenreaktion; quantitative polymerase chain reaction
REACH	Registration Evaluation and Authorization of Chemicals
RED	Steroid-5α-Reduktase; steroid-5α-reductase
Ri	Inferior reticular formation
RNA	Ribonukleinsäure; ribonucleic acid
RT	Reverse Transkription
T	Testosteron; testosterone
T3	Triiodthyronin; Triiodothyronine
TAM	Tamoxifen
TR	Schilddrüsenrezeptor; thyroid receptor
TREN	Trenbolon; trenbolone
TSH	Thyreoidea-stimulierendes Hormon; thyroid stimulating hormone
U.S.	United States of America
UHPLC-MS-MS	Ultra high performance liquid chromatography - tandem mass spectrometry
VIN	Vinclozolin
VST	Ventral striatum
VTG	Vitellogenin
YAS	Yeast Androgen Screen
YES	Yeast Estrogen Screen

Zusammenfassung

Viele umweltrelevante Chemikalien sind in der Lage, das endokrine System von wildlebenden Tieren aber auch das des Menschen zu beeinflussen. So genannte endokrine Disruptoren (ED) führen zu Schäden, indem sie Hormonrezeptoren stimulieren oder blockieren oder anderweitig in den Hormonhaushalt eingreifen (Sonnenschein and Soto 1998, Crews et al. 2000). Zu den ED gehören hauptsächlich anthropogene Substanzen wie z.B. Pflanzenschutzmittel oder Pharmazeutika, die unter anderem durch Abwässer in die Umwelt gelangen. Hier akkumulieren sie vor allem in Oberflächengewässern und beeinträchtigen dort die Physiologie und Entwicklung aquatischer (In-)Vertebraten, einschließlich Amphibien und Fische (Colborn et al. 1994, Tyler et al. 1998, Choi and Jeung 2003, Segner et al. 2003, Barata et al. 2004, Zala and Penn 2004, Clubbs and Brooks 2007, Rodríguez et al. 2007, Kloas et al. 2009, Contardo-Jara et al. 2011, Lewis and Ford 2012). Ein erhöhtes Augenmerk liegt hierbei auf Substanzen, die die Fortpflanzung von Wirbeltieren beeinflussen, indem sie in die Hypothalamus-Hypophysen-Gonaden-Achse (HHG-Achse) eingreifen (Kloas et al. 2009). ED, die in diese Achse eingreifen, imitieren oder hemmen die Wirkung der Sexualsteroide, wirken also (anti)androgen oder (anti)östrogen. Sie können in den negativen Rückkopplungsmechanismus eingreifen und zudem selbst auf Zielorgane wirken oder die Wirkung der natürlichen Sexualsteroide hemmen oder modulieren (Schantz and Widholm 2001, Clotfelter et al. 2004, Scott and Sloman 2004, Zala and Penn 2004, Kloas et al. 2009, Söffker and Tyler 2012).

Zum Nachweis und zur Beurteilung (anti)androgener und (anti)östrogener ED werden Biotests herangezogen. Die meisten dieser Testmethoden sind jedoch invasive Techniken, bei denen die Notwendigkeit besteht, die zu untersuchenden Tiere zu töten. Oder es handelt sich um *in vitro* Techniken, die limitiert sind, da sie nur spezifische Mechanismen darstellen können (z.B. YES, YAS) (Kloas et al. 1999, Kloas et al. 2009), Einflüsse der Stoffe auf den Gesamtorganismus jedoch völlig ignorieren. Zudem mangelt es den bestehenden Methoden an der nötigen Sensitivität, um umweltrelevante Konzentrationen der ED nach kurzzeitiger Exposition nachweisen zu können, sowie am Vermögen, alle vier Wirkmechanismen (androgen, antiandrogen, östrogen und antiöstrogen) mit einer einzelnen Testmethode feststellen und unterscheiden zu können. International anerkannte Prüfrichtlinien, die im Sinne der 3-R Strategie (replace, reduce, refine) auf nicht-invasiven, *in vivo* Testmethoden zur Beurteilung von umweltrelevanten ED basieren, gibt es bisher nicht. Einige Studien jedoch weisen darauf hin, dass das Reproduktionsverhalten von Wirbeltieren, vor allem von aquatischen Vertebraten als brauchbarer Endpunkt zur Detektion und Beurteilung (anti)androgener und (anti)östrogener ED herangezogen werden könnte (Colman et al. 2009, Saaristo et al. 2009a, Saaristo et al. 2009b, 2010b, a, Saaristo et al. 2013).

In jüngsten Studien konnten wir zeigen, dass das Paarungsrufverhalten von männlichen Südafrikanischen Krallenfröschen (*Xenopus laevis*) durch Exposition gegenüber (anti)androgene und (anti)östrogene ED in geringsten, umweltrelevanten Konzentrationen beeinflusst wird. Auf dieser Basis wurde eine nicht-invasive Nachweismethode für (anti)androgenen und (anti)östrogenen ED entwickelt (Hoffmann and Kloas 2010, Hoffmann 2012, Hoffmann and Kloas 2012b, c, a, d). Krallenfrösche sind optimale Modellorganismen für die Untersuchung von ED, da ihr endokrines System dem höherer Vertebraten ähnelt (Kloas et al. 2009). Zudem können sie durch ihre rein aquatische Lebensweise einfach den zu untersuchenden Substanzen im Umgebungsmedium ausgesetzt werden und sind ganzjährig fortpflanzungsbereit (Tinsley and Kobel 1996). *X. laevis* sind nachtaktiv und leben in dunklen, trüben Tümpeln, weshalb sie auf akustische Unterwasservokalisationen angewiesen sind, um ihren Paarungsstatus und ihren Standort zu vermitteln (Tinsley and Kobel 1996). Paarungswillige Männchen locken Weibchen an, indem sie Werberufe, so genannte „Advertisement calls“ und auch Chirping- Rufe von sich geben. Rezeptive Weibchen schwimmen daraufhin auf das Männchen zu (positive Phonotaxis). Treffen die beiden Geschlechter aufeinander, so umklammert das Männchen das Weibchen bis es zur Eiablage kommt und befruchtet dann den Laich (Tinsley and Kobel 1996). Männchen produzieren jedoch auch Rufe, die nicht zur Anlockung der Paarungspartner gedacht sind, sondern hauptsächlich während Männchen-Männchen-Interaktionen geäußert werden. Hierzu gehört das Growling,

das Ticking und das erst kürzlich entdeckte Rasping (Hoffmann and Kloas 2010, Hoffmann 2012). Jeder Ruftyp wird allein durch Muskelkontraktionen der Larynx erzeugt und besitzt spezifische spektrale und zeitliche Eigenschaften, welche die verschiedenen Ruftypen unterscheidbar machen.

Expositionsversuche (Hoffmann and Kloas 2010, Hoffmann 2012, Hoffmann and Kloas 2012b, c, a, d) ergaben, dass ED mit (anti)androgenen und (anti)östrogenen Wirkungsweisen das Rufverhalten der Männchen in unterschiedlicher Art und Weise beeinflussen. Frösche, die dem östrogen wirkenden Ethinylestradiol (EE2) oder dem antiandrogen wirkenden Vinclozolin (VIN) ausgesetzt waren (Hoffmann and Kloas 2010, Hoffmann 2012, Hoffmann and Kloas 2012c), äußerten z.B. deutlich weniger Advertisement calls als ihre nicht exponierten Artgenossen. Zudem veränderten sich auch spektrale und zeitliche Eigenschaften ihrer Werberufe. Unterschiede in östrogener und antiandrogener Behandlung konnten anhand der Verwendungshäufigkeit verschiedener, spezifischer Ruftypen ausgemacht werden (Hoffmann and Kloas 2010, Hoffmann 2012, Hoffmann and Kloas 2012c). EE2 exponierte Frösche äußerten vermehrt Rasping-Rufe, während VIN exponierte Tiere mehr Growling- Rufe produzierten. Männchen, die mit dem androgen wirkenden Methyldihydrotestosteron (MDHT) behandelt wurden, äußerten hingegen deutlich mehr Werberufe als die Kontrolltiere (Hoffmann 2012, Hoffmann and Kloas 2012b). Indes zeigten antiöstrogen wirkende Substanzen (Tamoxifen: TAM und Fulvestrant: ICI) keinen Einfluss auf das Paarungsrufverhalten der Frösche. Wurden Männchen jedoch gleichzeitig gegenüber dem östrogenen EE2 und dem antiöstrogen wirkenden ICI exponiert, löschten die Antiöstrogene die östrogenen Effekte aus (Hoffmann and Kloas 2010, Hoffmann 2012).

Die neu entwickelte, nicht-invasive Nachweismethode für (anti)androgene und (anti)östrogene ED (XENOCALL) ermöglichte erstmals eine Differenzierung der vier verschiedenen Wirkmechanismen allein anhand veränderter Rufparameter in einem einzigen Biomarkertest. Bei den bisherigen Testmethoden, wie z.B. der Sexualdifferenzierung von *X. laevis*, können nicht alle Wirkmechanismen eindeutig unterschieden werden, da östrogene und antiandrogene ED den gleichen Effekt, Feminisierung, zeigen (Kloas et al. 2009). Zudem erwies sich das neu entwickelte, nicht-invasive Testverfahren als schneller und sensitiver im Vergleich zu allen bestehenden *in vivo* Testmethoden. Obendrein konnte gezeigt werden, dass die durch EE2-Exposition veränderten Parameter nach einer expositionsfreien Zeit von sechs bis acht Wochen wieder Kontrollwerte erreichten (Hoffmann 2012, Hoffmann and Kloas 2012c). Dementsprechend wäre es potentiell möglich, Versuchstiere nach einer bestimmten Regenerationszeit in weiteren Testläufen erneut einzusetzen.

Die biologische Relevanz der veränderten Rufparameter wurde in zusätzlichen Weibchenwahl-Versuchen ermittelt (Hoffmann 2012, Hoffmann and Kloas 2012c). Es stellte sich heraus, dass rezeptive Weibchen die stereotypen Paarungsrufe der Kontrollmännchen deutlich vorziehen gegenüber durch östrogene ED veränderte Paarungsrufe. Außerdem ergaben die Versuche, dass Weibchen in Reaktion auf Paarungsrufe von östrogen-exponierten Männchen generell nur selten Paarungsbereitschaft zeigten. Infolgedessen könnten die mit östrogenen ED kontaminierten, männlichen *X. laevis* in der Natur größere Schwierigkeiten bei der Partnerfindung und somit bei der Reproduktion haben als nicht kontaminierte Männchen (Hoffmann and Kloas 2010, 2012c). Auch bei weiblichen *X. laevis* deuten erste Ergebnisse darauf hin, dass diese – wenn auch nicht so sensibel und eindeutig wie ihre männlichen Artgenossen – auf ED-Kontaminationen reagieren (Hoffmann and Kloas 2012b). Supraphysiologische Konzentrationen des androgen wirksamen MDHT, zum Beispiel, reduzierten das phonotaktische Verhalten der Weibchen, während physiologische und umweltrelevante Konzentrationen zu einem Anstieg des Phonotaxis-Verhaltens führte (Hoffmann 2012, Hoffmann and Kloas 2012b).

Das Ziel dieser Studie ist es daher, die Frage zu beantworten, ob das Rufverhalten männlicher Krallenfrösche als brauchbarer Endpunkt für die Detektion und Beurteilung (anti)androgener und (anti)östrogener ED im Rahmen der Umweltrisikobewertung herangezogen werden kann. Hierfür mussten diverse grundlegende wissenschaftliche Untersuchungen durchgeführt werden. Als erstes sollten die potentiellen biologischen und ökologischen Auswirkungen einer Veränderung des Rufverhaltens von Amphibien im Allgemeinen und *X. laevis* im Speziellen ermittelt werden. Es wurde bereits gezeigt, dass Weibchen veränderte Rufe weniger

attraktiv finden (Hoffmann and Kloas 2012c). Ob ein verändertes Rufverhalten aufgrund einer ED-Exposition negative Auswirkungen auf den individuellen Paarungserfolg und somit auch auf die reproduktive Fitness ganzer Populationen haben kann, ist noch nicht ausreichend erforscht. Es stellte sich daher die Frage, wie stark sich das Rufverhalten der Frösche ändern muss, um als adverser Endpunkt eingestuft zu werden. Darüber hinaus sollte geklärt werden, ob externe Faktoren wie Wassertemperatur, Regen, Luftfeuchte, etc. ebenfalls das Paarungs(ruf)verhalten von Fröschen beeinflussen können. Die bestehende XENOCALL-Methode sollte zudem in ein Durchflusssystem übertragen werden, in welchem die Expositionskonzentrationen über den kompletten Versuchszeitraum konstant gehalten werden können. Wir untersuchten, unter welchen Testbedingungen und für welche Chemikalien Veränderungen im Rufverhalten männlicher *X. laevis,* vor allem im Vergleich zu anderen Endpunkten bei Fischen und Amphibien, besonders sensitiv nachzuweisen sind. Hierfür untersuchten wir die Auswirkungen weiterer Substanzen mit (anti)androgener und (anti)östrogener Wirkungsweise auf diesen Endpunkt, um sicherzustellen, dass Chemikalien mit gleichem Wirkmechanismus auch identische Effekte hervorrufen. Zusätzlich testeten wir sogenannte umweltrelevante Negativsubstanzen, also Chemikalien, welche keine (anti)androgene oder (anti)östrogene Wirkungsweise innehaben, um festzustellen, ob die gezeigten Veränderungen im Rufverhalten der Tiere tatsächlich wirkspezifisch sind oder ob auch nicht (anti)androgen bzw. (anti)östrogen wirkende Stoffe dieses Verhalten beeinflussen können.

Dieses Projekt war in drei Arbeitspakete gegliedert, welche im Folgenden beschrieben sind.

Arbeitspaket 1 - Literaturrecherche

In diesem Arbeitspaket sollte anhand einer Literaturrecherche geklärt werden, welche biologische und ökologische Relevanz der Endpunkt Rufverhalten von Amphibien, speziell von Krallenfröschen, hat und wie bzw. in welchem Ausmaß sich das Rufverhalten ändern muss, damit es als Endpunkt relevant wird. Des Weiteren sollte ermittelt werden, unter welchen Testbedingungen eine durch Chemikalien verursachte Beeinträchtigung des Rufverhaltens messbar und besonders sensitiv ist und ob externe Faktoren wie z.B. Wassertemperatur, Regen, Anwesenheit von Algen, Nahrungsverfügbarkeit etc., das Paarungs- und Rufverhalten der Tiere verändern können. Zusätzlich sollte die Sensitivität des Endpunktes Rufverhalten mit anderen, bisher verwendeten Biomarkern bei Amphibien und Fischen verglichen werden.

Die Literaturrecherche erfolgte mittels diverser Schlüsselworte (z.B. calling behavior, courtship behavior, advertisement calling, anuran, amphibian, endocrine disrupting chemicals, biomarkers, estrogen, antiestrogen, androgen, antiandrogen, rain, temperature, external factors, environmental factors, etc.) auf den folgenden Webseiten:

1. Web of Science (Thomson Reuters), www.webofknowledge .com
2. Science Direct, www.sciencedirect.com
3. Pubmed, www.pumed.com
4. Google Scholar, https://scholar.google.de.

Die Literaturstudien ergaben, dass für die meisten Amphibienspezies v.a. eine exakte zeitliche Abfolge der einzelnen Rufelemente, aber auch charakteristische spektrale Parameter der Werbegesänge unabdingbar sind, damit paarungswillige Weibchen den Gesang der Männchen als arteigen erkennen und ggf. sogar eine Individuenerkennung stattfinden kann (Loftus-Hill 1971, Picker 1983, Klump and Gerhardt 1992, Schwartz 1994, Bibikov and Nizamov 1996, Gerhardt et al. 1996, Gerhardt and Schul 1999, Gerhardt et al. 2000, Gerhardt 2001, Schwartz et al. 2001, Bush et al. 2002, Schul and Bush 2002, Beckers and Schul 2004, Deily and Schul 2004, Gerhardt 2005b, Vignal and Kelley 2007, Bush et al. 2009, Deily and Schul 2009, Gerhardt and Brooks 2009, Gordon and Gerhardt 2009, Klump and Gerhardt 2013). Schon geringe Abweichungen der

stereotypen Rufe kann dazu führen, dass die Weibchen nicht mehr auf die anlockenden Paarungsrufe der Männchen reagieren und es somit nicht zur Paarung kommt (Gerhard 1974, Gerhardt 1991, Bibikov and Nizamov 1996, Gerhardt et al. 1996, Murphy and Gerhardt 1996, Murphy and Gerhardt 2000, Beckers and Schul 2004, Gerhardt 2005a, Deily and Schul 2006, Vignal and Kelley 2007, Gordon and Gerhardt 2009). Außerdem wurde in früheren Studien ermittelt, dass v.a. die Luft- und Wassertemperatur, aber auch die Luftfeuchtigkeit dabei eine Rolle spielen, wann die Paarungssaison und damit auch der Paarungsgesang beginnen. Unterschiedliche Temperaturen können zudem verschiedene Parameter des Werbegesangs einiger Froscharten beeinflussen (Gerhardt and Mudry 1980, Yamaguchi et al. 2008). In diesen Fällen kommt es in der Natur jedoch zu einem Phänomen, dass man "temperature coupling" nennt, wobei sich die Präferenz der Weibchen den sich temperaturabhängig ändernden Parametern der männlichen Rufe anpasst (Gerhardt 1977, Pires and Hoy 1992a, Pires and Hoy 1992b). Da vor allem der Werbegesang von Fröschen hauptsächlich durch Androgene gesteuert wird (Wada and Gorbman 1977, Schmidt 1983, Wetzel and Kelley 1983, Penna et al. 1992), liegt es nahe, dass dieses Verhalten auch durch ED, v.a. androgene und antiandrogene ED beeinflusst werden könnte.

Arbeitspaket 2 – Optimierung der bestehenden Methode

Zur Optimierung des bestehenden Verhaltenstests mussten einige Kriterien des bestehenden Versuchsaufbaus, sowie der Durchführung und Analyse der XENOCALL-Methode optimiert und weiterentwickelt werden. Zum einen wurde in den vorherigen Tests ein semistatischer Ansatz gewählt, bei dem alle zwei Tage das Umgebungswasser, sowie die Expositionssubstanzen erneuert wurden. Es ist jedoch empfehlenswert, eine konstante Konzentration während der Exposition zu gewährleisten, was auch die Sensitivität der Testmethode weiter steigern könnte. Hierfür wurden erste Testläufe ohne ED durchgeführt, um die grundlegenden Testbedingungen zu ermitteln, unter denen die Frösche ihr Paarungsrufverhalten uneingeschränkt zeigen, wie z.B. die ideale Beckengröße und der optimale Abstand zweier Becken voneinander. Im Rahmen dieses Arbeitspaktes sollte zudem geklärt werden, ob die ohnehin schon kurze Expositionszeit noch weiter reduziert werden könnte, z.B. durch Weglassen der Eingewöhnungszeit. Dies wurde durch statistische Analysen umgesetzt, bei denen das Rufverhalten der einzelnen Frösche in jeder Versuchsnacht separat ausgewertet wurde, um mögliche Einsparungspotentiale zu identifizieren. Obendrein sollte eine Analysesoftware entwickelt werden, die eine standardisierte Analyse aller wichtigen Parameter des Rufverhaltens der männlichen *X. laevis* vollständig automatisch auswertet. Zusätzlich wurde eine chemische Analytik zu jeder untersuchten Substanz etabliert.

Arbeitspaket 3 – Experimentelle Testung

Dieses Arbeitspaket umfasste die experimentelle Testung. Die zu bearbeitenden Fragestellungen waren hierbei die Untersuchung

1) der ökologischen Relevanz des Endpunktes. Hierbei sollte experimentell festgestellt werden, welchen Einfluss das veränderte Rufverhalten auf die Weibchen und damit auf den Paarungserfolg und somit auch auf das Bestehen der Population hat.
2) weiterer fünf Testsubstanzen und deren Auswirkungen auf das Rufverhalten männlicher *X. laevis*. Da sich die bisherigen Untersuchungen auf einige wenige Substanzen mit einem bekannten endokrinen Wirkmechanismus beschränken, sollten diese weiteren Substanzen im Rahmen dieses Arbeitspaktes getestet werden, um allgemeingültige Aussagen zur Anwendbarkeit dieses Endpunktes für die Erfassung hormonell wirksamer Substanzen im Rahmen der Umweltrisikobewertung ableiten zu können.

3) der Wiederverwendbarkeit von Testindividuen. Im Rahmen dieses Arbeitspaketes sollte analysiert werden, ob es prinzipiell möglich ist, Versuchstiere nach Beendigung der Versuche für weitere Untersuchungen erneut einzusetzen.

4) von Negativsubstanzen, also Chemikalien, die nicht (anti)androgen bzw. (anti)östrogen wirken. Hier sollte herausgefunden werden, ob solche Substanzen möglicherweise identische Effekte im Rufverhalten der Tiere auslösen können, wie die nachzuweisenden Testsubstanzen.

Bei allen Versuchen sollten obendrein die Konzentrationen der jeweiligen Testsubstanz in den Testbecken, sowie der Konzentrationsverlauf über die jeweilige Versuchsdauer durch eine entsprechende Analytik überprüft werden.

1) Biologische und ökologische Relevanz des Endpunktes

A) Langzeit-Exposition gegenüber dem östrogenen EE2

Um die biologische und ökologische Relevanz der durch ED hervorgerufenen Änderungen im Rufverhalten von Amphibien zu beurteilen, exponierten wir männliche *X. laevis* in einem Langzeitexperiment (28 Tage) gegenüber verschiedenen Konzentrationen des östrogenen EE2. Vor der Exposition wurden alle Männchen einmalig mit 100 Units humanem Choriongonadotropin angespritzt, um ihr Paarungsrufverhalten zu induzieren (Russell 1960). Wir analysierten das Rufverhalten der Männchen während der Exposition und führten anschließend zwei Paarungsexperimente mit nicht-exponierten Weibchen durch. Die hieraus entstandenen Kaulquappen zogen wir für 9 Tage auf und untersuchten die Schlupf- und Überlebensrate in den einzelnen Testgruppen. Die Ergebnisse dieses Versuchs sind bereits in einem Manuskript zusammengefasst worden, welches im Journal *Hormones and Behavior* veröffentlicht werden soll. Eine Detaillierte Versuchsbeschreibung, sowie die ausführliche diskutierten Ergebnisse können diesem Manuskript entnommen werden (Anhang 1).

Zusammengefasst konnten wir zeigen, dass höhere EE2 Konzentrationen (10^{-8} M) drastische, negative Effekten auf den sexuellen Erregungszustand der Tiere haben können. Exponierte Tiere zeigten weniger Werberufverhalten und auch ein geringeres Umklammerungsverhalten als Kontrolltiere. Diese EE2-exponierten Frösche stellten ihr Rufverhalten nach einer Expositionsdauer von ca. 14 Tagen sogar völlig ein. Infolgedessen schlüpften auch deutlich weniger Kaulquappen in den Becken, die die 10^{-8} M EE2 exponierten Vatertiere beherbergten. Zudem war die Überlebensrate in diesen Becken ebenfalls erniedrigt im Vergleich zu den Kontrollbecken.

Frösche, die 10^{-10} M bzw. 10^{-12} M EE2 ausgesetzt waren, riefen in den ersten zwei Versuchswochen ebenfalls weniger Werberufe und einen größeren Anteil an Rufen, die einen nicht-erregten Zustand der Männchen widerspiegeln. Gegen Ende der Exposition jedoch unterschied sich das Rufverhalten der Tiere in seiner Zusammensetzung nicht mehr von dem der Kontrolltiere. 10^{-10} M EE2 exponierte Männchen umklammerten ihre Weibchen deutlich kürzer als Männchen der 10^{-12} M EE2-Behandlung, wenn sich die Tiere in einer Konkurrenzsituation befanden. Dieses Ergebnis deutet auf eine monotone EE2-Dosis-abhängige Erniedrigung der sexuellen Erregung der Versuchstiere hin. Fehlte die Konkurrenz jedoch und die Männchen waren nur mit einem Weibchen zusammen im Aquarium, umklammerten die Männchen der 10^{-10} M EE2 Behandlungsgruppe länger als ihre Artgenossen der anderen Versuchsgruppen, inklusive der Kontrolltiere. Dieser Anstieg der sexuellen Erregung 10^{-10} M EE2 exponierter Frösche stimmen mit der erhöhten Umklammerungsaktivität der Frösche im nicht-kompetitiven Paarungsversuch überein. Dementsprechend scheint eine monotone EE2-Dosisabhängigkeit des Umklammerungsverhaltens nur bei Anwesenheit eines Rivalen aufzutreten. Entsprechend schlüpften und überlebten ähnlich viele Nachkommen der 10^{-10} M EE2 bzw. 10^{-12} M EE2 behandelten Väter, wie in der Kontrollgruppe.

10^{-8} M EE2-exponierte Frösche produzierten einen signifikant geringeren Anteil an Advertisement calls, sowie einen höheren Prozentsatz an Rasping, was belegt, dass die exponierten Tiere weniger sexuell erregt sind als die Kontrolltiere (Hoffmann and Kloas 2010, 2012c). Eine solche Verminderung der Paarungsbereitschaft könnte durchaus zu einem geringeren Paarungs- und damit Reproduktionserfolg führen (Gerhardt et al. 2000, Murphy and Gerhardt 2000, Rosso et al. 2006, Gerhardt and Brooks 2009). Ähnlich wie bei den früheren semi-statischen EE2-Versuchen (Hoffmann and Kloas 2012c), zeigten auch hier die Frösche, die gegenüber 10^{-8} M EE2 exponiert wurden, Veränderungen in spektralen und temporalen Parametern ihrer Werberufe (geringere Klickdauer und geringere Anzahl betonter Klicks) über den gesamten Versuchsverlauf hinweg. Diese veränderten Werberufe sind für nicht-exponierte, wählende Weibchen weniger attraktiv als Kontrollrufe (Hoffmann and Kloas 2012c). Darüber hinaus konnten wir darlegen, dass männliche *X. laevis*, die 10^{-8} M EE2 ausgesetzt waren, nach ca. 14 Tagen keine Rufe mehr von sich gaben. Übereinstimmend umklammerten diese Tiere ihr Weibchen in beiden Paarungsversuchen nicht oder nur vereinzelt und kurz. In dieser Behandlungsgruppe schlüpften keine bzw. nur vereinzelte Kaulquappen, auch wenn die dazugehörigen nicht-behandelten Weibchen ebenso viele Eier legten wie die Weibchen der Kontrollgruppe. Zudem zeigte der Nachwuchs der 10^{-8} M EE2 exponierten Männchen eine signifikant geringere Überlebensrate als die Kontrollen (Mittelwerte: 33% versus 94%).

Frösche, die gegenüber 10^{-10} M und 10^{-12} M EE2 exponiert waren, produzierten ebenfalls weniger Advertisement calls und mehr Rasping. Diese Parameter glichen sich jedoch gegen Ende der 28-tägigen Versuchslaufzeit immer mehr den Kontrollwerten an. Diese Verringerung der Wirksamkeit von EE2 auf das Rufverhalten der Frösche im Verlauf des Expositionszeitraums könnte für eine Art Adaptations- oder Habituationseffekt sprechen. Allerdings zeigten sich einige veränderte Parameter während der gesamten Versuchsdauer, nämlich die spektralen und zeitlichen Modifikationen. Trotz einer möglichen Gewöhnung und Abschwächung einiger durch EE2 ausgelösten Effekte, könnten gerade diese persistenten spektralen und temporalen Veränderungen der Werberufe negative Auswirkungen auf den Paarungserfolg dieser Frösche haben. Männchen, die EE2 Konzentrationen von 10^{-10} M ausgesetzt waren, umklammerten ihr Weibchen kürzer als die Kontrollfrösche oder Frösche, die gegenüber 10^{-12} M EE2 exponiert waren. Dieser Effekt scheint also EE2-dosisabhängig zu sein, jedoch nur, wenn ein Konkurrent mit im Testbecken saß. Überraschenderweise umklammerten die Männchen, die zuvor 10^{-10} M EE2 ausgesetzt war ihr Weibchen länger als alle anderen Frösche, inklusive der Kontrollen, wenn kein Rivale mit im Becken saß. Dieses gesteigerte Umklammerungsverhalten deckt sich mit dem ebenfalls gesteigerten Werberufverhalten dieser *X. laevis* in der letzten Expositionswoche. Es spielt demnach bei ED-Expositionen eine Rolle, ob ein aktiver Paarungskonkurrent anwesend ist, denn unter solchen, realeren Bedingungen konnten monotone Dosis-abhängige Effekte auf das Umklammerungsverhalten festgestellt werden. Zukünftige Studien sollten dieses Phänomen stärker fokussieren und hinterfragen, ob ED-Effekte ggf. stärker oder auch schwächer ausgeprägt werden, wenn die Versuchsumgebung realitätsnähere Situationen widerspiegeln, wie z.B. die Anwesenheit von Paarungskonkurrenten. Ähnlich wie bei 10^{-10} M EE2 exponierten Tieren war das Umklammerungsverhalten auch bei 10^{-12} M EE2 exponierten Tieren im gleichen Bereich wie bei den Kontrolltieren, wenn kein Paarungskonkurrent im Testbecken saß, sondern das Männchen nur mit einem einzelnen, unbehandelten Weibchen getestet wurde. Auch dies deckt sich wieder mit den ansteigenden Anteilen an Werberufen, die diese Tiere gegen Ende der Expositionszeit geäußert haben. Übereinstimmend konnten bei diesen beiden Behandlungsgruppen (10^{-10} M und 10^{-12} M) auch keine Unterschiede in der Schlupf- und Überlebensrate der Nachkommen im Vergleich zur Kontrollgruppe gefunden werden.

Da das Paarungsrufverhalten männlicher *X. laevis* androgen-abhängig ist (Kelley and Pfaff 1976, Wetzel and Kelley 1983, Tobias et al. 1993, Kelley 2002), könnte eine geringere Bioverfügbarkeit von Sexualsteroiden bei Tieren, die EE2 ausgesetzt waren, dazu geführt haben, dass sich diese Frösche vor allem zu Beginn der Expositionszeit in einem Zustand verringerter sexueller Erregung befunden haben. In dieser Studie war die Expression von RED1 und RED2 durch EE2 Dosis-abhängig herabgesetzt, während die Expression der Aromatase erhöht war. Da RED1 und 2 T zu DHT konvertieren und ARO T in E2 verwandelt, führt ein

durch EE2 hervorgerufener Anstieg der ARO-Genexpression und eine verringerte Expression von RED1 und 2 zu einer erhöhten E2-Verfügbarkeit und geringen T und DHT-Konzentrationen im Blut der exponierten Tiere. Dies wiederum hat eine Erniedrigung der sexuellen Erregung exponierter Tiere zur Folge.

B) *Simultane Exposition gegenüber EE2 und MDHT*

Des Weiteren untersuchten wir die Auswirkungen einer Exposition männlicher *X. laevis* gegenüber einem Gemisch des androgenen MDHT und des östrogenen EE2, um herauszufinden ob eine solche simultane Exposition zu additiven Effekten oder zur Auslöschung einzelner ED-Effekte führt. Hierfür exponierten wir männliche *X. laevis* (3 Jahre; n = 10) gegenüber umweltrelevanten Konzentrationen MDHT und EE2 in verschiedenen Mischungsverhältnissen (10^{-9} M MDHT + 5*10^{-9} M EE2 (MDHT+EE2) und 10^{-9} M EE2 + 5*10^{-9} M MDHT (EE2+MDHT) und einer Lösemittelkontrolle. Die Tiere wurden erst vier Nächte ohne Stimulation mit hCG exponiert. Daraufhin wurde ihr Rufverhalten mittels hCG-Injektion angeregt und die Tiere wurden für weitere vier Nächte gegenüber dem ED-Gemisch exponiert. Die Ergebnisse dieses Versuchs sind bereits in einem Manuskript zusammengefasst worden, welches im Journal *Hormones and Behavior* veröffentlicht werden soll. Eine Detaillierte Versuchsbeschreibung, sowie die ausführliche diskutierten Ergebnisse können diesem Manuskript entnommen werden (Anhang 2).

Zusammengefasst konnten wir zeigen, dass das östrogene EE2 effektiver war als das androgene MDHT und potentielle stimulierende, androgen-typische Effekte des MDHT auf das Rufverhalten der Frösche völlig unterdrückte. Außerdem wurden zusätzliche Effekte auf das Rufverhalten der Tiere gefunden, welche nicht durch eine Exposition der adulten *X. laevis* gegenüber den Einzelstoffen EE2 bzw. MDHT ausgelöst wurden. Zudem führte eine simultane Exposition gegenüber EE2 und MDHT zu einer EE2-konzentrationsabhängig erhöhten Expression des östrogenen Biomarkers Vitellogenin, während bei Endpunkten wie der Expression von Genen, die im Hämstoffwechsel eine Rolle spielen, additive bzw. synergistische Wirkungsweisen der beiden ED nachgewiesen werden konnten. ED-Gemische mit unterschiedlichen Wirkungsweisen können demnach verschiedene Effekte bei Individuen hervorrufen. Eine Kombination von antagonistischen und synergistischen Effekten ist jedoch mittels Expositionen gegenüber Einzelstoffen, wie sie meist im Rahmen der Risikobewertung durchgeführt werden, schwer abschätzbar. Da viele der betroffenen Endpunkte aber für eine erfolgreiche Fortpflanzung und die Erhaltung der Population und Spezies eine große Rolle spielen, ist es wichtig, dass zukünftige Studien sich vermehrt darauf fokussieren, zu verstehen, wie genau ED-Gemische wirken und solche kombinierten Effekte bei aquatischen Vertebraten hervorrufen. Nur so kann eine adäquate Risikobewertung und der Schutz von aquatisch lebenden Tieren in der Umwelt sichergestellt werden.

2) Untersuchung weiterer ED mit (anti)androgener und östrogener Wirkungsweise (5 Substanzen)

Um einen direkten Vergleich der XENOCALL-Methode im semi-statischen System (Hoffmann and Kloas 2010, 2012b, c, a, d) und der im Durchflusssystem anstellen zu können, wurden die Effekte weiterer fünf (anti)androgener und (anti)östrogener ED auf das Rufverhalten männlicher *X. laevis* untersucht. Zwei der im Durchfluss getesteten Substanzen (EE2 und VIN) wurden bereits im semi-statischen System verwendet (Hoffmann and Kloas 2010, 2012b, c, a, d) und drei Substanzen wurden erstmals untersucht. Hierzu gehörte das antiandrogene Dichlorodiphenyldichlorethen (DDE), ein Metabolit des Insektizides Dichlorodiphenyltrichlorethan (DDT), das androgene Steroid Trenbolone (TREN), welches v.a. in den USA bei Nutztieren und Vieh zum Muskelwachstum und Appetitanregen verwendet wird, als auch das vermeintlich androgen wirkende Bisphenol A (BPA), welches als Weichmacher in diversen Plastikprodukten gefunden werden kann.

Bei der Untersuchung von EE2 und VIN wurden acht Testtiere pro Behandlung eingesetzt und drei verschiedene Testkonzentrationen untersucht. In den anderen Tests (DDE, TREN, BPA) wurden zehn Tiere pro Behandlung eingesetzt, um die Wahrscheinlichkeit, signifikante Effekte zu entdecken, zu erhöhen. Jedoch wurden hier pro Substanz nur zwei verschiedene Testkonzentrationen untersucht. Alle Tests wurden in der Durchflussanlage des IGB-Berlin durchgeführt. Die Testdauer eines Versuches betrug 96 h. Vor jedem Experiment wurde den Versuchstieren hCG injiziert (100 Units / 50 µl, wie bereits für EE2+MDHT behandelte Tiere beschrieben), um ein grundlegendes Rufverhalten auszulösen.

Um die Vorratslösungen herzustellen wurden die Testchemikalien in 99% DMSO (bzw. Ethanol bei DDE) gelöst (Konzentration der Stammlösung: 10^{-2} M). Die Vorratslösungen (je 10 L, Lösungsmittelkonzentration: 0,00001%) waren immer 100x höher konzentriert als die gewünschte Stoffkonzentration in den Testbecken, da in den sogenannten Mixing-Chambers der Durchflussanlage des IGB-Berlin eine 100-fache Verdünnung der jeweiligen Stammlösung mit aufbereitetem Leitungswasser (für Details: (Lutz et al. 2008) durchgeführt wurde. Die Konzentration des Lösungsmittels in den Versuchsaquarien betrug immer 0,0000001 %. Alle Chemikalien wurden bei Sigma Aldrich (Steinheim, Deutschland) bestellt. Während des Versuchs wurden die Frösche alle zwei Tage mit einem gängigen Fischfutter (Metabolica, Aller Aqua, Golßen, Germany; 2 Pellets) gefüttert und die Wassertemperatur in den Becken gemessen. Der Hell:Dunkel-Zyklus betrug 12:12 h. Nach dem Experiment wurden die Tiere zurück in die institutseigene Haltung gebracht und dort nach Behandlungsgruppen getrennt in 60 L Wasserbehältern gehalten. EE2 behandelte Tiere wurden nach einer Erholungszeit ohne ED-Exposition erneut getestet, um feststellen zu können, ob Versuchstiere mit der XENOCALL-Methode mehrfach verwendet werden können (siehe Punkt 3).

Für die statistischen Analysen der Verhaltensdaten wurden erneut General Linear Mixed Models (GLMM) herangezogen (Hoffmann 2012), inklusive nachfolgender Sidak Post-hoc Tests (SPSS 20, IBM, Ehningen, Germany). Normalverteilungen wurden mittels Kolmogorov-Smirnov Tests sichergestellt. Für die chemisch-analytischen Untersuchungen wurden Wasserproben zu Beginn und zum Ende der Exposition aus den Testbecken und Vorratsbehältern entnommen und untersucht. Die Proben wurden aufkonzentriert (mittels C18-Kartuschen, siehe Garmshausen et al. (2015) (Anhang 3) und mittels UHPLC-MS-MS oder Gaschromatographie (GC) untersucht. Alle Experimente wurden vom Landesamt für Gesundheit und Soziales genehmigt (LAGeSo, Nr.: 0093/13).

Im Folgenden sind die Ergebnisse der einzelnen Tests aufgeführt.

EE2-Exposition

Im Vergleich zu den Kontrollen äußerten alle EE2-behandelten *X. laevis* einen geringeren Prozentsatz an Advertisement calls und einen höheren Anteil an Rasping. Allerdings waren diese Effekte nur signifikant, wenn man ausschließlich die höchstkonzentrierte EE2-Behandlung (10^{-10} M EE2) und die Kontrollgruppe betrachtete. Sobald man die Behandlungen mit den niedrigeren EE2-Konzentraionen 10^{-13} M EE2 und 10^{-12} M EE2 in die Berechnungen einbezieht, sind diese Unterschiede nicht mehr signifikant. Zudem produzierten EE2 exponierte Frösche grundlegend einen höheren Anteil an Growling. Kein anderer Ruftyp wurde durch eine EE2-Exposition beeinflusst. Bezüglich der spektralen und temporalen Parameter der Werberufe, wurde nur die Frequenzbandbreite durch 10^{-10} M EE2 erhöht, jedoch war dieser Effekt erneut nach Einbezug aller Behandlungsgruppen nicht signifikant.

BPA-Exposition

In beiden durchgeführten Versuchen produzierten männliche *X. laevis*, die dem BPA in einer Konzentration von 10^{-8} M ausgesetzt waren, überraschenderweise einen signifikant höheren Anteil an Advertisement calls und einen etwas geringeren Prozentsatz an Rasping verglichen mit den Kontrollen. 10^{-9} M BPA exponierte

Frösche produzierten im Durchfluss ebenfalls einen höheren Anteil an Werberufen und einen niedrigeren Anteil an Rasping, jedoch waren diese Effekte statistisch nicht signifikant. Spektrale und temporale Parameter der Werberufe wurden zudem nicht durch eine BPA-Exposition beeinflusst. Die Expression des Östrogenbiomarkers VTG, sowie die Konzentrationen der Sexualsteroide T, DHT und E2, sowie deren Verhältnisse wurden ebenfalls nicht durch eine BPA-Exposition beeinflusst. Und auch die Expression des Gonadotropins FSH im Gehirn und der gonadalen steroidkonvertierenden Enzyme wurde nicht durch BPA verändert. Die Expression von LH war jedoch signifikant unterdrückt.

TREN-Exposition

Wie erwartet äußerten alle männlichen *X. laevis*, die gegenüber TREN exponiert wurden, einen deutlich höheren Anteil an Werberufen als die Kontrolltiere, jedoch war dieser Effekt nur hinsichtlich der höheren TREN-Konzentration (10^{-9} M) signifikant. Frösche der TREN-Behandlungsgruppen tendierten überdies dazu einen geringeren Anteil an Ticking-Rufen zu produzieren. Bei einer Einzelexposition der Frösche mit dem androgenen TREN wurde eine signifikant höhere Peak-Frequenz der langsamen und schnellen Trills der Werberufe exponierter Männchen detektiert. Zudem enthielten die langsamen Trills der Werberufe weniger Klicks, wenn die Frösche 10^{-11} M TREN ausgesetzt waren. Keine weiteren spektralen und temporalen Parameter der Advertisement calls wurden durch TREN verändert.

VIN-Exposition

Frösche, die einer VIN Konzentration von 10^{-10} M ausgesetzt waren, produzierten weniger Werberufe und mehr Ticking als die Kontrolltiere. Werberufe 10^{-10} M VIN exponierter Tiere wiesen signifikant längere ICIs und eine geringere Klickrate im langsamen Teil der Rufe (slow Trill) auf, während der schnelle Teil der Werberufe (fast Trill) eine höhere Frequenz zeigte als bei den Werberufen der Kontrolltiere. Die beiden niedriger konzentrierten VIN-Behandlungen (10^{-11} M and10^{-12} M VIN) hingegen, beeinflussten das Rufverhalten der Tiere nicht. Demnach konnte für den Durchfluss eine lowest observed effect concentration (LOEC) von 10^{-10} M VIN festgestellt werden.

DDE-Exposition

DDE- behandelte Tiere äußerten, wie erwartet, weniger Advertisement calls. Darüber hinaus produzierten diese Tiere auch einen größeren Anteil an Rasping. Diese Effekte waren jedoch nur statistisch signifikant bezüglicher der 10^{-11} M DDE Behandlung. Der Anteil an Ticking war bei DDE-exponierten Männchen deutlich, jedoch nicht signifikant erhöht. Spektrale und temporale Parameter der Werberufe wurden nicht signifikant durch DDE beeinflusst.

Diskussion

Zusammenfassend kann gesagt werden, dass eine Exposition gegenüber allen getesteten (anti)androgenen und (anti)östrogenen ED, mit Ausnahme des BPA, in wirkspezifischen Effekten resultierten, die denen glichen, welche bereits in XENOCALL-Versuchen im semi-statischen System gezeigt werden konnten (Hoffmann and Kloas 2010, Hoffmann 2012, Hoffmann and Kloas 2012b, c). Androgene führten zu einer Erhöhung der sexuellen Erregung der Tiere, während eine Exposition gegenüber antiandrogenen und östrogenen ED zu einer Verminderung der Paarungsbereitschaft der Tiere führte. Darüber hinaus konnten die bereits im semi-statischen System festgestellten spezifischen Unterschiede, die antiandrogene und östrogene ED im Rufverhalten der Männchen auslösen (Hoffmann 2012, Hoffmann and Kloas 2012b, c) auch im Durchflusssystem festgestellt werden.

Eine Ausnahme dieser Regel stellte das vermeintlich östrogen wirkende BPA dar. Eine Exposition mit BPA führte nicht zu den östrogen-spezifischen Effekten, wie eigentlich erwartet, sondern rief die für androgene ED typische Erhöhung des Werbegesangs und der sexuellen Erregung der Tiere hervor. Auch die fehlende Induktion des östrogenen Biomarkers VTG deutet darauf hin, dass BPA in adulten, männlichen *X. laevis* nicht als östrogener ED wirkt. Jedoch führten auch dort unterschiedliche Konzentrationen der Testsubstanz zu unterschiedlich starken Effekten („U-Shape"). Es wäre dementsprechend also möglich, dass BPA in unterschiedlichen Konzentrationen und in verschiedenen Entwicklungsstadien der Frösche verschiedene Wirkmechanismen auslösen kann, welche u.a. auch zu den androgen-typischen Effekten im Rufverhalten der adulten, männlichen Krallenfrösche führten. Es ist zudem nicht auszuschließen, dass verschiedene Endpunkte unterschiedlich durch BPA beeinflusst werden können.

Des Weiteren muss in zukünftigen Studien ebenfalls geklärt werden, ob Antiöstrogene die östrogen-spezifischen Effekte auch im Durchfluss auslöschen können, ebenso wie es bereits im semi-statischen System gezeigt wurde (Hoffmann 2012, Hoffmann and Kloas 2012a).

3) Wiederverwendbarkeit von Testtieren

Wir konnten in früheren Studien zeigen, dass die durch EE2-Exposition veränderten Rufparameter nach einer expositionsfreien Zeit von sechs bis acht Wochen wieder Kontrollwerte erreichten (Hoffmann 2012, Hoffmann and Kloas 2012c). Dementsprechend wäre es potentiell möglich, Versuchstiere nach einer bestimmten Regenerationszeit in weiteren Testläufen wiederzuverwenden. Dementsprechend wurden die Tiere, die für 96 h EE2 bzw. VIN ausgesetzt waren, nach einer rund achtwöchigen Phase unter Kontrollbedingungen erneut in der Durchflussanlage getestet, diesmal jedoch ohne ED-Kontamination. So sollte festgestellt werden, ob die durch die ED ausgelösten Veränderungen im Rufverhalten der Tiere tatsächlich reversibel oder doch längerfristig persistent sind. Am Ende dieser Experimente wurden die Tiere wiederum der Haltung des IGB-Berlins zugeführt und nach Behandlungsgruppen getrennt in 60 L Becken gehalten. Sollten die EE2- bzw. VIN-typischen Effekte auf das Rufverhalten der *X. laevis* tatsächlich verschwinden, würden die Testtiere nach einer weiteren achtwöchigen expositionsfreien Zeit erneut gegenüber dem jeweiligen ED exponiert werden, um zu ermitteln, ob die ED-Effekte erneut auftreten.

Wie bereits im Abschnitt „Untersuchung weiterer ED mit (anti)androgener und östrogener Wirkungsweise (5 Substanzen)" beschrieben, konnten wir zeigen, dass eine 96-stündige EE2 Exposition im Durchflusssystem zu den östrogen-spezifischen Veränderungen im Rufverhalten der Männchen führt. Im ersten "Erholungsversuch" ohne EE2 oder andere ED im Expositionswasser, näherten sich die durch EE2 veränderten Rufparameter der Frösche (erniedrigte Anteil an Werberufen und erhöhte Anteil an Rasping) bei den Behandlungsgruppen 10^{-10} M EE2 und 10^{-13} M EE2 wieder den Kontrollwerten an. Bei den Tieren der 10^{-12} M EE2-Behandlung hingegen konnte keine „Erholung" der Effekte der ersten Exposition mit EE2 festgestellt werden. Bei diesen Tieren war vielmehr ein starker und statistisch signifikanter Unterschied im Rufverhalten im Vergleich zu den Kontrollen zu sehen. Tiere dieser Behandlung zeigten weiterhin die östrogen-spezifischen Veränderungen in ihrem Rufverhalten, äußerten also im Vergleich zur Kontrollgruppe einen geringeren Anteil an Werberufen und einen höheren Anteil an Rasping. Die spektralen und temporalen Parameter der Werberufe hingegen, unterschieden sich nach der Erholungszeit nicht mehr von den Kontrollrufen. Bei einer weiteren EE2-Exposition (mit Testsubstanz) nach ca. 8 Wochen zeigten diesmal Tiere beider Behandlungsgruppen (10^{-10} M EE2 und 10^{-12} M EE2) die typischen östrogen-charakteristischen Effekte in ihrem Rufverhalten: sie produzierten weniger Werberufe und mehr Rasping. Diese Effekte schienen sogar stärker ausgeprägt als bei der ersten Exposition gegenüber der Testsubstanz, wofür evtl. potenzierte Wirkung (Potenzierung) aufgrund der wiederholten Exposition verantwortlich sein könnte, ein Phänomen, das bereits bei anderen Spezies nachgewiesen werden konnte (Li et al. 1989, Diamond et al. 1994, Taylor and Jentsch 2001). Auch die in dieser wiederholten Exposition zusätzlich vermehrt geäußerten Ruftypen Ticking und Growling, welche nicht unmittelbar östrogen-spezifisch sind, könnten auf einen solchen Potenzierungseffekt zurückzu-

führen sein. Nach weiteren 8 Wochen „Erholungszeit“ unterschied sich das Rufverhalten der vormals EE2-exponierten Frösche jedoch nicht mehr im direkten Vergleich zur Kontrollgruppe.

Es scheint demnach so, dass Tiere, die bereits einmal gegenüber ED exponiert waren nicht grundsätzlich erneut in Testläufen eingesetzt werden können. Eine verlängerte, expositionsfreie Zeit (z.B. drei Monate) könnte dazu führen, dass durch ED hervorgerufene Effekte tatsächlich wieder verschwinden. Da dies jedoch nicht immer gesichert scheint, müsste das Rufverhalten eines jeden Testindividuums vor einem erneuten Testlauf noch einmal ohne ED-Exposition untersucht werden, um sicherstellen zu können, dass die Tiere tatsächlich kein verändertes Rufverhalten mehr zeigen.

4) Untersuchung von Negativsubstanzen

Sogenannte “Negativsubstanzen” sind Chemikalien, die keine (anti)androgene oder (anti)östrogene Wirkungsweise aufweisen, welche aber trotz allem durchaus in der Lage sein könnten, das Rufverhalten männlicher *X. laevis* zu beeinflussen. Um sicherzugehen, dass solche Stoffe das Paarungsrufverhalten der Tiere nicht oder zumindest in nicht identischer Weise beeinflussen wie (anti)androgene und (anti)östrogene ED, testeten wir die Auswirkungen der folgenden drei Negativsubstanzen auf das Paarungsrufverhalten männlicher *X. laevis* im Durchflusssystem: Diclofenac [DCF], Triiodthyronin [T3] und Metoprolol [METO]. Hierfür exponierten wir männliche Krallenfrösche (3 Jahre, n = 10) gegenüber zwei verschiedenen Konzentrationen der jeweiligen Chemikalien (DCF: 10^{-8} M und 10^{-10} M; T3 und METO: 10^{-9} M und 10^{-10} M) und einer Lösemittelkontrolle (0.00001 % DMSO) im Durchflusssystem, wie oben beschrieben.

Da wir die potentiell erregungssteigernde Wirkungen der Chemikalien, als auch eine mögliche Erniedrigung der sexuellen Erregung der Tiere durch die Negativsubstanzen untersuchen wollten, exponierten wir die Frösche zunächst für 96 h ohne vorherige Stimulation des Paarungsverhaltens mit hCG. Anschließend wurden jeweils 100 Units hCG (gelöst in 50 µL Aqua destillata) in den dorsalen Lymphsack eines jeden Frosches injiziert, um ein grundlegendes Paarungsverhalten der Tiere auszulösen (Russell 1960). Daraufhin wurden die Frösche erneut für 96 h simultan den jeweiligen Substanzen im Durchflusssystem ausgesetzt.

Nach der Exposition wurden die Frösche mittels MS222 (0.01%) anästhetisiert und per Genickschnitt getötet. Wie bereits oben beschrieben, wurde ihr Körpergewicht und –länge gemessen und Gewebeproben (Gehirn, Gonaden, Leber und Milz) entnommen, aus welchen die totale RNA extrahiert und mittels RT in cDNA umgeschrieben wurde. Die darauffolgenden Real-Time PCRs (qPCR) wurden, wie bereits oben, sowie in Efosa et al. (2016) (Anhang 4) und Garmshausen et al. (2015) (Anhang 3) beschrieben, für die Gonadotropine in den Gehirnproben, für RED1, RED2 und ARO in den Gonadenproben und für VTG in den Leberproben durchgeführt. Zusätzlich wurde auch hier die Expression einiger Gene des Häm-Stoffwechsels (HO1, HO2 und BLVRA) in den Leber- und den Milzproben ermittelt.

Für die statistischen Analysen der Verhaltens- und Genexpressionsdaten wurden General Linear Mixed Models (GLMM) herangezogen (Hoffmann 2012) mit nachfolgenden Sidak Post-hoc Tests (SPSS 20, IBM, Ehningen, Germany). Normalverteilungen wurden mittels Kolmogorov-Smirnov Tests sichergestellt.

Für die chemisch-analytischen Untersuchungen wurden Wasserproben zu Beginn und zum Ende der Exposition aus den Testbecken und Vorratsbehältern entnommen und untersucht. Die Proben wurden aufkonzentriert (mittels C18-Kartuschen; Garmshausen et al. (2015); Anhang 3) und Messungen der Stoffkonzentration wurden mittels UHPLC-MS-MS festgestellt.

Im Folgenden sind die Ergebnisse der einzelnen Tests aufgeführt.

DCF-Exposition

Die detailliert beschriebenen und diskutierten Resultate dieses Versuches können dem Manuskript "The pharmaceutical diclofenac interferes with the hypothalamus-pituitary-gonad axis of *Xenopus laevis*" entnommen werden, welches im Journal *Environmental Science and Technology* veröffentlicht werden soll (Anhang 4).

Zusammengefasst konnten wir zeigen, dass Diclofenac (DCF) leicht bis moderat östrogen wirken kann. Eine Exposition gegenüber DCF führte z.B. zu östrogen-ähnlichen Effekten im Rufverhalten der Frösche: die exponierten Tiere riefen einen geringeren Anteil an Werberufen und einen erhöhten Anteil an Rasping, jedoch nur, bevor die Männchen mittels hCG stimuliert wurden. Die stimulierende Wirkung des hCG, welche dazu führt, dass vermehrt Sexualsteroide gebildet werden (Forest et al. 1979, Rasar and Hammes 2006), könnte diesen leicht östrogenen Einfluss des DCF maskiert haben, weshalb nach der Stimulation keine Unterschiede im Rufverhalten im Vergleich zu den Kontrolltieren festgestellt werden konnten. Zudem konnten wir einen Dosis-abhängigen Einfluss von DCF auf die Induktion des östrogenen Biomarkers VTG nachweisen, was ebenfalls auf eine leichte bis moderate direkte östrogene Wirkungsweise von DCF hindeutet. Obendrein konnten wir jedoch auch eine zusätzliche pharmakologische Wirkung des DCF auf die gonadale Steroidgenese feststellen, welche zu einem Ungleichgewicht der Konzentrationsverhältnisse der einzelnen Sexualsteroide führt. Da DCF in beachtlichen Konzentrationen in Oberflächengewässern gefunden werden kann (Ternes 1998, Sacher et al. 2001, Heberer 2002), könnte es aufgrund der durch DCF hervorgerufenen Veränderungen im Rufverhalten der männlichen *X. laevis* zu einem herabgesetzten Paarungserfolg exponierter Tiere kommen, auch wenn der östrogene Einfluss von DCF auf das Verhalten der Frösche eher gering bis moderat ausfiel.
DCF sollte demnach nicht als „Negativsubstanz" angesehen bzw. behandelt werden, sondern als östrogen wirkender ED. Bezüglich der zu entwickelnden Methode XENOCALL kann also gesagt werden, dass die geringe bis moderate, östrogene Wirkungsweise von DCF mittels dieser Nachweismethode detektiert werden konnte.

Triiodthyronin (T3)-Exposition

Wir konnten zeigen, dass T3 das gesamte Rufverhalten, also jegliche Vokalisation der Tiere, reduziert, vor allem wenn sich die Frösche in einem sexuell erregten Zustand (+hCG) befanden und einer höheren Konzentration an T3 ausgesetzt waren. Diese Effekte waren jedoch statistisch nicht signifikant. Das Rufverhalten der Frösche wurde darüber hinaus nicht von einer T3-Exposition beeinflusst. Diese Verminderung der Motivation der Tiere zu rufen, könnte auf einen erhöhten Stresspegel der Tiere zurückzuführen sein, ein Effekt, welcher bereits bei anderen Tierarten in Zusammenhang mit dem Schilddrüsen-Stoffwechsel gebracht werden konnte (Bianco et al. 1987, Mason et al. 1994). Die Genexpressionsprofile von LH, FSH, ARO, RED1 und 2 unterschieden sich nicht von denen der Kontrollgruppe. Des Weiteren konnten wir zeigen, das T3-behandelte Tiere wie erwartet das Schilddrüsen-stimulierende Hormon (TSH) nicht exprimieren und die Expression des Schilddrüsenrezeptors beta (TRβ) stark erhöhen, was eine Bioakkumulation und Verstoffwechselung des T3 verifiziert.

Metoprolol (METO)-Exposition

Eine METO-Behandlung beeinflusste das Paarungsrufverhalten der *X. laevis* nur, wenn sich diese mittels hCG im sexuell stimulierten Zustand befanden. In diesem Fall führte eine METO-Exposition (beide Konzentrationen) zu einem generell erhöhten Rufverhalten. Die Tiere produzierten mehr Rufe, jedoch war dieser Effekt statistisch nicht signifikant. Die weiteren Rufparameter wurden nicht durch METO beeinflusst. Darüber hinaus konnten wir zeigen, das METO weder einen Einfluss auf die Expression der Gonadotropine im

Gehirn, noch auf die der gonadalen Enzyme in den Hoden der Tiere hatte. Auch die VTG-Expression unterschied sich nicht von der der Kontrollfrösche.

Diskussion

Wie sich herausstellte, stellt DCF gar keine „Negativsubstanz" dar, sondern ist tatsächlich ein leicht bis moderat östrogen wirkender ED. Die geringe Östrogenität dieses Stoffes konnte mittels XENOCALL eindeutig nachgewiesen werden, da exponierte Tiere im Vergleich zu den Kontrollen vor der stimulierenden hCG-Injektion die östrogen-typischen Auswirkungen (geringerer Anteil an Werberufen und höheren Anteil an Rasping) zeigten. Darüber hinaus sollte das als Negativsubstanz getestete DCF den Ergebnissen zufolge nicht als Negativsubstanz betrachtet werden (z.B. erhöhte VTG Expression), sondern vielmehr als leichter östrogen wirkender ED. XENOCALL konnte diese leichte Östrogenität des DCF eindeutig nachweisen, da diese Substanz zu den östrogen-charakteristischen Auswirkungen im Rufverhalten der Tiere führte: exponierte Frösche sangen weniger Werberufe und mehr Rasping. Keine der weiteren getesteten Negativsubstanzen (T3 und METO) veränderten das Rufverhalten der Frösche in einer androgen-, antiandrogen- oder östrogen-spezifischen Weise, denn weder das Werberufverhalten (Steigerung: typisch androgen; Abfall: typisch antiandrogen bzw. östrogen), noch der Anteil an Rasping (typisch östrogen) oder Ticking (typisch antiandrogen) wurden durch diese Substanzen beeinflusst.

Schlussfolgerungen

Zusammengefasst kann man sagen, dass die grundlegende XENOCALL-Methode auch erfolgreich in einem Durchflusssystem durchgeführt werden und folglich als effektiver Biomarker für den Nachweis und die Beurteilung (anti)androgener und (anti)östrogener ED verwendet werden kann. Eine Exposition gegenüber allen getesteten (anti)androgenen und (anti)östrogenen ED, mit Ausnahme von BPA, führten zu wirkspezifischen Effekten, die bereits in XENOCALL-Versuchen im semi-statischen System auftraten (Hoffmann and Kloas 2010, Hoffmann 2012, Hoffmann and Kloas 2012b, c). Die Auswirkungen von antiöstrogen wirksamen Substanzen, welche die östrogen-spezifischen Effekte auslöschen müssten (Hoffmann 2012, Hoffmann and Kloas 2012a), müssen in zukünftigen Studien noch detailliert untersucht werden. Nichtsdestotrotz resultierte eine Exposition gegenüber den weiteren getesteten (anti)androgenen und östrogenen ED wie erwartet in wirkspezifischen Effekten im Rufverhalten von *X. laevis*. Eine Ausnahme dieser Regel stellt das vermeintlich östrogen wirksame BPA dar, welches keine östrogen-spezifischen Auswirkungen zeigte, sondern androgen-spezifische Effekte im Rufverhalten der Tiere hervorrief. Da das BPA bei den adulten Tieren jedoch auch nicht zu der für Östrogene typischen Erhöhung der Expression des VTG führte, kann man davon ausgehen, dass diese Substanz bei adulten, männlichen *X. laevis* keine östrogenen Wirkmechanismen auslöst, sondern anderweitig die gefundenen androgen-typischen Effekten im Rufverhalten der Tiere auslöst.

Eine Exposition gegenüber einem ED-Gemisch mit unterschiedlichen Wirkungsweisen, aber auch gegenüber einzelnen ED, wie z.B. EE2 oder VIN, führen zu starken negativen Effekten im Ruf- und Umklammerungsverhalten von männlichen Fröschen, sowie einen herabgesetzten reproduktiven Erfolg. Diese Ergebnisse legen die Vermutung nahe, dass ED-Kontaminationen, wie sie bereits in der Natur, z.B. in Berliner Oberflächengewässern (Hoffmann 2015), gefunden werden, auch in der Umwelt zu einer Herabsetzung des Reproduktionserfolgs und damit der Fitness exponierter Tiere führen können. Diese Folgen könnten demnach einen Beitrag zum globalen Amphibiensterben leisten.

Eine Exposition gegenüber den getesteten Negativsubstanzen (T3 und METO) rief hingegen keine der für (anti)androgene und östrogene ED charakteristischen Effekte hervor, denn weder das Werberufverhalten (Steigerung: androgen; Abfall: antiandrogen bzw. östrogen), noch der Anteil an Rasping (Steigerung: östrogen) oder Ticking (Steigerung: antiandrogen) wurden durch diese Substanzen beeinflusst. DCF hingegen kann nicht als „Negativsubstanz" herangezogen werden, da diese Substanz leichte bis moderate östrogene

Wirkungsweisen aufweist. Mittels XENOCALL konnte diese geringe bis moderate, östrogene Wirkungsweise von DCF eindeutig nachgewiesen werden, da die Tiere die östrogen-charakteristischen Veränderungen im Rufverhalten, einen erniedrigten Anteil an Werberufen und einen erhöhten Prozentsatz an Rasping, zeigten.

Im Durchfluss scheint die XENOCALL-Methode weniger sensitiv zu sein als im semi-statischen System. Stoffkonzentrationen, die im semi-statischen zu hoch signifikanten Auswirkungen führten, waren im Durchflusssystem meist nicht signifikant. Gründe für diese geringere Sensitivität von XENOCALL im Durchfluss könnten die lauteren Hintergrundgeräusche, sowie das regelmäßig in die Becken laufende Wasser und die dadurch entstehenden Strömungen in den Testbecken sein. Denn vor allem wenn Expositionswasser in die Becken floss, konnte gezeigt werden, dass die Frösche sich deutlich gestört fühlten. Sie hörten meist abrupt auf zu vokalisieren und fingen oft auch erst nach geraumer Zeit wieder mit dem Gesang an. Hierbei scheint jedoch die Herkunft der verwendeten Frösche ausschlaggebend zu sein. Frösche aus der institutseigenen Zucht reagierten weniger schreckhaft auf die Testbedingungen als jene Frösche, die von außerhalb (z.B. USA) erworben wurden. Ein Grund hierfür könnte u.a. die Angehörigkeit der Frösche zu unterschiedlichen Unterarten sein, wobei genetische Analysen hierzu noch leider nicht vorliegen. Aber auch unterschiedliche Aufzuchtsbedingungen könnten hier eine Rolle spielen. Wenn die XENOCALL-Experimente also mit einer deutlich geringeren Durchflussrate oder sogar rein semi-statisch bzw. unter Anwendung einer Verknüpfung von Durchfluss (tagsüber) und semi-statischem System (nachts) durchgeführt würden, könnte dieser Biomarker möglicherweise effektiver sein, da er sensitivere Ergebnisse liefern würde. Die unterschiedliche Reaktion der IGB- und NASCO-Frösche auf Stress, wie z.B. dem zulaufenden Wasser, scheint zudem eine große Rolle zu spielen. Sollte sich herausstellen, dass die IGB- und die NASCO-Frösche unterschiedlicher Herkunft sind, bzw. unterschiedlichen Stämmen angehören, könnte eine ausschließliche Verwendung des Stress-resistenteren Stammes zu eindeutigeren und sensitiveren Ergebnissen führen. Auch striktere Validitätskriterien, wie z.B. eine grundlegende, minimale Vokalisationsdauer in jeder einzelnen Expositionsnacht, könnte zu einer erhöhten Sensitivität des XENOCALL Tests führen. Jedoch müssten demnach ggf. deutlich mehr Tiere getestet werden, um diese Validitätskriterien einhalten zu können.

Darüber hinaus ist es nicht grundlegend möglich, Testtiere in mehreren Versuchsdurchläufen wiederzuverwenden, da die durch ED hervorgerufenen Effekte nicht zwangsläufig nach expositions-freien Zeit verschwinden, sondern sogar ausgeprägter werden können. Versuchstiere müssten generell vor jeder Wiederverwendung ohne eine ED-Exposition getestet werden, um sicherzustellen, dass die hervorgerufenen ED-Effekte tatsächlich ausgelöscht sind. Die Umsetzbarkeit solcher “Zwischentests” in der praktischen Anwendung ist jedoch fraglich, da die Tiere in der Zwischenzeit gehältert, gefüttert und versorgt werden müssen (Platz- und Personalbedarf).. Außerdem müssten solche “Zwischentests” ebenfalls im Testsystem durchgeführt und daraufhin auch analysiert werden, was ebenfalls zeit- und kostenaufwendig wäre. Dementsprechend ist die Umsetzbarkeit einer solchen Wiederverwendung von Testtieren zu umständlich und kostspielig. Nichtsdestotrotz sollte das Potential der XENOCALL Methode, bereits verwendete Tiere erneut einsetzen zu können, nicht vollständig verworfen werden. Vielmehr sollte eine längere, grundlegende, Minimaldauer, nach der in keinem Fall die durch EDC hervorgerufenen Effekte mehr nachweisbar sind, gefunden werden, so dass auf die o.g. „Zwischentests“ vollständig verzichtet werden könnte. Allerdings müsste die Tatsache, dass die Tiere während dieser „Erholungszeit“ trotz allem Raum- und Ressourcen-fordernd gehalten und verpflegt werden müssen, weiterhin beachtet werden, was für die Umsetzung der Wiederverwendbarkeit der Testtiere weiter einen limitierenden Faktor darstellen könnte.

Summary

Endocrine disrupting chemicals (EDC) are exogenous chemicals or chemical mixtures that "alter the structure or function(s) of the endocrine system and cause adverse effects at the level of the organism, its progeny, the population, or subpopulations of organisms" (U.S.EPA 1998). EDC include natural substances such as phytohormones and many pharmaceuticals, industrial chemicals and biocides. EDC can mimic endogenous hormone actions, block hormone receptors (Sonnenschein and Soto 1998, Crews et al. 2000) and impact hormone synthesis and metabolism, respectively (Crisp et al. 1998, Vos et al. 2000, Lintelmann et al. 2003). Thereby EDC can adversely affect the physiology and development of invertebrates (Segner et al. 2003, Barata et al. 2004, Clubbs and Brooks 2007, Rodríguez et al. 2007, Contardo-Jara et al. 2011, Lewis and Ford 2012) and vertebrates (Colborn et al. 1994, Tyler et al. 1998, Choi and Jeung 2003, Zala and Penn 2004, Kloas et al. 2009). A main focus is on EDC that interfere with the hypothalamus-pituitary-gonad axis (HPG-axis) and thus affect the reproductive system, including reproductive development, physiology and behavior of (in)vertebrates. Those EDC primarily elicit (anti)androgenic and (anti)estrogenic modes of action (MOA), respectively (Schantz and Widholm 2001, Clotfelter et al. 2004, Scott and Sloman 2004, Zala and Penn 2004, Kloas et al. 2009, Söffker and Tyler 2012).

Biotests are used to detect and assess such EDC. However, most of these testing methods are invasive techniques, in which all tested animals have to be sacrificed or they are *in vitro* techniques, which are limited because they only demonstrate specific mechanisms (e.g. YES, YAS) (Kloas et al. 1999, Kloas et al. 2009) but neglect impacts on the whole organism. Furthermore, these methods lack the required sensitivity to detect environmentally relevant concentrations of EDC after short-term exposure and the ability to detect and distinguish all 4 MOA (androgenic, antiandrogenic, estrogenic and antiestrogenic) within one test. Internationally approved testing methods, which are based on non-invasive, *in vivo* techniques and conform to the 3R-principles in animal testing (replace, reduce, refine), do not exist yet, although recent studies demonstrated that the reproductive behavior of vertebrates, especially aquatic vertebrates might be used as suitable endpoint for detection and assessment of (anti)androgenic and (anti)estrogenic EDC (Colman et al. 2009, Saaristo et al. 2009a, Saaristo et al. 2009b, 2010b, a, Saaristo et al. 2013). However, so far only antiandrogenic as well as estrogenic EDC were shown to result in measurable behavioral impacts.

In recent studies we examined the impacts of (anti)androgenic and (antiestrogenic) EDC on the mate calling behavior of male South African clawed frogs (*Xenopus laevis*) and, on this basis, developed a non-invasive biomarker for the detection and assessment of (anti)androgenic and (anti)estrogenic EDC (Hoffmann and Kloas 2010, Hoffmann 2012, Hoffmann and Kloas 2012b, c, a, d). *X. laevis* is an ideal model organism for the investigation of EDC impacts, because its endocrine system is similar to that of higher vertebrates. Since South African clawed frogs are strictly aquatic, they can be easily exposed to EDC by dissolving the substance in the surrounding water. *X. laevis* is a nocturnal species, living in dark and turbid ponds, thus individuals rely on underwater vocalizations to convey location and mating status (Tinsley and Kobel 1996). Sexually active males attract females by producing advertisement calls and chirping. Receptive females respond to these serenades by swimming directly towards the male (positive phonotaxis). When both individuals encounter each other, the male starts to clasp the female until oviposition and then inseminates the eggs (Tinsley and Kobel 1996). Besides advertisement calling, male *X. laevis* also produce other call types, like growling, ticking and the recently discovered call type rasping (Hoffmann and Kloas 2010, Hoffmann 2012). Most of these call types are mainly produced during male-male-interactions (Tobias et al. 1998), but might also serve other, yet unknown functions.

Recent experimental studies (Hoffmann and Kloas 2010, Hoffmann 2012, Hoffmann and Kloas 2012b, c, a, d) yielded that exposure to environmentally relevant concentrations of EDC with (anti)androgenic and (anti)estrogenic MOA affect the male calling behavior in specific ways, differentiating between the different MOA. Specific effects of exposure of male frogs to the androgenic methyldihydrotestosterone (MDHT), for instance, were elevated proportions of advertisement calls and lower proportions of the call type rasping

(Hoffmann 2012, Hoffmann and Kloas 2012b). Frogs exposed to the antiandrogen vinclozolin (VIN) and the estrogen ethinylestradiol (EE2), respectively, advertised less and several spectral and temporal features of their advertisement call were altered, such as click duration and the number of accentuated clicks (Hoffmann and Kloas 2010, Hoffmann 2012, Hoffmann and Kloas 2012c). Specific features of antiandrogenic exposure were higher proportions of the call type growling (Hoffmann and Kloas 2010, Hoffmann 2012), while elevated proportions of the call type rasping were a unique characteristic features of estrogen exposed animals (Hoffmann 2012, Hoffmann and Kloas 2012c). The antiestrogen fulvestrant (ICI), on the other hand, did not result in altered calling behavior. But if males were co-exposed to EE2 and ICI, the antiestrogen extinguished all estrogenic effects (Hoffmann 2012, Hoffmann and Kloas 2012a).

Hence, the newly established non-invasive testing method differentiates between different MOA within one biomarker test when detecting (anti)androgenic and (anti)estrogenic EDC, solely on the basis of altered calling parameters. Currently used biomarker tests, such as the sexual differentiation of *X. laevis* cannot discriminate properly between each MOA, since estrogenic and antiandrogenic EDC, for example, show the same effects of feminization (Kloas et al. 2009). Furthermore, the new non-invasive procedure proved to be faster (4 instead of at least 21 days of exposure) and probably more sensitive compared to existing testing methods.

The biological relevance of altered calling parameters were tested in female choice experiments (Hoffmann 2012, Hoffmann and Kloas 2012c). Receptive females preferred the advertisement calls of non-exposed control males over the calls of EE2 exposed males. Moreover, females decreased their phonotactic mating behavior when presented with advertisement calls of EE2 exposed males (Hoffmann 2012, Hoffmann and Kloas 2012c). Thus, alterations of temporal or spectral parameters due to EE2 contamination might disable females to discriminate properly within and between *Xenopus* species or let exposed males appear less attractive (Hoffmann 2012, Hoffmann and Kloas 2012c). Since *X. laevis* particularly depend on acoustic advertisement during reproductive seasons, (anti)androgenic and (anti)estrogenic EDC contamination of their aquatic habitats might have dramatic consequences on reproductive success and population dynamics. First experimental studies showed, that also female *X. laevis* can be affected by (anti)androgenic and (anti)estrogenic EDC contamination, however, effects are less prominent and occur only at higher concentrations (Hoffmann 2012, Hoffmann and Kloas 2012b). Supraphysiological concentrations of the androgen MDHT, for example, decreased the phonotactic behavior of females, while physiological concentrations of the same substance resulted in an increase of phonotactic behavior (Hoffmann 2012, Hoffmann and Kloas 2012b).

The aim of the present study was to resolve the question whether the calling behavior of male *X. laevis* is a suitable endpoint for the detection of endocrine active substances in the context of the environmental risk assessment. For this purpose further basic research investigations were necessary. First of all, the potential ecological impact of this endpoint needed to be further clarified. It was shown that female *X. laevis* are less attracted to altered calls uttered by potential mates in laboratory experiments (Hoffmann and Kloas 2012c). However, does that mean that an altered calling behavior due to an EDC exposure can adversely affect the individual mating success and, thus, might even influence the fitness of whole populations? And moreover, what other external factors can influence this behavior (e.g. water temperature, rainfall, etc.)? In this regard, we also needed to examine how intensively the calling behavior has to change in order to classify this endpoint as relevant and adverse. Furthermore, we wanted to evaluate whether the existing XENOCALL method can be transferred to a flow-through system in which the exposure concentrations can be kept constant over the whole experimental period. We examined under which test conditions the calling behavior of male *X. laevis* can be measured particularly sensitively, e.g. we varied the size of the test tanks as well as their relative distance of the test tanks to prevent cross-talk. We additionally tested for what kind of chemicals the calling behavior can be used as particularly sensitive endpoint compared to other (adverse) endpoints and existing biomarkers in amphibians. For this purpose, we tested a number of substances with (anti)androgenic and (anti)estrogenic MOA, that were previously tested in the semi-static system, as well as

further test substances to ensure that such substances cause identical effects. Moreover, we also tested so called negative substances, chemicals which do not elicit (anti)androgenic or (anti)estrogenic MOA to ascertain that such chemicals do not evoke the MOA-specific EDC-effects.

Previous studies (Hoffmann and Kloas 2010, Hoffmann 2012, Hoffmann and Kloas 2012b, c, a, d) indicated that XENOCALL test individuals potentially could be used in several test trials. First tests showed that the alterations in the calling behavior e.g. due to an estrogenic EDC exposure persisted for four to six weeks under control conditions and reached control levels after six to eight weeks without EDC exposure (Hoffmann 2012, Hoffmann and Kloas 2012c). Therefore, it seemed to be possible to use experimental animals in more than one test trial. However, more tests were needed to be conducted to ascertain this hypothesis.

The project was divided in to three work packages, which are described in the following.

Work package 1 – Literature research

In this work package, an intensive literature research was performed to clarify the biological and ecological relevance of, as well as the natural and anthropogenic causes for an alteration of the calling behavior of amphibians in general, and *X. laevis* in particular. It further was investigated whether this endpoint can be affected by EDC at very low, environmentally relevant concentrations and, hence, might be used as a sensitive biomarker for the detection and assessment of (anti)androgenic and (anti)estrogenic EDC. The literature search was performed using various keywords (e.g. calling behavior, courtship behavior, advertisement calling, anuran, amphibian, endocrine disrupting chemicals, biomarkers, estrogen, antiestrogen, androgen, antiandrogen, rain, temperature, external factors, environmental factors, etc.) on the following websites:

1. Web of Science (Thomson Reuters), www.webofknowledge .com
2. Science Direct, www.sciencedirect.com
3. Pubmed, www.pumed.com
4. Google Scholar, https://scholar.google.de.

The literature search revealed that in many amphibian species especially temporal and spectral features of the advertisement calls are necessary for the females to recognize species affiliation and to discriminate between individuals (Loftus-Hill 1971, Picker 1983, Klump and Gerhardt 1992, Schwartz 1994, Bibikov and Nizamov 1996, Gerhardt et al. 1996, Gerhardt and Schul 1999, Gerhardt et al. 2000, Gerhardt 2001, Schwartz et al. 2001, Bush et al. 2002, Schul and Bush 2002, Beckers and Schul 2004, Deily and Schul 2004, Gerhardt 2005b, Vignal and Kelley 2007, Bush et al. 2009, Deily and Schul 2009, Gerhardt and Brooks 2009, Gordon and Gerhardt 2009, Klump and Gerhardt 2013). Previous experiments further suggest that especially temperature but also rainfall and humidity can affect the onset of the mating season and thus also the beginning of the calling behavior (Gerhardt and Mudry 1980, Henzi et al. 1995, Yamaguchi et al. 2008). Furthermore, some aspects of the calling behavior of single amphibian species can also be affected by different temperatures (Gerhardt and Mudry 1980, Yamaguchi et al. 2008). In those cases the preference of the females changes accordingly with the varying temperatures and call parameters, an effect called “temperature coupling” (Gerhardt 1977, Pires and Hoy 1992a, Pires and Hoy 1992b).

Since especially the advertisement calling of many amphibians is androgen-dependent (Wada and Gorbman 1977, Schmidt 1983, Wetzel and Kelley 1983, Penna et al. 1992), it is likely that this behavior might also be affected by EDC, especially EDC with androgenic and antiandrogenic MOA.

Work package 2 – Optimization of the existing method

This work package focused on the optimization and refinement of the semi-static XENOCALL method, including the adaptation of this method to a flow-through system. In a flow-through system, the concentration of the exposure chemicals can be kept constant throughout a whole test run. Therefore, initial XENOCALL tests without exposing the male experimental *X. laevis* to EDC were performed in the flow-through system of the IGB and various parameters were examined. To elicit a sufficient amount of calling behavior of the male frogs, the ideal size of the test aquaria and the optimal distance between two test tanks was determined, to ensure an acoustic and visual isolation of the test animals. The optimization of the XENOCALL method further included an investigation of whether the duration of a test performance can be shortened, e.g. by omitting the habituation period or decreasing the exposure time. This was done by conducting statistical analyses on the calling behavior of the frogs in each individual exposure night during actual test runs to look for the potential to reduce the duration of the test trials. Furthermore, computer software ought to be developed, which enables an automated analysis of all important parameters of the frog calling behavior in a standardized fashion. To assure a steady effect concentration, a sensitive chemical analysis was established for each test substance.

Work package 3 – Experimental testing

In the frame of this work package the actual experimental tests were performed to determine

1) the biological and ecological relevance of the endpoint calling behavior of amphibians.
2) the effects of further substances with (anti)androgenic and (anti)estrogenic MOA on the male calling behavior of *X. laevis* in a flow-through system to ensure the general validity of the XENOCALL testing method.
3) whether already used experimental animals can be reused after an appropriate amount of time without EDC contamination. Previous studies suggested this possibility, since the typical EDC effects on the male calling behavior were shown to vanish after 6-8 weeks under control conditions (Hoffmann 2012, Hoffmann and Kloas 2012c).
4) potential effects of various negative substances on the calling behavior of the male frogs, to be able to exclude that other chemicals than EDC with (anti)androgenic and (anti)estrogenic MOA are able to affect the male calling behavior of *X. laevis* in a similar way.

Furthermore, a sensitive analytical analysis of the test concentrations in each test aquarium have been established for every test trial and test chemical, to be able to assure a steady effect concentration of each test substance.

1) Biological and ecological relevance of the endpoint amphibian calling behavior

A) Long-term EE2 exposure

To be able to assess the biological relevance of an altered calling behavior of male *X. laevis* due to endocrine disruption, we performed a long-term EE2 exposure experiment with subsequent mating experiments. The fertilization rate of treated and untreated males and the quality and quantity of the resulting offspring were recorded. Detailed description of the methods and results, as well as a discussion of the findings can be found in the manuscript “Estrogens can disrupt amphibian mating success” intended for submission to the journal *Hormones and Behavior*. The manuscript can be found in the appendix (Appendix 1).

In short, we found that higher EE2 concentrations (10^{-8} M) can lead to dramatic adverse effects on the male sexual arousal, indicated by a diminished and spectrally altered advertisement calling and clasping behavior. Those EE2 exposed frogs ceased their mate calling behavior entirely after 14 days of exposure and only occasionally individuals clasped their female at all. Consequently, considerably fewer tadpoles hatched in the tanks comprising the progeny of those EE2 exposed males (10^{-8} M) and, more importantly, fewer tadpoles survived until the end of the observation period compared to the tanks containing offspring descending from males of the lower treatment groups, including the controls (mean: 33% versus 94%).

Frogs exposed to 10^{-10} M and 10^{-12} M EE2, respectively, also uttered a lower percentage of advertisement calls and a higher amount of calls indicating a sexually unaroused state, during the first two exposure weeks. However, those parameters returned to control levels towards the end of the 28-day exposure. Males exposed to 10^{-10} M EE2 also clasped their female shorter than 10^{-12} M treated frogs in the competitive mating situation, indicating a monotonic, dose-dependent decrease in sexual arousal. Male *X. laevis* exposed to 10^{-10} M EE2, however, clasped their female longer during the non-competitive mating experiment when compared to males of the control group. This increase in clasping activity coincides with the increased mate calling behavior of those animals during the last exposure week. Hence, a monotonic dose-dependent effect of EE2 on the clasping behavior seems to be only present, when a competing party is involved. Similarly, the hatching and survival rate of tadpoles descending from males that were exposed to 10^{-10} M and 10^{-12} M EE2, respectively, was in similar ranges when compared to the controls. We further could show, that the gene expression of the RED 1 and 2 in the gonads of male *X. laevis* decreased with ascending EE2 exposure concentrations, while the gonadal ARO gene expression increased.

10^{-8} M EE2 exposed frogs uttered a significantly lower amount of advertisement calls, as well as a higher amount of rasping compared to control frogs, indicating a drastic decrease in sexual arousal (Hoffmann and Kloas 2010, 2012c), which might cause a lower reproductive success of EE2 exposed males (Gerhardt et al. 2000, Murphy and Gerhardt 2000, Rosso et al. 2006, Gerhardt and Brooks 2009). Similarly to our previous study (Hoffmann and Kloas 2012c), the advertisement calls of 10^{-8} M EE2 exposed male *X. laevis* used in this study also showed differences in temporal and spectral parameters (call accentuation and click duration) during the whole experiment. Those alterations were previously shown to reduce the sexual attractiveness of those calls toward females (Hoffmann and Kloas 2012c). We could further demonstrate that male *X. laevis* exposed to EE2 at 10^{-8} M cease their mate calling behavior around exposure day 14 entirely. Accordingly, 10^{-8} M EE2 treated *X. laevis* displayed a diminished clasping behavior during the competitive and non-competitive mating situation compared to the controls. Consequently, considerably less tadpoles hatched in the tanks comprising the progeny of those EE2 exposed males (10^{-8} M). Moreover, fewer tadpoles survived until the end of the observation period compared to the other treatment groups, including the controls.

Frogs exposed to 10^{-10} M and 10^{-12} M EE2, respectively, called a lower percentage of advertisement calls and a higher amount of rasping during the first two exposure weeks. However, those parameters returned to control levels towards the end of the 28-day exposure. This decrease in effectiveness of EE2 on the calling behavior of those frogs might indicate an adaption or habituation effect of the test chemical. However, the call accentuation and the duration of clicks of the advertisement calls were severely reduced throughout the whole experimental period. These temporal and spectral alterations can probably also reduce the attractiveness of EE2 exposed males toward females and thus lead to a lowered reproductive success, as it was previously suggested (Hoffmann and Kloas 2012c), although the total vocal output was not affected. Males exposed to 10^{-10} M EE2 clasped their female shorter than 10^{-12} M treated frogs in the competitive mating situation, indicating a monotone dose-dependent decrease in sexual arousal. However, male *X. laevis* exposed to 10^{-10} M EE2 clasped their female longer during the non-competitive mating experiment compared to males of the control group. This increase in clasping activity coincides with the increased mate calling behavior of those animals during the last exposure week. Hence, a monotonic dose-dependent effect of EE2 on the clasping behavior seems to be only present, when a competing party is involved. Future studies should focus on this phenomenon, and address whether EDC effects are more pronounced in situations, which reflect natural

species-specific settings compared to laboratory-generated experiments. Similarly, the hatching and survival rate of tadpoles descending from males that were exposed to 10^{-10} M and 10^{-12} M EE2, respectively, was in similar ranges when compared to the controls. This is again in accordance with the enhanced mate calling behavior of those individuals towards the end of the exposure period and the increased clasping behavior in the second mating experiment.

The mate calling and clasping behavior of male *X. laevis* is androgen-dependent (Kelley and Pfaff 1976, Wetzel and Kelley 1983, Tobias et al. 1993, Kelley 2002). Thus, lower availability of plasma T and DHT levels in EE2 exposed animals, especially at the beginning of the experiment, might have caused the reduction in mating behavior in EE2 treated males. In the present study, the gene expression of the RED 1 and 2 in the gonads of male *X. laevis* decreased with increasing EE2 exposure concentrations, while the gonadal ARO gene expression increased. RED 1 and 2 convert T to DHT and ARO converts T to E2. Therefore, an increase in ARO and decrease in RED 1 and 2 gene expression might have resulted in accumulated bioavailability of E2 and a reduced presence of T and DHT in all EE2 treated males. This might have causes the EE2 induced alterations in courtship behavior.

B) *Simultaneous exposure to EE2 and MDHT*

We further examined whether an exposure of male frogs to a mixture of an estrogenic and an androgenic EDC leads to accumulated, additive effects of the individual substances or results in similar or decreased impacts compared to the individual EDC effects. We therefore exposed adult male *X. laevis* to environmentally relevant concentrations of mixtures of the androgenic MDHT and the estrogenic EE2 (10^{-9} M MDHT + $5*10^{-9}$ M EE2 (MDHT+EE2) and 10^{-9} M EE2 + $5*10^{-9}$ M MDHT (EE2+MDHT), respectively) and a solvent control. Animals were first exposed to the EDC mixture for 96 h, then injected with hCG to stimulate a basic mate calling behavior and again exposed to the mixture for another 96h. A detailed description of the methods and results, as well as the discussion of the findings can be found in the manuscript "Co-exposure to the estrogen ethinylestradiol and the androgen methyldihydrotestosterone causes antagonistic, independent and synergistic impacts on male mate calling behavior of *Xenopus laevis*, vitellogenin induction and heme metabolism, respectively" intended for submission to the journal *Proceeding of the Royal Society, Part C*. The manuscript can be found in appendix 2.

The obtained results clearly demonstrate that at similar concentrations the estrogenic EE2 is much more effective than MDHT regarding the male mate calling behavior, overcoming any stimulatory impact of MDHT. Moreover, additional effects on the calling behavior of the frogs emerged during the co-exposure, which were not detected during sole MDHT and EE2 exposures, respectively. Exposed frogs, for instance, uttered longer advertisement calls with a higher number of clicks, and the frequency of those longer calls was also higher compared to the controls. Furthermore, the estrogen-characteristic enhanced expression of the vitellogenin gene was detected, while the EDC mixture did not affect the expression of gonadotropins and steroidogenic gonadal enzymes. Concerning other endpoints such as heme metabolism, both EDC EE2 and MDHT, have synergistic impacts by decreasing heme oxigenase expression.

Taken together, mixtures of EDC with different MOA can have different outcomes on individuals, because the combination of antagonistic and synergistic effects is hard to predict by single component exposures. Many of those affected endpoints, such as temporal and spectral parameters of the advertisement calls, are relevant at a population level. Thus for adequate risk assessment and protection of aquatic wildlife, there is need for more research to help understanding the various mechanisms of combined EDC mixture effects on aquatic vertebrate behavior and physiology.

2) Testing of further EDC with (anti)androgenic and estrogenic MOA (5 substances)

To be able to perform a direct comparison of the XENOCALL method performed in a semi-static system as previously done (Hoffmann and Kloas 2010, 2012b, c, a, d) and a flow-through system, the effects of some further (anti)androgenic and (anti)estrogenic EDC on the calling behavior of the males were tested using the flow-through system of the IGB-Berlin. The effects of five test substances were analyzed, whereof two chemicals were tested in the semi-static system before: the estrogenic contraceptive EE2 and the antiandrogenic pesticide VIN. The other three test substances were used in XENOCALL experiments for the first time. Those chemicals included the antiandrogenic dichlorodiphenyldichloroethylene (DDE) a metabolite of the insecticide dichlorodiphenyltrichloroethane (DDT), the androgenic steroid trenbolone (TREN), which is widely used on livestock to increase muscle growth and appetite, as well as the supposedly estrogen active synthetic compound bisphenol A (BPA), which can be found in certain plastics and epoxy resins.

For testing EE2 and VIN, we used 8 male individuals per treatment group and tested three different EDC concentrations, whereas an n of 10 was employed, when DDE, TREN and BPA concentrations were examined (two exposure concentrations). All individuals were tested in the flow-through system as described above for 96 h. All test animals received an hCG injection (100 units / 50 µl, as described for EE2+MDHT treated males) prior to the experiment to elicit a basic mate calling behavior.

Exposure chemicals were dissolved in 99% DMSO or ethanol to prepare stock solutions and DMSO concentrations in the stocks were 0.00001%, while solvent concentrations in the test tanks were 0.0000001%. All chemicals were obtained from Sigma Aldrich (Steinheim, Germany). During the experiment, frogs were fed a commercial fish diet (Metabolica, Aller Aqua, Golßen, Germany; 2 pellets) every second day. Water temperature in the test tanks was measured daily. The light:dark cycle was 12:12 h. At the end of the exposure period, all frogs were returned to the animal husbandry of the institute and transferred to different 60 L tanks, according to their assigned treatment. Animals of the EE2 and VIN treatment groups were tested again after a basic recovery time without EDC exposure to determine whether experimental animals can be reused (see below).

For statistical analyses GLMM were applied for the behavioral data as described previously (Hoffmann 2012) and subsequent Sidak post-hoc tests (SPSS 20, IBM, Ehningen, Germany) were performed. Normal distribution of data or residuals was ensured with Kolmogorov-Smirnov tests. For verifying the desired test concentrations, water samples of each tank were taken and analyzed at the beginning and the end of the experiment (Tab. 1).

The results of the single experiments are described in the following.

Exposure to EE2

Compared to the controls, all EE2 treated *X. laevis* uttered a lower percentage of advertisement calls and a higher percentage of rasping. However, implementing all three EE2 treatments, these effects were not significant. If only the 10^{-10} M EE2 treatment was considered, both endpoints differed significantly from the controls. In addition, EE2 exposed male frogs generally tended to utter more growling than the controls. No other call type was affected by an EE2 exposure. Regarding the temporal and spectral parameters of the advertisement calls, only the bandwidth was increased due to a 10^{-10} M EE2 treatment, however implementing all 4 treatment groups in the statistical analysis, this effect was not significant.

Exposure to BPA

Male frogs exposed to BPA at 10^{-9} M uttered a significantly higher amount of advertisement calls and a lower proportion of rasping compared to the control group. 10^{-11} M treated frogs exhibited the same effects, however, differences were not significant. None of the other measured parameters were affected by any BPA treatment. Spectral and temporal parameters of the advertisement calls were not affected by a BPA exposure. Moreover, neither the estrogenic biomarker VTG induction, nor the expression of gonadal steroidogenic enzymes and the gonadotropin FSH were affected by BPA. LH gene expression, on the other hand, was significantly reduced in BPA treated frogs.

Exposure to TREN

Male frogs exposed to TREN at both concentrations vocalized a higher amount of advertisement calls and a lower percentage of rasping compared to the controls. However, this effect was only statistically significant regarding the higher concentrated TREN treatment (10^{-9} M) and was not significant in the 10^{-11} M TREN treatment group. Advertisement calls of frogs exposed to TREN at 10^{-11} M contained significantly less clicks and the frequency of the slow and fast trills of the advertisement calls produced by male frogs of both EDC treatments was significantly higher. No further spectral and temporal parameters were affected by TREN.

Exposure to VIN

Frogs exposed to VIN at 10^{-10} M produced significantly less advertisement calls and more ticking compared to control animals. Advertisement calls of frogs exposed to VIN at 10^{-10} M had significantly longer ICIs and a lower click rate within the slow trill part of the calls, whereas the fast trills were higher in frequency compared to the controls. The other two treatment groups (10^{-11} M and10^{-12} M VIN) did not affect the calling behavior of the frogs. Hence, a LOEC of 10^{-10} M could be determined for the antiandrogenic VIN using the XENOCALL method.

Exposure to DDE

DDE treated animals uttered a lower percentage of advertisement calls and a higher percentage of rasping compared to the controls. This effect was only significant regarding the 10^{-11} M DDE treatment. The percentage of ticking was increased in all DDE treated frogs but differences were not significant. Spectral and temporal parameters of the advertisement calls were not significantly affected by DDE exposure.

Discussion

Taken together, all but one of the tested EDC with (anti)androgenic and estrogenic MOA evoked the mode-specific effects, which were previously also detected applying XENOCALL in a semi-static system (Hoffmann and Kloas 2010, Hoffmann 2012, Hoffmann and Kloas 2012b, c). Androgenic EDC resulted in enhanced sexual arousal of the frogs, indicated by the production of higher percentages of advertisement calls. Both, estrogenic and antiandrogenic EDC lowered this percentage and thus the sexual arousal of the exposed males (Hoffmann and Kloas 2010, 2012c). Moreover, the previously detected differences in calling behavior of estrogen and antiandrogen exposed male *X. laevis* (Hoffmann 2012, Hoffmann and Kloas 2012b, c), which made it possible to distinguish between EDC with these two MOA, were also detectable in the flow-through system. One exception was the industrial chemical BPA. An exposure to BPA caused typical androgen-specific effects in the calling behavior of the amphibians. BPA also did not evoke an increased gene expression of the estrogen biomarker VTG. Thus, it seems as if BPA does not elicit estrogenic MOA in

adult male *X. laevis*, although previous studies suggested an estrogenic MOA for BPA in developing *X. laevis* (BPA exposure led to feminization (Levy et al. 2004)). However, in that study different BPA concentrations lead to varying effects (hormesis effect). Thus it might be possible, that an exposure of *X. laevis* to different concentrations of BPA results in distinct effects at different developmental stages, e.g. the androgen-characteristic effects on the calling behavior in adult males. Furthermore, it cannot be ruled out that different endpoints are affected differently by an exposure to BPA, for instance via different pathways and mechanisms.

Antiestrogenic EDC, which are expected to cancel out estrogenic impacts on the male calling behavior (Hoffmann 2012, Hoffmann and Kloas 2012a) need to be tested in the flow-through system in future experiments and projects.

3) Re-usability of experimental animals

Previously, we could show that the EE2 induced alterations of the calling behavior persist for four weeks under control conditions but can reach control levels again after six to eight weeks without EDC exposure (Hoffmann 2012, Hoffmann and Kloas 2012c). This finding suggests that it might be possible to use experimental animals in more than one test trial. Therefore, male frogs which were prior exposed to EE2 and VIN (see above), were kept under control conditions for approximately 8 weeks after exposure. We then tested the calling behavior of the frogs again in the flow-through system, this time without EDC exposure, to examine whether the males can recover from the EDC effects in general or whether those effects persist. At the end of this experiment, all frogs were returned to the animal husbandry of the institute and transferred to different 60 L tanks, according to their assigned treatment. If the estrogen- and antiandrogen-specific effects vanished after the exposure-free period, we would re-expose the frogs after another 8 weeks under control conditions to the respective EDC and analyze their calling behavior once more, to see whether the typical MOA-specific effects return.

As mentioned in the paragraph “Testing of further EDC with (anti)androgenic and estrogenic MOA (5 substances)”, we could demonstrate that an exposure to environmentally relevant concentrations of EE2 in the flow-through system results in estrogen-specific alterations in the mate calling behavior of male frogs. However, these effects were not statistically significant. In the first recovery trial without EE2 exposure, the previous estrogen-specific impacts (decreased amount advertisement calling, increased proportion of rasping) could not be detected in the frogs of the 10^{-10} M and 10^{-13} M EE2 treatments. In the 10^{-12} M EE2 treatment, on the other hand, the estrogen-specific effects on the amount of different call types, which were visible but statistically insignificant during the EE2 exposure, were even more pronounced without EE2 in the surrounding water. These effects (lower amount of advertisement calls and higher percentages of rasping) were significantly different from the controls. Spectral and temporal parameters of the advertisement calls, on the contrary, did not differ between the treatments and the control group anymore. In a re-trial, where individuals were exposed to the test substance EE2 for 96 h once more, animals of both treatment groups (10^{-10} M EE2 und 10^{-12} M EE2) showed the estrogen-typical effects on the calling behavior again. They called a higher amount of rasping and a lower proportion of advertisement calls. Moreover, animals of both treatments further uttered a higher proportion of growling and ticking, respectively. All of those effects were even more pronounced than in the first EE2 exposure trial. A reason for this increased effectiveness of EE2 on the calling parameters might have been a potentiation effect due to the repeated exposure regime, a phenomenon previously demonstrated in other species (Li et al. 1989, Diamond et al. 1994, Taylor and Jentsch 2001). Accordingly, the call types that were also produced increasingly in the EE2 re-exposure, namely growling and ticking, might also be a result of this potentiation. During the second recovery trial, however, none of the parameters of the calling behavior of the frogs of the two EE2 treatment groups differed from the control treatment.

Hence, it seems that male frogs cannot necessarily be reused in more than one test trial. A prolonged exposure-free period (> 10 weeks) might resolve this problem, but animals definitely have to be tested for an altered calling behavior prior to every test trial to ensure that test animals recovered from potential enduring EDC effects.

4) Testing of negative substances

So called "negative substances" are chemicals, which do not trigger (anti)androgenic and (anti)estrogenic MOA but which still might be able to affect the calling behavior of the male *X. laevis*. To ensure that chemicals without (anti)androgenic and (anti)estrogenic MOAs do not affect this behavior at all or at least not in the demonstrated MOA-specific ways, we tested 3 negative substances (diclofenac [DCF], triiodothyronine [T3] and metoprolol [METO]) and evaluated their impacts on the male amphibian mate calling behavior. We therefore exposed male *X. laevis* (3 years of age; n = 10) to two concentrations of each of the chemicals (DCF: 10^{-8} M and 10^{-10} M; T3 and METO: 10^{-9} M and 10^{-10} M) and a solvent control (0.00001 % DMSO) in the flow-through system as described above. As in previous experiments, frogs were fed a commercial fish diet (Metabolica, Aller Aqua, Golßen, Germany; 2 pellets) every second day and water temperature in the test tanks was measured daily. The light:dark cycle was 12:12 h.

To study potential enhancing effects of the test chemicals on the male courtship behavior (Hoffmann and Kloas 2012b) as well as a possible reduction of this behavior (Hoffmann and Kloas 2012c), we exposed the males to the EDC without prior stimulation of their calling behavior with human chorionic gonadotropin (hCG) (Russell 1960, Hoffmann and Kloas 2010) for 96 h first. Afterwards frogs were injected with 100 units hCG (dissolved in 50 µL distilled water) in the dorsal lymph sack and again exposed to the respective EDC and solvent for 96 h in the flow-through system.

At the end of the exposure period, all frogs were anesthetized using MS 222 and sacrificed. Their body weight and length was measured and tissue samples (brain, gonads, liver and spleen) were taken and immediately shock-frozen in liquid nitrogen. RNA of tissue samples, RT and qPCRs were then performed as described above and in Efosa et al. (2016, submitted) (appendix 4) and Garmshausen et al. (2015) (appendix 3). The gene expression of LH and FSH was measured in brain samples, while gene expression profiles of RED1 and RED2, as well as of ARO were examined in gonad samples. VTG gene expression as well as the expression of HO1 and 2 and BLVRA was measured in liver samples. Primer pair sequences can be found in Efosa et al. (2016, submitted) (appendix 4) and Garmshausen et al. (2015) (appendix 3). Primer pair efficiencies were calculated as demonstrated in Urbatzka et al. (2010). Data was analyzed applying the $\Delta\Delta C_T$ method (Pfaffl 2001, Efosa et al. 2016, submitted) and the elongation factor 1 α (EF) was used as normalizing housekeeping gene. Statistical analyses comprised GLMM for the behavioral and gene expression data as described previously (Hoffmann 2012) with subsequent Sidak post-hoc tests (SPSS 20, IBM, Ehningen, Germany). Normal distribution of data or residuals was ensured with Kolmogorov-Smirnov tests.

To verify the desired test concentrations, water samples of each tank were taken and analyzed once at the beginning and a second time at the end of the experiment (Tab. 1). The experiments were approved by the Berlin State Office of Health and Social Affairs (LAGeSo, reference no. 0093/13).

The results of the single experiments are described in the following.

Exposure to DCF

The detailed results of the DCF exposure experiment can be found in the manuscript "Diclofenac exhibits direct estrogenic modes of action in male Xenopus laevis, and causes further side effects disturbing the hypo-

thalamus-pituitary-gonad axis and mating vocalizations" submitted for publication in the journal *Environmental Science and Technology*. The manuscript can be found in appendix 4.

In short, we could show that DCF elicits slight to moderate estrogenic MOA. An exposure to DCF resulted in the estrogen-characteristic alterations of the calling behavior of male *X. laevis*: exposed frogs uttered a lower percentage of advertisement calls and a higher amount of rasping compared to the controls before being sexually stimulated by hCG. The stimulating potency of the applied hCG treatment, which is known to promote increases in sex steroid production (Forest et al. 1979, Rasar and Hammes 2006) might have masked the decrease in sexual arousal of DCF exposed males. Moreover, exposure to DCF led to a dose-dependent induction of the estrogenic biomarker VTG gene expression. Furthermore, we could demonstrate that DCF can elicit further pharmacological impacts on gonadal steroidogenesis, imbalancing sex steroid ratios. Since DCF can be found in considerable amounts in surface waters (Ternes 1998, Sacher et al. 2001, Heberer 2002), the estrogen-typical alterations of the calling behavior of male *X. laevis* due to an exposure to DCF, might result in a decreased mating and reproductive success, although the estrogenic MOA of DCF was only slight to moderate.

Thus, DCF should not be considered as a negative substance, but rather as an estrogenic EDC. XENOCALL, however, was able to detect the slight to moderate estrogenicity of DCF.

Exposure to T3

T3 was shown to slightly but statistically insignificantly reduce the total vocal output of exposed male frogs, especially when their mating behavior was stimulated with hCG beforehand. No other call parameters were affected by a T3 exposure. The reduction in behavioral motivation might be the result of an elevated stress response to the administered T3, since stress was previously shown to be closely linked to the thyroid metabolism in different species (Bianco et al. 1987, Mason et al. 1994). LH, FSH, ARO, RED 1 and 2 levels did not differ between the treatments. Furthermore, we could show that T3 treated animals did not express the thyroid stimulating hormone (TSH) gene at all, but showed a dose-dependent elevation in thyroid hormone receptor beta (TRβ) gene expression, confirming the bioaccumulation and metabolization of T3 by the male *X. laevis*.

Exposure to METO

METO treatment only affected the calling behavior of the male *X. laevis* when animals where in a sexually stimulated condition (+ hCG). METO at both tested concentrations led to a higher total vocal output compared to the controls. However, these differences were not significant. No other parameters were affected by a METO exposure. Furthermore, METO neither affected gonadotropin gene expression in brain, nor the expression of RED1, RED 2 and ARO in gonads. VTG gene expression in liver samples was also not affected by any METO treatment.

Discussion

As it turned out, DCF does not represent a so called negative substance, but elicits slight to moderate estrogenic MOA. This slight estrogenicity could be precisely detected using XENOCALL, because DCF exposed frogs exhibited the estrogen-typical alterations of the calling behavior (higher amount of advertisement calls and lower amount of rasping).

None of the other two negative substances (T3 and METO) affected the calling behavior of the male *X. laevis* at all or in a similar way to the (anti)androgenic and (anti)estrogenic EDC, affecting the amount of advertisement calls or the call types ticking and rasping, respectively.

Conclusions

Taken all results obtained in this study together, it is reasonable to say that the basic XENOCALL method can be conducted successfully in a flow-through system and, thus, be used as an effective biomarker for the detection and assessment of (anti)androgenic and (anti)estrogenic EDC. All tested EDC, except BPA, caused the expected MOA-specific effects in the calling behavior of the male *X. laevis*. Antiestrogenic EDC, which should cancel out estrogenic impacts on the male calling behavior (Hoffmann 2012, Hoffmann and Kloas 2012a) still need to be tested in the flow-through system. Nevertheless, all other EDC with (anti)androgenic and estrogenic MOA were detectable and distinguishable from each other using XENOCALL. One exception to this rule is the supposedly estrogenic BPA. An exposure to BPA caused typical androgen-specific effects in the calling behavior of the amphibians (elevated advertisement calling). The exposure to BPA did also not result in the estrogen-characteristic induction of the VTG gene expression. Hence, it seems that BPA does not elicit estrogenic MOA in adult male *X. laevis*, but rather induced the androgen-characteristic effects on the male calling behavior via different pathways or mechanisms.

An exposure of frogs to a mixture of EDC with different MOA, but also to single EDC, such as EE2 or VIN, resulted in strong adverse effects on the calling and the clasping behavior, as well as on the reproductive success of the males. The results suggest the possibility that such an EDC exposure can reduce the reproductive success of EE2 exposed animals and these effects might contribute to the global problem of amphibian decline.

Furthermore, the tested negative substances (T3 and METO) did not affect the calling behavior of the males at all or in a different way than the (anti)androgenic and estrogenic EDC: an exposure to these substances did not evoke an altered advertisement calling (increase: androgen-specific; decrease: estrogen- and antiandrogen-specific, respectively), rasping (increase: estrogen-specific) or ticking (increase: antiandrogen-characteristic) behavior. DCF on the contrary cannot be used as negative substance, since this chemical elicits slight to moderate direct estrogenic MOA, e.g. resulting the estrogen-characteristic alterations of the calling behavior, a decreased amount of advertisement calling and a higher percentage of rasping. Using XENOCALL, this slight estrogenicity could be detected decisively.

The XENOCALL method, however, seems to be less sensitive when performed in a flow-through system compared to the semi-static system. Exposure concentrations which led to significant effects semi-statically still resulted in visible and MOA-specific alterations of the calling behavior, but those alterations were mostly not significant. The reason for this lower sensitivity is probably the elevated background noise and especially the water drifts due to the running exposure water of the flow-through system, which both seemed to disturb the male experimental frogs, but especially the males obtained from NASCO (USA). Performing the experiments semi-statically or at least with an extremely low flow-through-rate might, hence, be more effective when testing for (anti)androgenic and (anti)estrogenic EDC using XENOCALL. Furthermore, differences in origin or ancestry might have been the reason for the obvious differences in stress-resistance of the frogs (IGB versus NASCO). Hence, it might be helpful to use the more stress-resistant frog strain for XENOCALL experiments. Moreover, stricter validity criteria, such as a minimum amount of time the individual spent calling during each of the recorded night, might also help to acquire a higher sensitivity of the XENOCALL method. To date the XENOCALL test counted as valid, if the individual was uttering any vocalization during at least one of the recorded nights.

Tests concerning the reusability of experimental animals revealed that male frogs cannot necessarily be reused. A prolonged exposure-free period might resolve that problem, but animals definitely have to be tested for an altered calling behavior prior to every test trial to ensure recovery from potential enduring EDC effects. However, the applicability and practicability of such an “in-between testing” needs to be carefully considered. Frogs need to be kept and fed during the period of time when the animals cannot be used in any test trial. Ecotoxicological laboratories would need to have enough space and resources for keeping the frogs

during those times, which might turn out to be time consuming and costly. Additionally, the "in-between testing" also needs to be performed in the test system under supervision and exposure as well as call analyses take time. Thus, it might be difficult, tedious and expensive to implement the reuse of test animals under practical conditions. Nevertheless, the potential of the XENOCALL method, utilizing already tested animals, should not be discarded entirely. Instead, it might be possible to determine a decisive amount of time after which the EDC-triggered effects are vanished for good, so that an additional "in-between testing" might not be necessary. However, the fact that the experimental animals have to be kept, fed and adequately supplied during the recovery phase cannot be neglected and might constitute a limiting factor.

1 Introduction

1.1 Endocrine disrupting chemicals (EDC)

Exogenous chemicals or chemical mixtures that "alter the structure or function(s) of the endocrine system and cause adverse effects at the level of the organism, its progeny, the population, or subpopulations of organisms" (U.S.EPA 1998) are classified as endocrine disrupting chemicals (EDC). Besides natural substances such as phytohormones, EDC include many anthropogenic chemicals like pharmaceuticals, industrial chemicals and biocides. EDC can mimic the action of endogenous hormones and block hormone receptors, respectively (Sonnenschein and Soto 1998, Crews et al. 2000). They can impact hormone synthesis and metabolism, as well as hormone secretion into the blood stream (Crisp et al. 1998, Crews et al. 2000, Vos et al. 2000, Lintelmann et al. 2003). Through those actions, EDC can adversely affect the physiology and development of various invertebrates (Segner et al. 2003, Barata et al. 2004, Clubbs and Brooks 2007, Rodríguez et al. 2007, Contardo-Jara et al. 2011, Lewis and Ford 2012) and especially vertebrates (Colborn et al. 1994, Tyler et al. 1998, Choi and Jeung 2003, Zala and Penn 2004, Kloas et al. 2009). Affected systems comprise the thyroid system (Kloas et al. 2009, Lorenz et al. 2011), the stress hormone system (Pottinger 2003) and the immune system (Chalubinski and Kowalski 2006, Inadera 2006). EDC that interfere with the hypothalamus-pituitary-gonad axis (HPG-axis) and thereby affect the reproductive system of vertebrates mostly trigger (anti)androgenic and (anti)estrogenic modes of action (MOA) (Schantz and Widholm 2001, Clotfelter et al. 2004, Scott and Sloman 2004, Zala and Penn 2004, Kloas et al. 2009, Söffker and Tyler 2012).

EDC have been detected especially in surface waters that are a main sink for those substances due to surface runoff, inland drainage and especially due to sewage discharge (Falconer et al. 2006, Benotti et al. 2008). Hence, aquatic vertebrates are intensively exposed to EDC and have to cope with the emerging effects (Kloas et al. 2009).

1.2 Endocrine regulation of the reproductive physiology and behavior in aquatic vertebrates

As above mentioned, (anti)androgenic and (anti)estrogenic EDC can interfere with the HPG-axis of vertebrates. The HPG-axis of aquatic vertebrates is organized similar to other vertebrates, e.g. mammals (Matsumoto and Ishii 1992, Kloas et al. 2009) (Fig. 1).

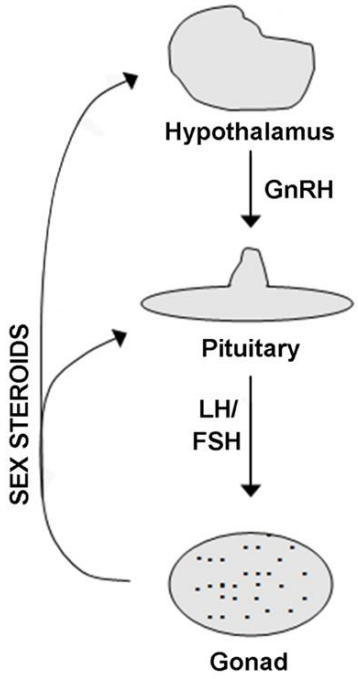

Figure 1: The hypothalamus-pituitary-gonad (HPG) axis. Gonadotropin releasing hormone (GnRH) is discharged from hypothalamic central nervous system to stimulate the secretion of gonadotropins from the pituitary gland. In response to gonadotropins (follicle stimulating hormone [FSH], luteinizing hormone [LH]) the gonads synthesize and secrete sex steroids causing feedback on pituitary and hypothalamus (Hoffmann 2012).

Within the hypothalamus, neurosecretory cells integrate exogenous and endogenous stimuli and regulate the production and secretion of the gonadotropin-releasing hormone (GnRH) accordingly (Zohar et al. 2010, Adler 2012, Norris and Carr 2013). GnRH in turn stimulates the production of the gonadotropins luteinizing hormone (LH) and follicle stimulating hormone (FSH) in the pituitary. LH and FSH are then released into the blood stream and lead to the production and secretion of sex steroids, which in turn regulate homeostasis through negative feedback on the hypothalamus and the pituitary (McEwen 1980, McCreery and Licht 1984, Kloas and Lutz 2006). LH promotes androgen and progesterone secretion, while FSH is responsible for estrogen secretion and lactation (Polzonetti-Magni et al. 1998, Norris and Carr 2013). Androgens, estrogens and progestagens are required for reproductive processes and are involved e.g. in final gamete maturation, ovulation and spermiation (Polzonetti-Magni et al. 1998, Norris and Carr 2013) and the induction of vitellogenesis (Ryffel et al. 1977). They are essential for the formation of secondary sexual characteristics and reproductive behaviors, including courtship and parental care (Beyer 1979, Wetzel and Kelley 1983, Borg 1994, Harvey et al. 1997, Munakata and Kobayashi 2010). In anurans and higher vertebrates testosterone (T) is usually converted in the more potent dihydrotestosterone (DHT) via sex steroid 5α-reductases type 1 and 2 (RED1 and RED2). In fish, however, the predominant androgen is 11-ketotestosterone (11-KT) (Tinsley and Kobel 1996, Kloas et al. 2009). The enzyme aromatase (ARO) converts T to the estrogen 17β-estradiol (E2) (Callard et al. 1978, Levavi-Sivan et al. 2006, Norris and Carr 2013). Those sex steroids act via their respective receptor and hence enable genomic pathways, but can also

activate non-genomic pathways (Aranda and Pascual 2001, Björnström and Sjöberg 2005, Watson et al. 2011).

1.3 Endocrine disruption in aquatic vertebrates with a focus on (anti)androgenic and (anti)estrogenic endocrine disrupting chemicals

EDC with (anti)androgenic and (anti)estrogenic MOA can bind to the respective sex steroid receptor and thereby mimic hormone action or block the receptor so that no other hormone can bind to it. Additionally, various (anti)androgen and (anti)estrogen active EDC can alter sex steroid synthesis and bioavailability indirectly e.g. by increasing or decreasing the availability of the gonadotropins LH and FSH or of enzymes involved in steroid production, such as ARO and RED1 and RED2. Hence, the HPG-axis offers ample target sites for EDC action (Kloas et al. 2009).

1.3.1 Effects of (anti)androgenic and (anti)estrogenic endocrine disrupting chemicals on the reproductive biology of aquatic vertebrates

While antiandrogenic and estrogenic EDC were shown to have feminizing effects in fishes and amphibians (Kloas et al. 1999, Bayley et al. 2002, Kloas 2002, Bögi 2003, Bögi et al. 2003, Brion et al. 2004, Oka et al. 2006), androgenic EDC cause masculinization of phenotypes (Kloas 2002, Bögi et al. 2003). Furthermore, androgen active chemicals were shown to cause germ cell necrosis and ovarian atresia in juvenile fish and adult amphibians (Hahlbeck et al. 2004, León et al. 2007). In adult amphibians, these effects were suggested to be evoked by a decrease of circulating and brain LH concentrations (Tsai et al. 2005, Urbatzka et al. 2007). In adult fish, on the other hand, androgenic EDC were demonstrated to stimulate Sertoli cells and spermatogenesis in males, whereas oogenesis and vitellogenesis were suppressed in females (van der Ven et al. 2003, Chikae et al. 2004, Bogers et al. 2006). Exposure to various antiandrogenic EDC can also cause malformations of male and female gonads in aquatic vertebrates (Bayley et al. 2002, Bögi et al. 2003, Kiparissis et al. 2003, León et al. 2007, Cevasco et al. 2008), reduce sperm count, fecundity and fertility (Makynen et al. 2000, Martinovic et al. 2008, Sebire et al. 2008) and diminish the expression of secondary sexual characteristics and courtship behavior (Baatrup and Junge 2001, Bayley et al. 2003, Behrends et al. 2010, Hoffmann and Kloas 2010). Estrogenic EDC were shown to result in abnormal gonadal function and morphology (Brion et al. 2004, Fenske et al. 2005, Oka et al. 2006, Cevasco et al. 2008, Xu et al. 2008), reduced expression of secondary sexual characteristics (Brion et al. 2004, Hayes et al. 2010) and impaired reproductive behavior (Bayley et al. 1999, Colman et al. 2009, Saaristo et al. 2009a, Saaristo et al. 2009b, Partridge et al. 2010, Saaristo et al. 2010b, Hoffmann and Kloas 2012c) in fish and amphibians. Furthermore, estrogens lead to an increased gene expression and elevated plasma levels of the egg yolk precursor protein vitellogenin (VTG) in various species (Denslow et al. 1999). These estrogenic effects were suggested to be caused by direct effects of the EDC on respective estrogen receptors (ER) or indirect effects, e.g. by upregulation of ARO action (Scholz and Gutzeit 2000, Kuhl et al. 2005, Hoffmann and Kloas 2012c). Antiestrogens can also act directly by blocking ER and indirectly by affecting the normal turnover of estrogens, e.g. by inhibiting ARO action (Chikae et al. 2004, Sun et al. 2009, Sun et al. 2011). Those effects were shown to result in phenotypic masculinization in fish (Andersen et al. 2004, Kitano et al. 2007) and underdeveloped, non-functioning gonads in fish and amphibians (Kloas 2002, Bögi et al. 2003, Rasmussen et al. 2005, Cevasco et al. 2008). Antiestrogenic EDC were furthermore shown to reduce or even neutralize some of the above mentioned estrogenic EDC effects on gonad morphology (Rastogi and Chieffi 1975) and courtship behavior (Hoffmann and Kloas 2012a).

1.3.2 Present *in vivo* methods for the detection and assessment of endocrine disrupting chemicals

The Organisation for Economic Co-operation and Development (OECD) develops standardized OECD test guidelines (OECD TG) for evaluating the endocrine disrupting potential of chemicals, which are applied correspondingly in all OECD member states e.g. in the EU within the frame of the REACH (registration, evaluation, authorization and restriction of chemicals), biocides or plant protection products regulation. The conceptual framework (CF) of the OECD for testing and assessment of EDC comprise 5 levels. At the first level, existing data and non-test information is gathered, for instance available (eco)toxicological data from standardized and non-standardized tests and information regarding physical and chemical properties of the respective chemical. The next level includes the testing of the chemicals using *in vitro* assays, which provide data about the endocrine mechanism(s) and/or pathways (e.g. receptor transactivation and binding affinities). The third, fourth and fifth level imply the testing of focus substances using *in vivo* methods. First of all, applying the third step, more data concerning selected endocrine mechanisms(s) and pathway(s) should be made available. In a next step, *in vivo* assays should provide evidence (if any) for adverse effects on relevant endpoints and at the level 5 the *in* vivo assays should provide more comprehensive data (if any) on adverse effects on relevant endpoints over extensive parts of the life cycle of the organisms. Information on a respective chemical gathered at a low level of the CF should be used to assess what kind of tests at higher CF-levels are needed to be able to evaluate whether this substance should be classified as EDC or not (OECD 2012).

In vivo assays assessing (anti)androgenic and (anti)estrogenic MOA include various tests examining mammals and birds (e.g. rodents: Hershberger Assay, Female/Male Pubertal Development Assay, Uteroptrophic Assay; birds: Avian Two-generation Toxicity Test), but also comprise tests on aquatic vertebrates, such as fishes and amphibians. One of these tests, for instance, is the Fish Short-Term Reproduction Assay (OECD TG No. 229), in which sexually mature male and spawning female fish are exposed to the respective chemical for 21 days. During that period survival, reproductive behavior and secondary sexual characteristics of the fish, as well as the fecundity and fertility are monitored (Ankley et al. 2001). Additionally, various histological and biochemical endpoints that are important for the HPG-axis are evaluated, e.g. the analysis of plasma VTG levels (OECD 2009). The Fish Sexual Development Test (OECD TG NO. 234) is used for the examination of potential endocrine disruptors as well. Fish are exposed from the stage of a newly fertilized egg until the completion of sexual differentiation (OECD 2011). The VTG plasma levels, as well as the genotypic/phenotypic sex ratio, including intersex and undifferentiated fish are measured as endpoints. The Medaka Extended One Generation Reproduction Test (MEOGRT, OECD TG No. 240), on the other hand, is an extended full life cycle assay using Japanese medakas (*Oryzias latipes*), which are exposed to the test chemical as adults (F0 generation). The exposure continues until successful reproduction and development of the F1 generation and until hatch of the F2 generation. The endpoints measured are similar to those of the Fish Short-Term Reproduction Assay and the Fish Sexual Development Test. The Larval Amphibian Growth and Development Assay (LAGDA, OECD TG No. 241) is a test evaluating the potential effects of chemicals on the HPG-axis of amphibians. Larval South African clawed frogs (*Xenopus laevis)* are exposed to the respective chemical until metamorphosis. Endpoints such as phenotypic/genotypic sex ratios, as well as gonad morphology, reproductive duct histopathology and biochemical endpoints like VTG gene expression are used to examine potential (anti)androgenic or (anti)estrogenic effects of the test substance.

One problem with the above mentioned assays is that each of the tests uses invasive techniques. The experimental animals are exposed to the respective test chemicals for a long time and eventually have to be sacrificed. Non-invasive alternatives, which are in accordance with the 3R-strategy (replace, reduce refine), do not exist yet. The 3R-strategy is an international demand for the development and implementation of alternatives of animal testing to avoid the use of live animals (replace) or at least reduce the number of test

animals (reduce) and minimize potential pain, suffering or distress (refine) (Russell et al. 1959). Nevertheless, there are first approaches regarding the implementation of some aspects of the 3R-strategy to the standardized testing of chemicals regarding their endocrine disrupting potential.

Baatrup and Junge (2001), for example developed a computer software that automatically quantifies various aspects of the courtship behavior of the male guppy (*Poecilia reticulata*), which are affected by antiandrogenic EDC. They demonstrated that a fish diet containing antiandrogenic EDC (0.1 – 100 µg/g fodder for 30 days) can cause an altered posturing behavior and sigmoid display, the most conspicuous courtship features in male guppies. By implementing software that analyzes the posturing behavior and the sigmoid display of the fish, any given antiandrogenic EDC can be detected and assessed automatically in a standardized fashion, without killing the experimental animals. Although this approach is a first step in the right direction, the method has one disadvantage; only antiandrogenic EDC can be detected, other EDC with androgenic, estrogenic and antiestrogenic MOA are neglected.

In a semi-static system, we could previously show that the calling behavior of male South African clawed frogs is affected by minimal, environmentally relevant concentrations of (anti)androgenic and (anti)estrogenic EDC (Fig. 2) (Behrends et al. 2010, Hoffmann and Kloas 2010, Hoffmann 2012, Hoffmann and Kloas 2012b, c, a, d).

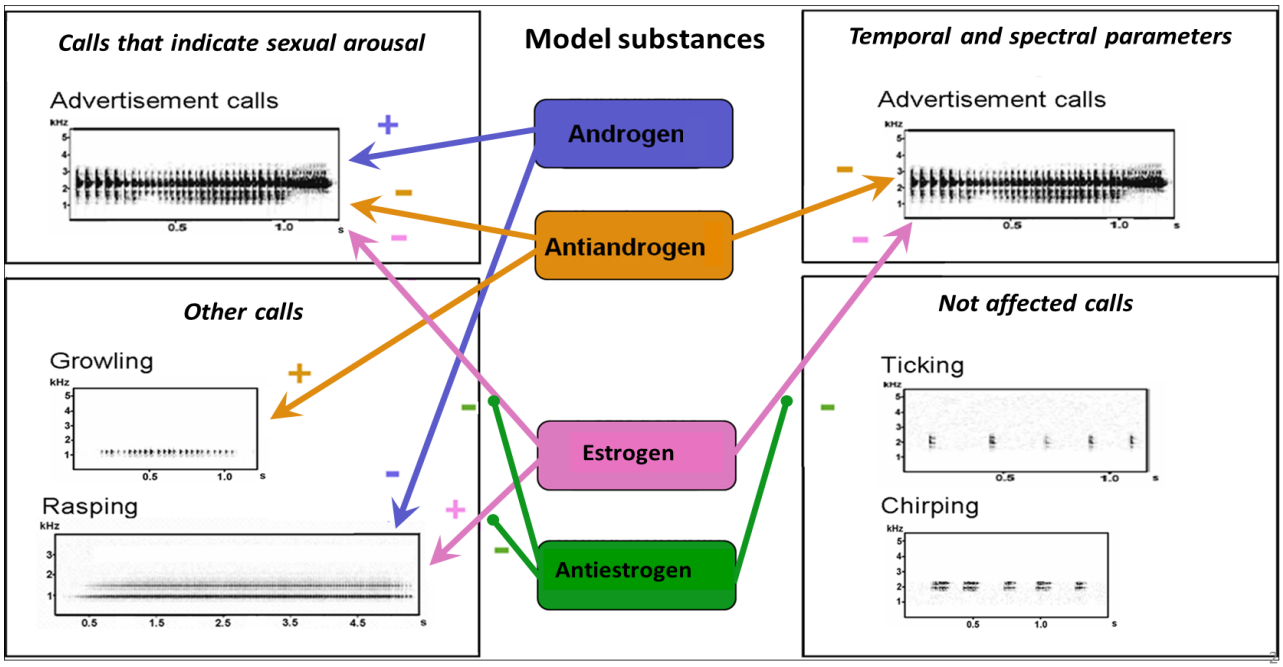

Figure 2: Schematic diagram of the various effects of (anti)androgenic and (anti)estrogenic endocrine disrupting compounds. While the androgenic methyldihydrotestosterone (MDHT, blue color) increases the percentages of advertisement calls uttered and decreases the percentages of the call type rasping, the antiandrogen vinclozolin (VIN, orange color) decreases the relative proportions of advertisement calls and elevates the percentages of the call type growling. VIN furthermore decreases the number of accentuated clicks, as well as the duration of clicks within advertisement calls (temporal and spectral analyses). The estrogenic 17alpha-ethinylestradiol (EE2, pink color) also lowers the relative amount of advertisement calls and reduces the number of accentuated clicks and the click duration. EE2, however, does not affect growling but lowers the call type rasping. When EE2 and the antiestrogenic fulvestrant (ICI, green color) are co-administered, ICI inhibits each EE2 effect (Hoffmann 2012)

The EDC-effects were highly characteristic, so that it was even possible to differentiate between the four different MOA. *X. laevis* is an ideal model organism for investigating EDC impacts, since its endocrine

system is more similar to that of higher vertebrates compared to fish (e.g. DHT as most potent androgen, not 11-KT). Furthermore, South African clawed frogs are aquatic and can be easily exposed to EDC by dissolving the substance in the surrounding water. *X. laevis* is a nocturnal species, living in dark and turbid ponds, which is why individuals rely on underwater acoustic vocalizations to convey location and mating status (Tinsley and Kobel 1996). Receptive females respond to these serenades by swimming directly towards the male (positive phonotaxis). When both individuals encounter each other, the male starts to clasp the female until oviposition and then inseminates the eggs (Tinsley and Kobel 1996). Male vocalizations comprise five different call types (Tobias et al. 1998, Tobias et al. 2004, Hoffmann and Kloas 2010) and each call type is "composed of repetitive trills, consisting of trains of click sounds, brief and noisy sound elements in a frequency range between 1 kHz and 3 kHz" (Hoffmann 2012). Those click sounds are produced by muscle contractions of the larynx (Fig. 3) (Yager 1982), which is innervated by several nuclei of the vocal (motor) pathway, distinct neural circuits in the central nervous system (CNS) of the frogs (Wetzel et al. 1985, Emerson and Boyd 1999, Brahic and Kelley 2003).

Figure 3: Larynx (vocal organ) of male *Xenopus laevis*. The major structural components of the larynx include hyaline cartilage (hc) which forms the cartilaginous box and arytenoids cartilage which forms the sound producing arytenoid disks (ad), surrounded by elastic cartilage (ec). Sounds are produced when the laryngeal bipennate muscles contract and, acting via tendinous insertions onto the arytenoids disks, pull the disks apart and thereby produce a click sound (modified from Kelley and Tobias (1999)).

Those highly stereotyped call types differ from each other in spectral and temporal parameters (Tobias et al. 1998, Hoffmann and Kloas 2010, 2012c). Sexually active males attract females by producing advertisement calls and chirping. Besides those calls, male *X. laevis* also produce other call types, like growling, ticking (Tobias et al. 1998) and the recently discovered call type rasping (Hoffmann and Kloas 2010, 2012c). Most of these call types are mainly produced during male-male-interactions (Kelley and Tobias 1999), but might also serve other, yet unknown functions (Hoffmann and Kloas 2010, 2012b, c, a). It was suggested that, the larynx of *X. laevis* is the effector organ for the male-specific vocalizations. Laryngeal muscle fibers express high levels of androgen receptors (AR) and also ER (Kelley and Pfaff 1976, Kelley 1980, Sassoon et al. 1987, Segil et al. 1987) and have been shown to be a direct target of androgen action (Kelley 1980, Gorlick and Kelley 1987, Kelley and Tobias 1999). Moreover, nuclei of the vocal pathway (Fig. 4) and neurons of the cranial nerve nucleus IX-X that innervate the larynx (Wetzel et al. 1985, Emerson and Boyd 1999, Brahic

and Kelley 2003) express AR and ER and therefore could also be direct targets of (anti)androgenic and (anti)estrogenic EDC action (Kelley and Pfaff 1976, Pérez et al. 1996, Pérez and Kelley 1996, Hoffmann and Kloas 2010, 2012b, c, a, d).

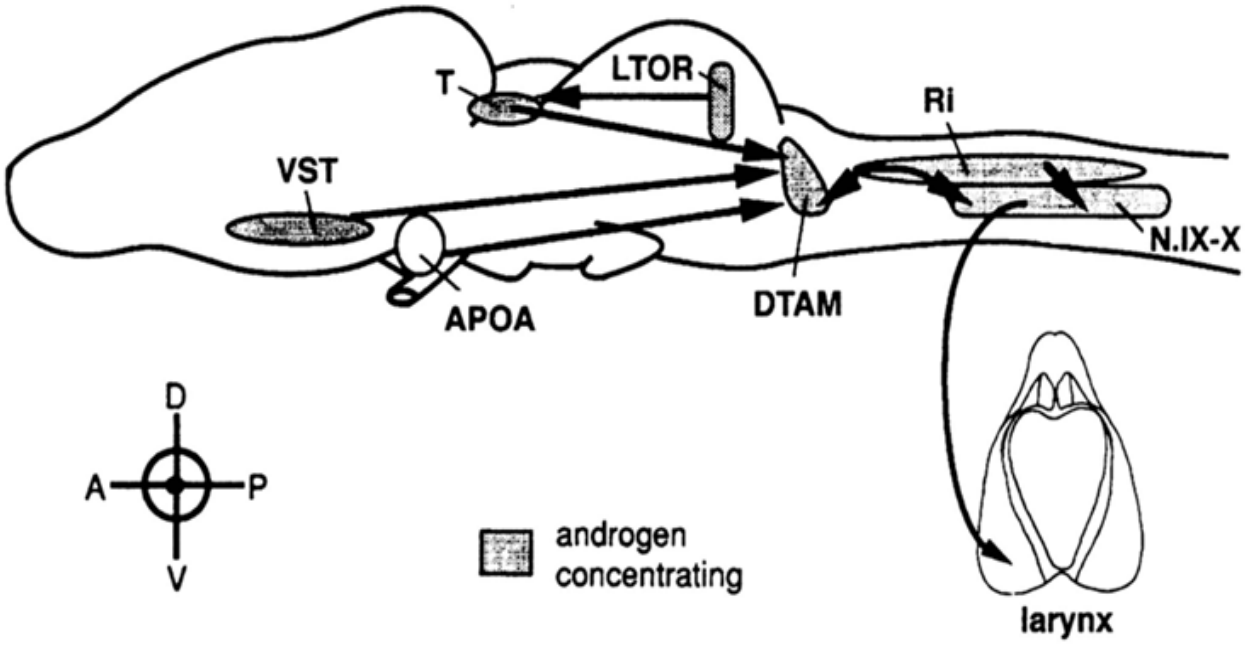

Figure 4: The vocal pathway of *Xenopus laevis*. Motor neurons in the cranial nerve nucleus IX-X (N. IX-X) project via the laryngeal nerve to the bipennate muscles. N IX-X contains motor neurons and interneurons and receives projections from adjacent inferior reticular formation (Ri) and a superior reticular nucleus, the dorsal tegmental area of the medulla (DTAM); in males this connection is reciprocal. DTAM receives input from the dorsal diencephalon (auditory thalamus: T) and ventral telencephalon (APOA: anterior preoptic area; VST: ventral striatum). The laminar nucleus of the torus semicircularis (LTOR) provides auditory input to the thalamus (from Kelley and Tobias (1999)).

Analyses of the calling behavior of short-term exposed male *X. laevis* (96 h) revealed that (anti)androgenic and (anti)estrogenic EDC cause characteristic alterations of calling behavior making it possible to discriminate between the tested EDC with different MOA (Fig. 2). Specific effects of an exposure of male frogs to the androgenic methyldihydrotestosterone (MDHT), for instance, were elevated proportions of advertisement calls and lower proportions of the call type rasping, indicating an increase in sexual arousal of exposed frogs (Hoffmann and Kloas 2012b). Frogs treated with the antiandrogen active fungicide vinclozolin (VIN) or the estrogenic ingredient of many contraceptives, 17α-ethinylestradiol (EE2), on the other hand, advertized less, indicating a lowered sexual arousal compared to untreated frogs (Hoffmann and Kloas 2010, 2012c). Moreover, advertisement calls of antiandrogen or estrogen exposed frogs both showed altered spectral and temporal features, such as lower click durations and a reduced number of accentuated clicks (Hoffmann and Kloas 2010, 2012c). Those altered call features were shown to result in a lower attractiveness of the exposed males towards females (Hoffmann and Kloas 2012c). Surprisingly, there were also obvious differences in to the effects of antiandrogenic and estrogenic EDC, respectively. A characteristic effect of an antiandrogenic exposure was a higher proportion of the call types growling and to some extent also ticking (Hoffmann and Kloas 2010), while an elevated proportion of the call type rasping was a unique feature of estrogen exposed animals (Hoffmann and Kloas 2012c). The antiestrogen fulvestrant (ICI), in contrast, did not result in any alterations of the calling behavior of male frogs. But if males were exposed to EE2 and ICI simultaneously, the antiestrogen reduced or even extinguished all estrogenic effects (Hoffmann and Kloas 2012a).

Remarkably, this new and non-invasive testing method, named XENOCALL, differentiates between the four different MOA within one test when detecting and assessing (anti)androgenic and (anti)estrogenic EDC only on the basis of altered calling parameters. It has also been proven to be faster (4 nights instead of at least 21 days of exposure) and considerably more sensitive compared to existing testing methods: concentrations up to 0.3 ng/L EDC (EE2) could be detected (Hoffmann and Kloas 2012c). In addition, alterations of the calling behavior, e.g. due to an estrogenic EDC exposure, persisted four weeks under control conditions but reached control levels again after six to eight weeks without EDC exposure (Hoffmann and Kloas 2012c). This finding suggests that it might be possible to test experimental animals in more than one test trial.

1.4 Aim of the project

The aim of the present study was to resolve the question whether the calling behavior of male *X. laevis* is a suitable endpoint for the detection of endocrine active substances in the context of environmental risk assessment. For this purpose basic research investigations were necessary. First of all, the potential ecological impact of this endpoint needed to be further clarified. It was shown that female *X. laevis* are less attracted to the altered calls uttered by potential mates in laboratory experiments (Hoffmann and Kloas 2012c). However, does that mean an altered calling behavior due to EDC exposure adversely affects the individual mating success and, thus, might even influence the fitness of whole populations? And moreover, what other external factors can influence this behavior (e.g. water temperature, rainfall, etc.)? In this regard, we also needed to examine how intensively the calling behavior has to change in order to classify this endpoint as relevant and adverse. Furthermore, we wanted to evaluate under which test conditions the calling behavior of male *X. laevis* is most sensitively measurable and to which extent those test conditions reflect environmentally realistic circumstances. We additionally examined for what kind of chemicals the calling behavior can be used as particularly sensitive endpoint compared to other (adverse) endpoints and existing biomarkers in amphibians. For this purpose, a number of further substances with (anti)androgenic and (anti)estrogenic MOA were tested, as well as environmentally relevant negative substances, without the above MOAs.

Within this project three work packages were defined, which are specified in the next chapter.

2 Performance description - the three work packages

2.1 Work package 1: Literature research

In this work package, a literature search ought to be performed, clarifying the biological and ecological relevance of an altered mate calling behavior e.g. due to an exposure to (anti)androgenic and (anti)estrogenic EDC in amphibians in general, and *X. laevis* in particular. Previous studies investigating the impact of external factors such as water temperature, humidity or rainfall on the mate calling behavior of amphibians should further shed light on the relevance of this behavior for the mating success of those animals. Additionally, the practicability and sensitivity of existing endpoints and biomarkers for the detection and assessment of (anti)androgenic and (anti)estrogenic EDC should be compared to the new (optimized) XENOCALL method.

2.2 Work package 2: Optimization of the existing method

In this work package, the existing semi-static XENOCALL method ought to be further optimized and refined. For this purpose, the experiments should be adapted to a flow-through system, in which the concentration of the exposure chemicals can be kept constant throughout a whole test run and possibly increase the sensitivity of the test system. To eliminate as many disturbing factors as possible, we needed to examine various parameters, such as the ideal size of the test aquaria, the optimal distance between to test tanks to ensure acoustic and visual isolation of the test animals, and the best flow-through volume. Moreover, to assure a steady effect concentration, a sensitive chemical analysis needed to be established for each test substance.

The optimization of the XENOCALL method also included the investigation, whether the duration of a test performance can further be shortened, e.g. by omitting a habituation period or decreasing the exposure duration. Furthermore, computer software ought to be developed, which enables a completely automatic and standardized analysis of all important parameters of the calling behavior, such as classification of call types and spectral and temporal features of the calls. This software should decrease the amount of time necessary for test analyses.

2.3 Work package 3: Experimental testing

Within the frame of this work package, the issue of the environmental relevance of the endpoint calling behavior of male *X. laevis* ought to be addressed. We should determine how intensively this behavior has to change in exposed animals in order to result in adverse effects, e.g. a reduced individual mating success, which might result in adverse population effects. Previous investigations are limited to one EDC per tested MOA (Hoffmann and Kloas 2010, 2012b, c, a). Thus, additional EDC with (anti)androgenic and (anti)estrogenic MOA should be tested to ensure the general validity of the XENOCALL method (3 – 5 substances) especially with regard to the environmental risk assessment. Furthermore, potential effects of various negative substances (3 chemicals) on the calling behavior of the males had to be examined to further exclude, that chemicals other than (anti)androgenic and (anti)estrogenic EDC are able to affect the considered parameters of the male calling behavior of *X. laevis.* A sensitive chemical analysis of the test concentrations in each test aquarium had to be performed, to be able to assure a steady effect concentration of each test substance. Previous studies suggest that the EDC effects on the male calling behavior vanish after 6-8 weeks under control conditions (Hoffmann 2012, Hoffmann and Kloas 2012c). Hence, we wanted

to determine whether experimental animals can be reused after an appropriate time without EDC contamination.

3 Work package 1: Literature research – Methods, results and discussion

3.1 Methods

The literature search was performed using various keywords (e.g. calling behavior, courtship behavior, advertisement calling, anuran, amphibian, endocrine disrupting chemicals, biomarkers, estrogen, antiestrogen, androgen, antiandrogen, rain, temperature, external factors, environmental factors, etc.) together with the following websites:

1. Web of Science (Thomson Reuters), www.webofknowledge.com
2. Science Direct, www.sciencedirect.com
3. Pubmed, www.pumed.com
4. Google Scholar, https://scholar.google.de.

3.2 Results and discussion

An intensive literature search revealed that in many amphibian species, including *X. laevis*, temporal features of the vocalizations are crucial for species and individual recognition (Loftus-Hill 1971, Picker 1983, Klump and Gerhardt 1992, Schwartz 1994, Bibikov and Nizamov 1996, Gerhardt et al. 1996, Gerhardt and Schul 1999, Gerhardt et al. 2000, Gerhardt 2001, Schwartz et al. 2001, Bush et al. 2002, Schul and Bush 2002, Beckers and Schul 2004, Deily and Schul 2004, Gerhardt 2005b, Vignal and Kelley 2007, Bush et al. 2009, Deily and Schul 2009, Gerhardt and Brooks 2009, Gordon and Gerhardt 2009, Klump and Gerhardt 2013). Spectral cues, on the other hand, seem to be more important for the degree of attractiveness of a respective call (Gerhard 1974, Gerhardt 1991, Bibikov and Nizamov 1996, Gerhardt et al. 1996, Murphy and Gerhardt 1996, Murphy and Gerhardt 2000, Beckers and Schul 2004, Gerhardt 2005a, Deily and Schul 2006, Vignal and Kelley 2007, Gordon and Gerhardt 2009). Even small differences in temporal and spectral characteristics of male mating vocalizations, e.g. due to an exposure to EDC (Hoffmann and Kloas 2010, 2012b, c, a) can result in a lower attractiveness towards females, among others shown by a lower amount of female phonotactic behavior (Dyson and Passmore 1988, Gerhardt and Klump 1988, Gerhardt 1991, Gerhardt et al. 2000, Bush et al. 2002, Lynch et al. 2005, Deily and Schul 2006, 2009, Gordon and Gerhardt 2009, Hoffmann and Kloas 2012c). If alterations are prominent, females might not even recognize the calling male as a potential mating partner (e.g. Fig. 5) (Vignal and Kelley 2007, Hoffmann 2012).

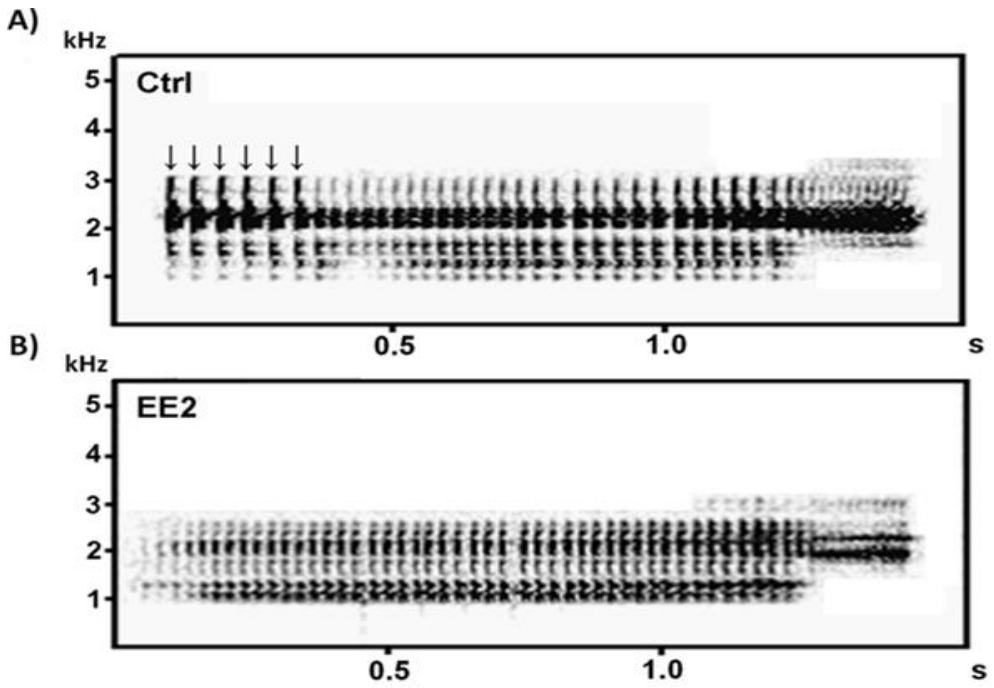

Figure 5: Spectrogram of an advertisement call of (A) an unexposed control male (CTRL) with six accentuated clicks at the beginning of the call (indicated by vertical arrows) and (B) an EE2 exposed male (10^{-8} M) with no accentuated clicks.

Previous experiments further suggest that water temperature and humidity have no or just negligible effects on the calling behavior of male *X. laevis* (Hoffmann 2012). However, studies investigating other amphibian species showed that especially temperature but also rainfall and humidity can affect this behavior (Gerhardt and Mudry 1980, Henzi et al. 1995, Yamaguchi et al. 2008). Nevertheless, temperature and other environmental factors almost exclusively affect the onset of advertising season as well as the daily beginning and duration of the calling (Blankenhorn 1971). Some aspects of calls uttered by male *Hyla* species, such as the trill rate and the frequency, however, might also be affected by different temperatures (Gerhardt and Mudry 1980, Yamaguchi et al. 2008). In those cases the preference of the females changed accordingly with the varying temperatures, an effect called "temperature coupling" which was shown in various species (Gerhardt 1977, Pires and Hoy 1992a, Pires and Hoy 1992b).

Previous studies could show that single EDC with (anti)androgenic and (anti)estrogenic MOA, respectively, can be detected *in vivo* using fish and amphibian species (Bell 2001, Veldhoen and Helbing 2001, Bell 2004, Pawlowski et al. 2004, Sun et al. 2007, Cevasco et al. 2008, Xu et al. 2008, Colman et al. 2009, Kloas et al. 2009, Salierno and Kane 2009, Saaristo et al. 2010b, Sun et al. 2011, Wang et al. 2011, Holbech et al. 2012, Lei et al. 2013, Baumann et al. 2014a). But all of those methods lack the sensitivity to detect and assess EDC at environmentally relevant concentrations and/or the ability to detect and distinguish all four MOA within just one test. Moreover, the exposure duration of all existing EDC biomarkers and detection methods is long (21to 28 days) and most applied techniques are very expensive. XENOCALL is so far the only method for the detection and assessment of (anti)androgenic and (anti)estrogenic EDC which is fast (96 h), comparably inexpensive and detects all four MOA within one test (Hoffmann 2012). Furthermore, XENOCALL is a very sensitive and most notably a non-invasive method implementing several aspects of the 3R-strategy. Hence, XENOCALL constitutes a potential biomarker for the detection and assessment of EDC comprising several advantages compared to the existing biomarkers.

4 Work package 2: Optimization of the existing method

4.1 Establishment of a flow-through system

The flow-through system at the IGB-Berlin, which was used for our experiments is described fully in Efosa et al. (2016, submitted) and Garmshausen et al. (2015) (appendix 4 and 3). Initial test trials in the flow-through system showed that a standardized size of glass tanks (9L tanks filled with 7L of reconstituted tap water) is sufficient to elicit the mate calling behavior of adult male *X. laevis* with one individual per tank. Male *X. laevis* older than 9 years of age, however, should not be used as test animals, since they do not produce any or in some cases just a few calls in the flow-through system compared to younger males. Moreover, experiments revealed that all test tanks need to be coated by acoustic foam plates (thickness 5 cm) and have to have a distance of about 20-30 cm to each other to prevent cross-talk and ensure a noise-reduced recording of the amphibian vocalizations. To further prevent noise interferences, silicon tubes were mounted to the flow-through hose system, reaching into the water of the test tanks to avoid impingement of the running water on the water surface.

In the semi-static system, animals were acclimatized for 3 days. The implemented experiments, however, suggest that this acclimation period can be reduced to several hours or can even be omitted. A further shortening of the duration of a test trial, on the other hand, was not possible although previous finding indicated such a possibility (Hoffmann 2012). The remaining noise and especially the periodically inflowing water of the mixing chambers of the flow-through system seemed to disturb the male experimental frogs, demonstrated by abrupt interruptions coinciding with inflow (Fig. 6). These interruptions, often followed by a longer period of silence, made it harder to detect possible alterations of the calling behavior due to the lower amount of calls uttered, in general. To still be able to detect environmentally relevant concentrations of EDC, the test animals had to be exposed to the respective chemical for at least 96 h, the same amount of time as they were exposed semi-statically. Nevertheless, the test substances EE2 and vinclozolin (VIN) provoked the same effects in the flow-through system as they did in the semi-static experimental setup (Hoffmann and Kloas 2010, 2012c). However, the sensitivity of the new test system turned out to be lower compared to the previously used semi-static approach.

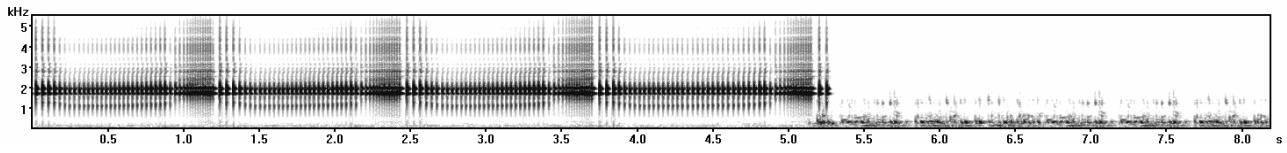

Figure 6: Spectrogram of interrupted calling behavior. Noise and particularly the suddenly inflowing water from the mixing-chamber of the flow-through system caused abrupt interruptions in male frog calling behavior which were often followed by a longer period of silence.

4.2 Establishment of chemical analytics

After concentrating the water samples with C18- cartridges (Garmshausen et al. 2015) (appendix 3), the exact concentrations of the test substances were determined using different methods and were then compared to the expected exposure concentration (Tab. 1). We could show that the measured concentrations just slightly differed from the desired exposure concentrations or were in the range of the recovery rate of the respective reference method (Tab. 1).

Triiodthyronin (T3) concentrations in the supply tanks of the flow-through system were adequate, but T3 concentrations in the test tanks were only about 10 – 30% of the expected concentration (Tab. 1). This was

probably the result of bioaccumulation processes. Male frogs incorporated the T3 out of the water into their bodies and then metabolized and accumulated it. Gene expression analyses on those test animals also showed significant T3 effects on the gene expression of thyroid-specific genes, such as the thyroid receptor beta (TRβ) and the thyroid stimulating hormone (TSH), which ensured that T3 was in fact in test tanks at considerable concentrations.

During the first exposure of male frogs to VIN, we could only analyze the higher concentrated supply tank of 10^{-10} M treatment of the flow-through system, since test concentrations in the aquaria were below the detection limit of the old gas chromatograph (GC), used at the institute at that time (10^{-10} M; recovery rate: ~ 80%). The solution in the supply tanks was directly used to produce the exposure solution in the test tanks (200-fold dilution, amount of dilution water measured daily), thus it is safe to assume that the recovery rate of the exposure concentrations in the test tanks was around 80% as well. Before conducting the second VIN-exposure, the IGB purchased a new GC, which was able to measure VIN standard concentrations as low as 10^{-13} M. However, due to tremendous background noise when testing water out of the test tanks (probably due to excretions of the frogs), we were unable to measure accurate VIN exposure concentrations, and were still dependent on measurements of the VIN concentrations in the supply tanks. Nevertheless, the recovery rate of the VIN concentrations in the supply tanks was satisfactorily (~92%), thus, we can assume that the VIN concentrations in the test tanks also ranged within that recovery rate.

Additionally, we measured the oxygen, nitrate and ammonium concentrations, as well as the conductivity and the hardness of the water regularly to ensure a good water quality in the test tanks.

Table 1 Detection methods of the test substances and the respective measured recovery rates in the supply and test tanks. ELISA – Enzyme-linked immunosorbent assay; YES – Yeas estrogen screen, UHPLC-MS-MS – Ultra high performance liquid chromatography-tandem mass-spectrometry, GC – Gas chromatography.

Test substance	Method	Recovery rate in supply tanks [%]	Recovery rate in test tanks [%]
Ethinylestradiol (EE2) 1st exposure	ELISA (Kit)	88	85
Ethinylestradiol (EE2) 2nd exposure	YES-Assay	80	79
Methyldihydrotestosterone (MDHT)	UHPLC-MS-MS	104	103
Diclofenac (DCF)	UHPLC-MS-MS	85	86
Metoprolol (METO)	UHPLC-MS-MS	91	88
Triiodothyronine (T3)	UHPLC-MS-MS	80	25
Vinclozolin (VIN) 1st exposure	GC	84	not possible (< detection limit)
Vinclozolin (VIN) 2nd exposure	GC (new)	92	not possible (background noise)
Bisphenol A (BPA)	UHPLC-MS-MS	77	76
Trenbolone (TREN)	UHPLC-MS-MS	127	107
Dichlorodiphenyldichloroethylene (DDE)	UHPLC-MS-MS	63	55

4.3 Establishment of an analysis software

Dr. Henning Thielemann, a professional computer programmer developed analysis software, which automatically categorizes the five different call types of the male *X. laevis* and measures spectral and temporal features of the different calls. However, a direct comparison of manually determined data and software generated data of the same test trials revealed mixed results. Some of the software generated data matched the manually ascertained data up to 100%, while automatic data of other frogs and trails did not match the manual results at all (100% discrepancy) or had a very low predictive accuracy (Tab. 2).

One reason for the problematic distinct categorization of the call types is the high background noise and sounds of the frogs in the tanks (e.g. swimming around in the tank or against the hydrophone). Those noises are hard to distinguish from the frog calls in the analysis when using the software (Fig. 7) and Dr. Thielemann could not entirely resolve this categorization problem.

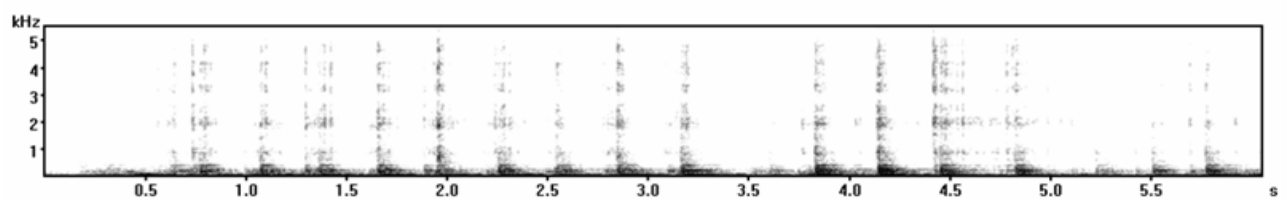

Figure 7: Spectrogram of background noise of the flow-through system. The visual components of these spectrograms differ greatly from male frog calls but spectral components are hard to discriminate by the software.

In some cases however, no obvious reasons for the faulty categorization of the calls could be found: no obvious visible differences in the recording and call quality could be found when inspecting the spectrograms. At the moment, Dr. Thielemann is working on a “supervised training” of the model to potentially increase the matching rate. With this training the model is supposed to specifically learn which sequences of amplitude and frequency represent what kind of calls. However, initial tests resulted in an even lower matching rate between the automatically generated data and the manually determined data compared to the previously generated data (Tab. 3). A detailed description of the software model, technical background and application of the software can be found in appendix 5.

Accordingly, a computerized analysis of the calling behavior of the frogs was not possible during the duration of the project and all call analyses had to be conducted manually, which was much more time-consuming (manual analysis: ~4 weeks per short-term experiment [96 h]; automatic analysis: ~48 h per short-term experiment [96 h]). Hence, the project schedule was noticeably postponed.

Table 2: Comparison of software analysis data (including unsupervised training) and manual analyses of the calling behavior of male *X. laevis*.

Individual	Advertisement calls (Software) [s]	Advertisement calls (manually) [s]	Deviation from manual results [s]	Advertisement calls (Software incl. unsupervised Training) [s]	Deviation from manual results [s]
DCF 10^{-8}M-1	597.50	1,087.40	489.90	43.60	1,043.80
DCF 10^{-8}M -2	136.90	798.00	661.10	62.10	735.90
DCF 10^{-8}M -3	2.20	30.20	28.00	9.50	20.70
DCF 10^{-8}M -4	0.00	11.70	11.70	0.00	11.70
DCF 10^{-10}M-2	2,836.70	5,320.50	2,483.80	264.60	5,055.90
DCF 10^{-10}M -3	1,026.50	1,034.30	7.80	1,026.50	7.80
DCF 10^{-10}M -4	1,127.34	1,122.50	4.84	1,011.60	110.90
DCF Co1	3,404.70	5,503.70	2,099.00	478.90	5,024.80
DCF Co2	121.50	565.90	444.40	28.30	537.60
DCF Co3	137.10	402.30	265.20	8.80	393.50
DCF Co4	2.80	27.10	24.30	0.00	27.10

Table 3: Comparison of new software analysis data (including supervised training) and manual analysis of the calling behavior of male *X. laevis*.

Individual	Total vocal output (Software) [s]	Total vocal output (manually) [s]	Deviation from manual results [s]	Advertisement calls (Software incl. supervised training) [s]	Advertisement calls (manual) [s]	Deviation from manual results [s]
DCF 10-8M-1	11.00	0.00	11.00	0.00	0.00	0.00
DCF 10-8M-2	1.05	894.00	892.95	805.50	798.00	7.50
DCF 10-8M-3	19.00	0.00	19.00	0.00	0.00	0.00
DCF 10-8M-4	42.00	37.20	4.80	5,675.00	30.20	5,644.80
DCF 10-10M-1	16.00	0.00	16.00	0.00	0.00	0.00
DCF 10-10M-2	5.72	5,395.50	5,389.78	5,145.80	5,320.50	174.70
DCF Ko1	6.34	5,568.70	5,562.36	5,199.80	5,503.70	303.90
DCF Ko 2	687.00	572.90	114.10	598.80	565.90	32.90
DCF Ko 3	498.00	406.30	91.70	445.70	402.30	43.40

5 Work package 3: Experimental testing

5.1 Biological and ecological relevance of the endpoint calling behavior of male *X. laevis*

To be able to further assess the biological relevance of an altered calling behavior of male *X. laevis* due to endocrine disruption, we performed a long-term exposure experiment with subsequent mating experiments. Fertilization rate of treated and untreated males, as well as the quality and quantity of the resulting offspring were determined. We further examined whether a simultaneous exposure of male frogs to an estrogenic and an androgenic EDC lead to the same effects as the individual substances or whether the simultaneous exposure results in synergistic (additive) effects or decrease or even diminish single EDC effects.

5.1.1 Long-term exposure of male *X. laevis* to the estrogenic 17alpha-ethinylestradiol

In this experiment, we exposed male *X. laevis* to three different concentrations of EE2 for 28 days (10^{-8} M, 10^{-10} M and 10^{-12} M EE2). During the exposure we recorded and analyzed the mate calling behavior of the frogs. Afterwards we performed two mating experiments. The first experiment focused on the behavior of the frogs in a competitive situation. We placed one male of the control group together with a male of the 10^{-8} M EE2 treatment group and an untreated female or a male of the 10^{-10} M EE2 treatment together with a male treated with 10^{-12} M EE2 and an untreated female, respectively. We then observed the courtship and clasping behavior of the frogs for two hours. Subsequently, we paired each male frog with one new and untreated female for the rest of the night (11 h) and again recorded their courtship and clasping behavior. The next morning, we sacrificed the male frogs and examined gene expression profiles of various genes involved in the HPG-axis. We also counted the number of eggs laid by the females, counted and reared the hatched tadpoles until day 9 after oviposition (post oviposition day = pod) and recorded the hatching and survival rate as well as visible developmental alterations. Details of this study can be obtained from the respective manuscript “Chronic 17α-ethinylestradiol exposure of male *Xenopus laevis* can impair amphibian mating success” (Garmshausen et al. 2016, in prep.), which will be submitted for publication in *Hormones and Behavior* and can be found in the appendix 1.

In short, we found that 10^{-8} M EE2 exposed frogs uttered a significantly lower amount of advertisement calls, as well as a higher amount of rasping than control frogs, indicating a drastic decrease in sexual arousal (Hoffmann and Kloas 2010, 2012c). This might result in a lower reproductive success of EE2 exposed males (Gerhardt et al. 2000, Murphy and Gerhardt 2000, Rosso et al. 2006, Gerhardt and Brooks 2009). Similarly to our previous study (Hoffmann and Kloas 2012c), the advertisement calls of 10^{-8} M EE2 exposed male *X. laevis* used in this study showed differences in temporal and spectral parameters (call accentuation and click duration) continuously during the whole experiment. Those alterations were previously shown to reduce the sexual attractiveness toward females (Hoffmann and Kloas 2012c). We could further demonstrate that male *X. laevis* exposed to EE2 at 10^{-8} M ceased their mate calling behavior around exposure day 14 entirely. Accordingly, 10^{-8} M EE2 treated *X. laevis* displayed a diminished clasping behavior during the competitive and non-competitive mating situation compared to the controls. Consequently, considerably less tadpoles hatched in the tanks comprising the progeny of those EE2 exposed males (10^{-8} M) and, more importantly, less tadpoles survived until the end of the observation period (pod 9) compared to the other treatment groups, including controls.

Frogs exposed to 10^{-10} M and 10^{-12} M EE2, also called a lower percentage of advertisement calls and a higher amount of rasping during the first two exposure weeks. However, those parameters returned to control levels

towards the end of the 28-day exposure. This decrease in effectiveness of EE2 on the calling behavior of those frogs might indicate some sort of adaption or habituation effect of the test chemical. However, the call accentuation and the duration of clicks of the advertisement calls were severely reduced throughout the whole experimental period. These alterations could, hence, still reduce the attractiveness of EE2 exposed males toward females and thus lead to a lowered reproductive success of exposed males, although the total vocal output was not affected. Males exposed to 10^{-10} M EE2 also clasped their female shorter than 10^{-12} M treated frogs in the competitive mating situation, indicating a monotonic, dose-dependent decrease in sexual arousal. Surprisingly, male *X. laevis* exposed to 10^{-10} M EE2 clasped their female longer during the non-competitive mating experiment when compared to males of the control group. This increase in clasping activity coincides with the increased mate calling behavior of those animals during the last exposure week. Hence, a monotonic, dose-dependent effect of EE2 on the clasping behavior seems to be only present, when a competing party is involved. Future studies should focus on this phenomenon, and address whether EDC effects are more profound in situations, which reflect natural species-specific settings compared to laboratory-generated experiments. Similarly, the hatching and survival rate of tadpoles descending from males that were exposed to 10^{-10} M and 10^{-12} M EE2, was in similar ranges when compared to the controls, which is again in accordance with the enhanced mate calling behavior of those individuals towards the end of the exposure period and the increased clasping behavior in the second mating experiment. However, whether concentration-dependent effects also involve the hatching and survival success of the resulting offspring after a competitive mating experiment needs to be tested in future studies.

The mate calling and clasping behavior of male *X. laevis* is androgen-dependent (Kelley and Pfaff 1976, Wetzel and Kelley 1983, Tobias et al. 1993, Kelley 2002). Thus, a lower availability of plasma T and DHT levels in EE2 exposed males might have caused the reduction in mating behavior, especially at the beginning of the experiment. In the present study, the gene expression of the RED 1 and 2 in the gonads of male *X. laevis* decreased with increasing EE2 exposure concentrations, while the gonadal ARO gene expression increased. Since RED 1 and 2 convert T to DHT and ARO converts T to E2, the increase in ARO and the decrease in RED 1 and 2 gene expression might have resulted in accumulated bioavailability of E2 and a reduced presence of T and DHT in all EE2 treated males. This might be the cause for the EE2 induced alterations in courtship behavior.

We furthermore noted a green coloring of the blood plasma of EE2 exposed males, which was absent in the controls. We subsequently investigated this phenomenon and found out that the green substance is biliverdin, a product which is formed during heme catabolism. After further investigations, we could demonstrate that EE2 can also impair the heme catabolism in male *X. laevis* by interfering with the gene expression of various genes involved in heme degradation (heme oxigenases 1 and 2 (HO 1 and 2), biliverdin reductase A (BLVRA)). Details can be found in the manuscript by Garmshausen et al. (2015) (appendix 3), published in *Comparative Biochemistry and Physiology, Part C*.

5.1.2 Simultaneous exposure to an androgenic and an estrogenic EDC

In a parallel project, funded by the Berlin State Office for Urban Development and Environment (SenStadtUm) and the European Fonds for Regional Development (EFRE) we could show that Berlin surface waters primarily elicit an androgenic and estrogenic activity, but contain no or at least untraceable concentrations of antiandrogenic and antiestrogenic EDC (Hoffmann 2015). Furthermore, EDC mixtures were previously shown to result in additive or decreased EDC effects (Canesi et al. 2008, Kortenkamp 2008, Sun et al. 2009). Hence, to examine the effects of environmentally relevant mixtures of EDC on the courtship behavior of amphibians, we exposed male *X. laevis* to a mixture of estrogenic (EE2) and an androgenic EDC (MDHT) and observed their calling behavior. By using different concentrations of EE2 and MDHT (1:5 and 5:1), we wanted to obtain insights whether a exposure of male frogs to these mixtures causes synergistic effects, additive effects of the individual substances or causes attenuation or even

extinction of single EDC effects. Details of this study can be obtained from the respective manuscript "Co-exposure to the estrogen ethinylestradiol and the androgen methyldihydrotestosterone causes antagonistic, independent and synergistic impacts on male mate calling behavior, vitellogenin induction and heme metabolism of *Xenopus laevis*, respectively", which will be submitted for publication in *Proceedings of the Royal Society B* and can be found in appendix 2.

In short, we could show that that a co-exposure of adult male *X. laevis* to EE2 and MDHT at similar concentrations can result in additive effects, but also in individual EDC impacts, which could not be detected during a sole EE2 or MDHT exposure. Furthermore, the parallel exposure also led to a reduction of single EDC effects, e.g. regarding the calling behavior of the males. The obtained results, for instance, clearly demonstrate that at similar concentrations, the estrogen EE2 is much more effective than MDHT considering the male calling behavior, canceling out any stimulatory impact of MDHT. Additional effects on the calling behavior of the frogs e.g. included several spectral components of the advertisement calls, such as the duration of as well as the number of clicks within those calls and the peak frequency. All affected parameters might lead to a reduced mating success of simultaneously exposed *X. laevis*, since especially spectral and temporal parameters of the advertisement calls are necessary for species and individual recognition (Vignal and Kelley 2007). Such alterations can reduce the attractiveness of the calls towards females (Hoffmann and Kloas 2012c). Additionally, an estrogen-characteristic and concentration-dependent induction of the expression of the vitellogenin gene was detected, while the expression of gonadotropins and steroidogenic gonadal enzymes was not affected. Concerning other endpoints such as heme metabolism, both EDC evoked synergistic impacts by decreasing heme oxigenase expression.

EDC usually do not occur individually in the environment (Crews et al. 2000). Thus, the investigation of combined effects of environmentally relevant EDC mixtures on different endpoints in various aquatic species is necessary to be able to detect and assess potential risks of such EDC mixtures. As our data illustrate, mixtures of EDC with different MOA can have various outcomes when examining different endpoints. For an adequate risk assessment of aquatic wildlife, further studies should be conducted to shed light on the various mechanisms of EDC mixture effects on aquatic vertebrate behavior and physiology.

5.2 Test conditions under which an alteration of the calling behavior of male *X. laevis* is measurable particularly sensitively and to what extent those test are realistic

A direct comparison of the results obtained in this study and the results of the previous, semi-static experiments (Hoffmann and Kloas 2010, Hoffmann 2012, Hoffmann and Kloas 2012b, c, a, d) revealed that the basic XENOCALL method is also transferrable to a flow-through system. However, EDC effects in the semi-static exposure system were detected at lower concentrations than in the flow-through system. Semi-statically, most effects already occurred within the first night of exposure, suggesting the potential of a reduction of the testing period (< 96 h). A reduction of the test duration, however, was not applicable in the flow-through system, since EDC effects were generally less marked compared to the semi-static system. Hence, differences in calling behavior were more difficult to detect. One reason for this difference in test sensitivity is the enhanced background noise, especially at times when the exposure solution is flowing into the tanks (approximately every 10 minutes), as well as the water drifts due to the running water of the flow-through system. Those parameters seemed to irritate the male experimental frogs, indicated by abrupt interruptions in the calling behavior, especially at the moments the exposure solution is flowing into the tanks (Fig. 7). These interruptions, which were often followed by longer periods of silence, resulted in a lower total vocal output and thus made it harder to detect possible alterations of the calling behavior. To still be able to detect environmentally relevant concentrations of EDC, the test animals had to be exposed to the respective testing chemical for at least 96 h in the flow-through system, the same amount of time as they

were exposed semi-statically (Hoffmann and Kloas 2010, Hoffmann 2012, Hoffmann and Kloas 2012b, c, a, d). A testing method applying a semi-static EDC exposure consequently might be more sensitive and hence, more suitable. Moreover, a semi-static approach might also better reflect a natural, realistic environment, since *X. laevis* usually prefer small, dark and turbid ponds without water movement as habitats (Tinsley and Kobel 1996).

5.3 Testing of further substances with different MOA (5 chemicals)

To be able to perform a direct comparison of the XENOCALL method performed in a semi-static system as previously done and a flow-through system, the effects of further (anti)androgenic and (anti)estrogenic EDC on the calling behavior of the males were tested, using the flow-through system of the IGB-Berlin. The effects of five test substances were analyzed, whereof two chemicals were tested in the semi-static system before (EE2 and VIN) and three test substances were tested for the first time (DDE, TREN, BPA).

5.3.1 Exposure to the estrogenic ethinylestradiol (EE2)

5.3.1.1 Introduction

EE2 is a main component of many classical contraceptives. In the EU and the US, around 140 kg of EE2 are produced per year, and the prescription rate of this drug is very high (Arcand-Hoy et al. 1998, Sanderson et al. 2004). EE2 inhibits ovulation (Emperaire and Greenblatt 1969, Shearman 1975) by suppressing FSH secretion and altering structures of the endometrium (Van Heusden and Fauser 2002, Prasad 2010). EE2, however, is excreted unmetabolized through feces and urine (Orme et al. 1983, Braun et al. 2003), enters the environment via wastewater effluents and accumulates in surface waters (Jones et al. 2001). EE2 elicits high estrogenic activity, thus low concentrations can already harm aquatic organisms (Purdom et al. 1994, Jobling et al. 1998). It has been detected in effluents (Stumpf M et al. 1996, Belfroid et al. 1999, Ternes et al. 1999) and in surface waters (Desbrow et al. 1998, Belfroid et al. 1999, Shen et al. 2001) at concentrations within the two- to three-digit ng/L range. Moreover, EE2 could even be detected in drinking water at low ng/L concentrations (Kuch and Ballschmiter 1999, Hoffmann 2012). EE2 was shown to impair reproductive behaviors of fishes and amphibians, including the mate calling behavior of *X. laevis* in a semi-static experimental setup (Bjerselius et al. 2001, Xu et al. 2008, Colman et al. 2009, Saaristo et al. 2009a, Saaristo et al. 2009b, Partridge et al. 2010, Saaristo et al. 2010b, a, Hoffmann 2012, Hoffmann and Kloas 2012c).

5.3.1.2 Material and methods

In this experiment we used adult male *X. laevis* (3 years of age) of the animal husbandry of the Leibniz-Institute of Freshwater Ecology and Inland Fisheries Berlin, where they were kept in groups of up to 12 individuals in 60 L tanks at a light:dark cycle of 12:12 h (Hoffmann 2012). At the beginning of the experiment one male frog was transferred into each of the 30 glass tests tanks (30 x 20 x 15.5 cm) of the flow through system, which is described in detail in Efosa et al. (2016, submitted) and Lutz et al. (2008). Each treatment comprised 8 test aquaria wrapped with acoustic foam plates (Hoffmann and Kloas 2010) to ensure visual and acoustic isolation of the frogs. A hydrophone (Sensor Technology SQ 26, Nauta, Milano, Italy), which was connected to a fire-wire audio interface (Saffire Pro 40, Focusrite, High Wycombe, United Kingdom) and a desktop computer, was placed in each tank. An automated and trigger-controlled acoustic recording (frequency range: 0.5 – 4.0 kHz) was conducted to record the amphibian vocalizations each night from 6 pm to 6 am using the Avisoft Recorder software (Avisoft, Berlin, Germany). The individual-specific recordings were stored as wave-files on the desktop computers until further analysis. Recordings were analyzed with Avisoft SasLab software (Avisoft, Berlin, Germany) as described in Hoffmann and Kloas

(2010) and Hoffmann and Kloas (2012c). The following call parameters were measured per frog and night: absolute calling activity, and the absolute and relative amount of each of the five call types. Temporal and spectral parameters of the advertisement calls were determined using newly developed software by Dr. Henning Thielemann. For this purpose, accurately classified audio files of the single frogs were gathered manually and then fed to the software, which analyzed the following parameters of those calls: the duration of the whole call, as well as the duration and the mean frequency of the slow trill and the fast trill part, the mean number of clicks, as well as accentuated clicks, the click rate (number of clicks per second in the slow trill part of the advertisement calls), the duration of clicks and pauses between clicks (ICI) and the bandwidth within the slow trill part, and the mean frequency and bandwidth of the fast trill part of the calls.

EE2 was previously shown to reduce the sexual arousal of male frogs after hCG-stimulation, indicated by the production of lower amounts of advertisement calls and higher amounts of rasping (Hoffmann 2012, Hoffmann and Kloas 2012c). Therefore in this experiment, male frogs were first injected with 100 units hCG and then exposed to EE2 for 96 h. Since EE2 is hardly dissolvable in water, it was dissolved in 99% DMSO to prepare stock solutions (0.00001 %). All chemicals were obtained from Sigma Aldrich (Steinheim, Germany). During the experiment, frogs were fed a commercial fish diet (Metabolica, Aller Aqua, Golßen, Germany; 2 pellets) every second day. Water temperature in the test tanks was 21.6 ± 0.2 °C throughout the whole experiment and the light:dark cycle was 12:12 h, with a light period starting at 7 am. At the end of the exposure period, all frogs were returned to the animal husbandry of the institute in different 60 L tanks, according to their assigned treatment. Animals were tested again after a basic recovery time without EDC exposure (5.4).

General Linear Mixed Model analyses (GLMM) with subsequent Sidak post-hoc tests (SPSS 20, IBM, Ehningen, Germany) were conducted to identify statistical differences between treatments as described previously (Hoffmann and Kloas 2010, Hoffmann 2012, Hoffmann and Kloas 2012b, c, a, d, Efosa et al. 2016, submitted). To account for repeated measures, animals were set as random factor. Weight and length was included as covariates. Normal distribution of data or residuals was ensured with Kolmogorov-Smirnov tests.

For verifying the desired test concentrations, water samples of each tank were taken and analyzed at the beginning and at the end of the experiment using enzyme-linked immune assays (ELISAs) according to the manufacturers' instructions (Garmshausen et al. 2015)(appendix 3; 5.1.1).

5.3.1.3 Results

Analytical water analyses

None of the control tanks contained traces of EE2. Water samples from the tanks of the 10^{-10} M EE2 treatment group (desired concentration: 29.6 ng/L EE2) contained 18.1 ng/L (16.4 ng/L–20.5 ng/L) EE2 (median (interquartile range). Samples from the tanks of the 10^{-12} M EE2 treatment group (0.27 ng/L EE2) and the 10^{-13} M EE2 treatment group (0.03 ng/L EE2) contained 0.26 ng/L (0.25 ng/L–0.28 ng/L) EE2 and 0.032 ng/L (0.030 ng/L – 0.034 ng/L) EE2. In the following sections the nominal test concentrations are used in the denotation of the particular treatments for simplification purposes.

Calling behavior

Compared to the controls, EE2 exposed male frogs called for a longer period of time during the first two exposure nights and a similar amount during the last two nights. Hence, the interaction of exposure night and treatment (treatment*night) resulted in a statistically high significance ($p = 0.005$; data not shown), while the effects of the treatments alone were not significant ($p > 0.05$). EE2 treated *X. laevis* called a lower percentage

of advertisement calls, however implementing all three EE2 treatments, this effect was not significant as well ($p = 0.089$; Fig. 8). Considering only the 10^{-10} M EE2 treatment, the effect resulted in a statistically significant value ($p = 0.026$).

Males exposed to EE2 also uttered a higher percentage of rasping compared to unexposed frogs, but again the effect was not significant ($p > 0.138$). However, considering only the 10^{-10} M EE2 treatment, this effect turned out to be significant ($p = 0.011$; Fig. 9). In addition, EE2 exposed male frogs generally tended to utter more growling than the controls ($p = 0.054$; data not shown). The interaction of night and treatment (treatment*night) was highly significant for advertisement calls, as well as for rasping in all treatment groups ($p = 0.002$).

Regarding temporal and spectral parameters of the advertisement calls, only the bandwidth was increased due to a 10^{-10} M EE2 treatment ($p = 0.022$), however implementing all 4 treatment groups in the statistical analysis, this effect was not significant anymore ($p = 0.123$, data not shown). The number of accentuated clicks and the duration of clicks in the slow trill of the advertisement calls were slightly reduced in 10^{-10} M EE2 treated frogs, however effects were not significant ($p > 0.05$).

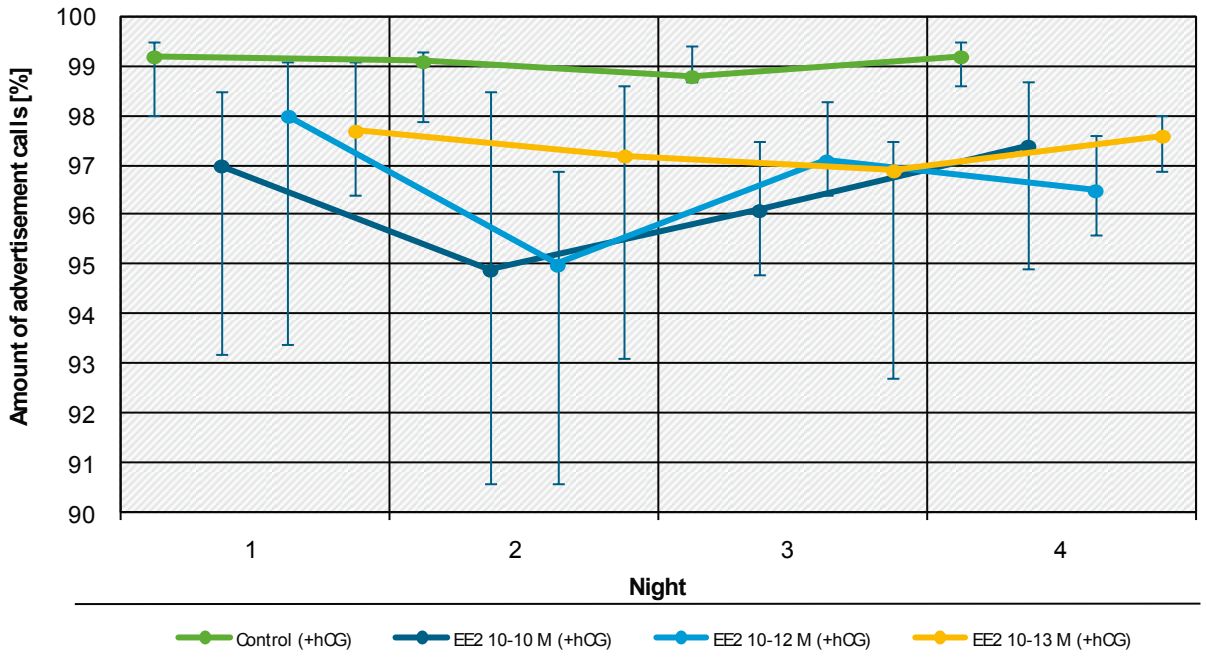

Figure 8 Line graph of the percentages of advertisement calls produced during the four exposure nights after stimulation with hCG (median and interquartile ranges). Animals that were exposed EE2, generally produced less of those calls than control animals, however this effect was not significant (GLMM: $p > 0.05$). If just those animals that were exposed to 10^{-10} M EE2 were considered in the statistical analysis, the percentage of advertisement calls was significantly lower in EE2 treated males compared to the controls.

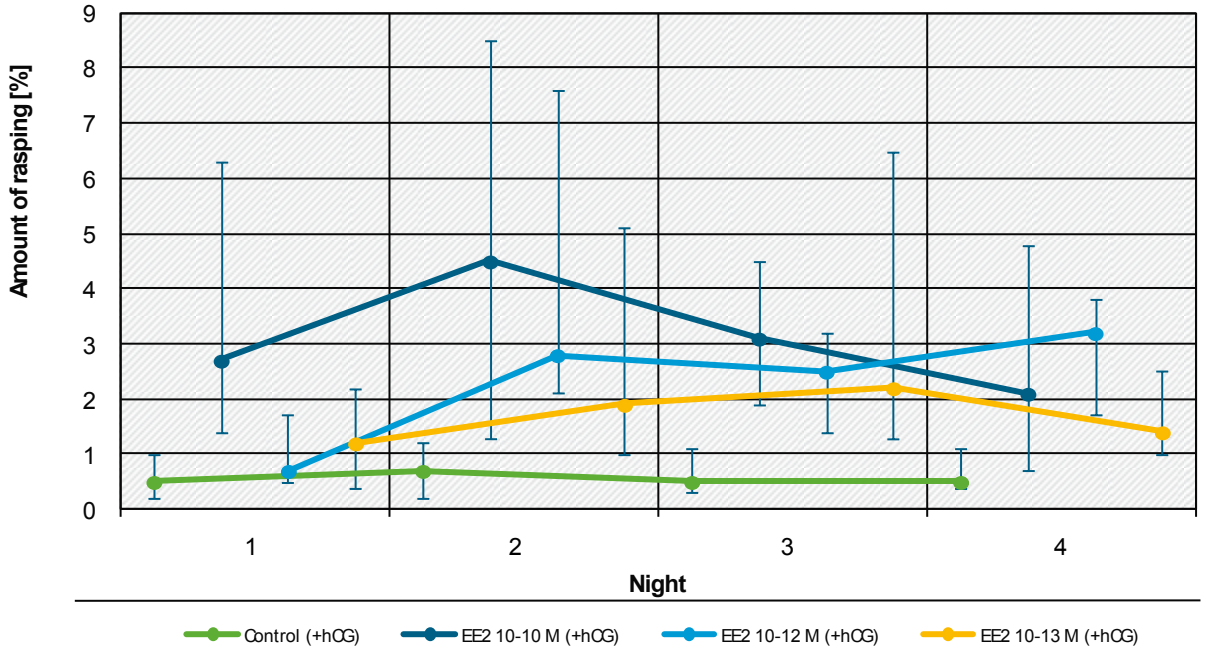

Figure 9 Line graph of the percentages of rasping uttered during the four exposure nights after stimulation with hCG (median and interquartile ranges). Animals that were exposed to 10^{-10} M EE2 produced a significantly higher percentage of rasping, when being the only treatment compared to the control (GLMM: $p = 0.011$). 10^{-12} M and 10^{-13} M EE2 exposed frogs also tended to produce more rasping than the controls, however if implementing all EE2 treatments, this effect slightly missed a statistically significant level ($p < 0.138$).

5.3.1.4 Discussion

Animals exposed to EE2 in the flow-through system experienced the same alterations in their calling behavior as male *X. laevis* exposed to EE2 in the semi-static system (Hoffmann 2012, Hoffmann and Kloas 2012c) (Fig. 10).

In both systems, animals produced a lower percentage of advertisement calls and a higher amount of rasping, indicating a lower sexual arousal of exposed males (Hoffmann 2012, Hoffmann and Kloas 2012c) (Fig. 10). These effects were statistically significant in the semi-static system even at concentrations as low as 10^{-12} M EE2. When implementing all treatments of the flow-through experiment in the GLMM, those effects slightly missed a significant level. On the other hand, when analyzing only the 10^{-10} M EE2 treatment, the effects were significant. Hence, this treatment concentration might represent the lowest observed effect concentration (LOEC). These results coincide with the findings mentioned in paragraph 4.1 and 5.2, stating that the increased background noise, the more or less constantly running water in the flow-through system and the periodic inflow of the exposure water in the test tanks prevent a similarly sensitive detection of the respective EDC. Those parameters represent an irritation for the male experimental frogs, indicated by e.g. abrupt interruptions in the calling behavior, especially at the moments when the exposure solution flows into the tanks (Fig. 7). These interruptions were mostly followed by long periods of silence, which eventually resulted in a lower total vocal output. The lower amount of calling made it more difficult to detect possible alterations of the calling behavior. To still be able to detect environmentally relevant concentrations of EDC, more test animals would have to be exposed to the respective testing chemical ($n \geq 10$) for at least 96 h in the flow-through system, the same amount of time as they were exposed semi-statically (Hoffmann and Kloas 2010, Hoffmann 2012, Hoffmann and Kloas 2012b, c, a, d). A testing method applying a semi-static EDC exposure consequently might be more sensitive and hence, more suitable. Moreover, a semi-static approach might also better reflect a natural, realistic environment, since *X. laevis* usually prefer small, dark and turbid ponds without water movement (Tinsley and Kobel 1996).

In the flow-through system another call parameter was affected by EE2 exposure, namely the amount of growling. This effect was previously found in antiandrogen exposed male *X. laevis* (Hoffmann and Kloas 2010). Hence, this call parameter might not only be antiandrogen-specific but rather a call type that is generally uttered at a higher percentage when animals exhibit low sexual arousal, e.g. due to an EDC exposure.

Besides a slightly lower amount of accentuated clicks and a slightly shorter duration of all clicks in the slow trill part, further effects on temporal and spectral parameters of the advertisement calls were discovered when frogs were exposed to EE2. The bandwidth of the advertisement calls was higher in 10^{-10} M EE2 treated males; however, this effect was again only significant, if only this treatment was considered in statistical analyses. A higher bandwidth was not discovered in the previous semi-static system (Hoffmann and Kloas 2012c). One reason why this effect was not detected in the semi-static system might be the method of data exploration and measurement. While only few calls per frog could be analyzed manually, many more advertisement calls could be analyzed using newly developed analysis software by Dr. Henning Thielemann. The analysis of more calls, hence, can result in more accurate data. Moreover, slight differences in parameter determination between the two software programs (semi-static: Avisoft, flow-through: Thielemann-Software) might also be the reason for the slightly deviating results. Since spectral characteristics of calls are important for the attractiveness of the call towards potential mating partners (Picker 1983, Schwartz 1994, Gerhardt et al. 1996, Schwartz et al. 2001, Bush et al. 2002, Beckers and Schul 2004, Gerhardt 2005b, Vignal and Kelley 2007, Bush et al. 2009, Gerhardt and Brooks 2009, Gordon and Gerhardt 2009), deviations of those stereotype parameters might result in a lower mating success of EE2 exposed frogs.

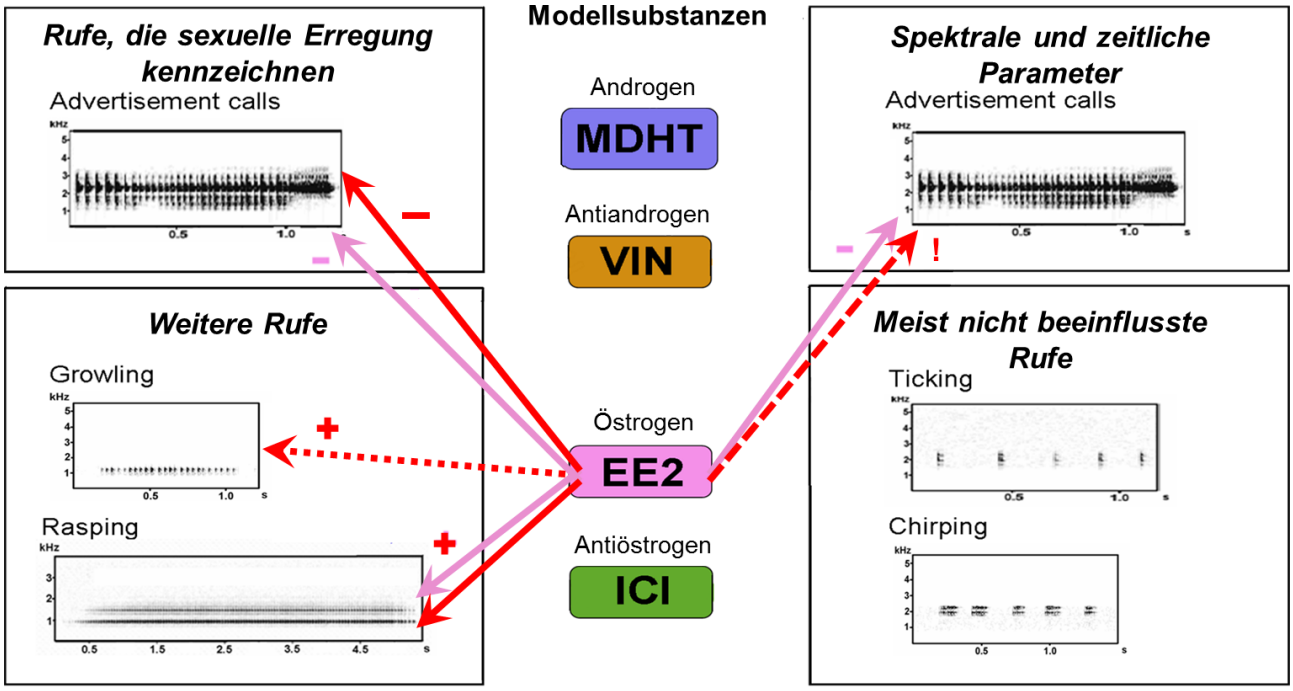

Figure 10 Schematic diagram of the effects of an exposure (96 h) to the endocrine disrupting chemical (EDC) ethinylestradiol (EE2) in the semi-static (pink arrows) and the flow-through (red arrows) system. The estrogenic EE2 lowered the relative amount of advertisement calls and reduced the number of accentuated clicks and the click duration in the semi-static system (Hoffmann and Kloas 2012c). A semi-static EE2 exposure, however, did not affect growling but increased the amount of the call type rasping. In the flow-through system, EE2 caused the same effects, although not at a significant level: lower amounts of advertisement calls and higher amounts of rasping. EE2 treated animals also produced a slightly higher percentage of growling (dotted red arrow), an effect missing in the semi-static system. Using spectral analyses, a higher bandwidth of 10^{-10} M EE2 treated frogs could be detected, but no further temporal and spectral parameter of the advertisement call was affected by EE2.

5.3.2 Exposure to bisphenol A (BPA)

5.3.2.1 Introduction

BPA can be found in various household and everyday items made of polycarbonate (e.g. CDs/DVDs, plastic baby bottles, plastic dishes, etc.), as well as in thermo paper and PVCs (Umweltbundesamt 2010). In 2006, 3.8 million tons of BPA were produced worldwide (Umweltbundesamt 2010) and BPA could be detected in the environment at alarming concentrations (RAR 2010, Huang et al. 2012, Hoffmann 2015). BPA, as most EDC accumulate in surface waters which are the sink of diverse effluents (U.S.EPA 1998, Europäische Kommission Scientific Committee on Toxicity and Environment 1999, Gehring et al. , Zühlke 2004, Bolong et al. 2009, Umweltbundesamt 2010). Bisphenol A (BPA) at considerable amounts can result in toxic effects (Benachour et al. 2007). BPA, moreover, elicits estrogenic MOA (Alonso-Magdalena et al. 2012) and thereby lead to adverse effects on the reproductive physiology and development of vertebrates, including humans (Umweltbundesamt 2010). Adverse effects of BPA include malformations of sexual organs, reduction in sperm quality, decreased reproductive success and feminization in birds, mammals and aquatic vertebrates, such as fishes, reptiles and amphibians (Iguchi and Sato 2000, Levy et al. 2004, Cheshenko et al. 2008, Brunström et al. 2009, Caliman and Gavrilescu 2009, Flint et al. 2012, Bhandari et al. 2014, Jandegian et al. 2015). Furthermore, BPA can also affect diverse behavioral traits in those species, especially behaviors regarding mating, reproduction and parental care (Cummings et al. 2010, Jones and Watson 2012, Blocker

and Ophir 2013, Bhandari et al. 2014, Johnsson et al. 2016, submitted). Whether BPA can also affect the mate calling behavior of male *X. laevis* was examined in this part of the project. Moreover, it was tested whether an exposure to BPA results in similar or even identical effects as the estrogenic EE2, to be able to assess the general validity of the XENOCALL method for the detection of estrogenic EDC.

5.3.2.2 Material and methods

Adult male *X. laevis* (5 years of age; weight: 72.9 g ± 8.2 g; length: 9.3 cm ± 0.6 cm) were exposed to two different BPA concentrations (10^{-8} M and 10^{-9} M; n = 10) in the flow-through system and their calling behavior was recorded and analyzed as described previously (5.1.2.1). Temporal and spectral parameters of the advertisement calls were determined using software newly developed by Dr. Henning Thielemann. For this purpose, accurately classified audio files of the single frogs were gathered manually and then fed to the software, which analyzed the following parameters of those calls: the duration of the whole call, as well as the duration and the mean frequency of the slow trill and the fast trill part, the mean number of clicks, as well as accentuated clicks, the click rate (number of clicks per second in the slow trill part of the advertisement calls), the duration of clicks and pauses between clicks (ICI) and the bandwidth within the slow trill part, and the mean frequency and bandwidth of the fast trill part of the calls. The estrogen EE2 was previously shown to reduce the sexual arousal of male frogs, indicated by the decreased production of advertisement calls and higher amounts of rasping after an hCG-stimulation (Hoffmann 2012, Hoffmann and Kloas 2012c). Therefore in this experiment, male frogs were likewise injected with 100 units hCG and then exposed to estrogenic BPA for 96 h. Exposure chemicals were dissolved in dimethyl sulfoxide (> 99%; DMSO) to prepare stock solutions and all chemicals were obtained from Sigma Aldrich (Steinheim, Germany). During the experiment, frogs were fed a commercial fish diet (Metabolica, Aller Aqua, Golßen, Germany; 2 pellets) every second day. Water temperature in the test tanks was 21.2 ± 0.1 °C throughout the experiment and the light:dark cycle was 12:12 h. At the end of the exposure period, all frogs were returned to the animal husbandry of the institute and transferred in different 60 L tanks, according to their assigned treatment.

To verify the obtained results of the flow-through experiment, another semi-static BPA exposure was conducted testing a single BPA concentration (10^{-8} M) of a new BPA batch and a solvent control using DMSO alone (0.00001%). Therefore, adult male frogs (five-years old) were exposed to BPA for 2x96 h. During the first 96h frogs were not stimulated by hCG. After this period of time, frogs were injected with 100 units hCG and then again exposed to BPA for another 96 h. For this purpose, individual frogs were transferred into 10 L glass tanks containing 7 L of reconstituted tap water composed of distilled water, supplemented with 5 g marine salt (Tropic Marin Meersalz, Tagis, Dreieich, Germany). We then dissolved BPA (Sigma-Aldrich, Dreieich, Germany) in DMSO and exposed the animals by adding the dissolved chemical to the ambient water (Hoffmann 2012). Call recordings and analyses were performed as described above. During the experiment, frogs were fed a fish diet (Fisch-Fit; Interquell, Wehringen, Germany; 2.5 g/frog) twice a week and rearing water and chemicals were renewed every second day. Animals were anesthetized after the experiment using MS 222 (tricaine methanesulfonate) and weight and body length (snout to cloacae length) were measured. Afterwards, anesthetized frogs were sacrificed and brain, gonad, spleen and liver samples were taken for gene expression analyses as described in (Efosa et al. 2016, submitted) (appendix 4).

For verifying the desired test concentrations of both experiments, water samples of each tank were taken, concentrated (Garmshausen et al. 2015) (appendix 3) and analyzed at the beginning and at the end of the experiment using HPLC-MS-MS (Tab. 1).

General Linear Mixed Model analyses (GLMM) were conducted as described previously (Hoffmann and Kloas 2010, Hoffmann 2012, Hoffmann and Kloas 2012b, c, a, d, Efosa et al. 2016, submitted) and subsequent Sidak post-hoc tests were performed (SPSS 20, IBM, Ehningen, Germany) to identify statistical

differences between treatments. Normal distribution of data or residuals was ensured with Kolmogorov-Smirnov tests.

5.3.2.3 Results

A) BPA exposure in the flow-through system

Male frogs exposed to BPA generally uttered higher amounts of advertisement calls and lower proportions of rasping compared to the control group, however, these effects were only statistically significant at an exposure concentration of 10^{-9} M BPA ($p = 0.027$; Fig. 11 and 12). None of the other measured parameters were affected by BPA. No temporal and spectral parameters of the advertisement calls were affected by a BPA exposure.

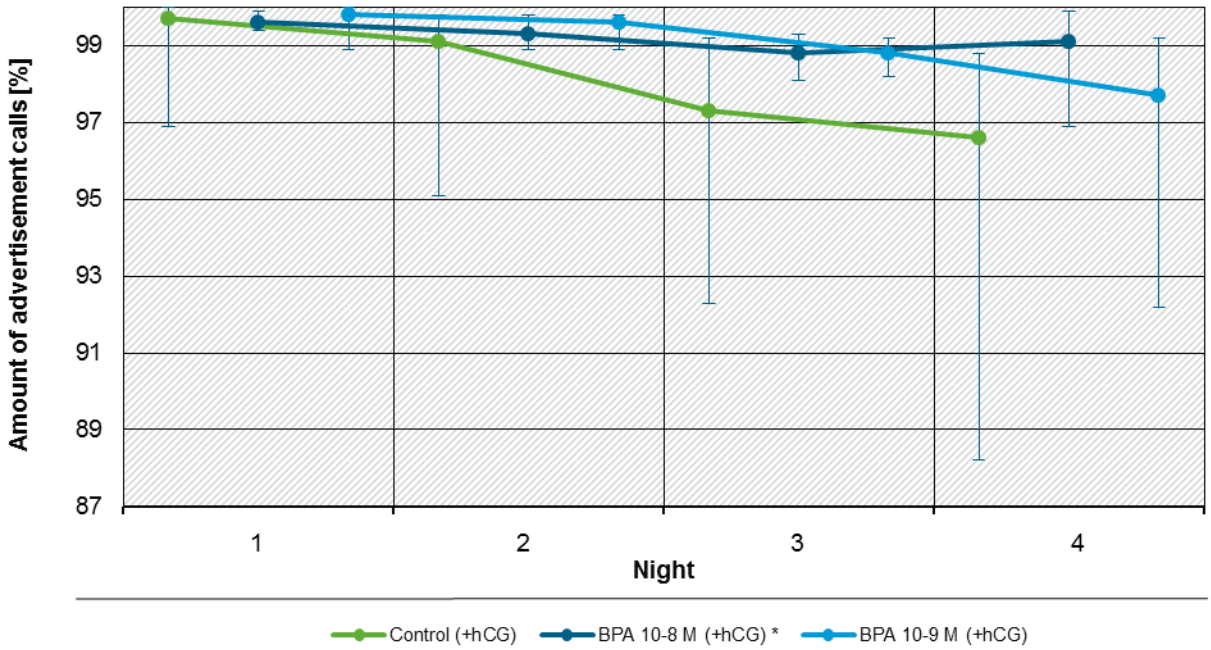

Figure 11 Line graph of the percentages of advertisement calls produced during the four exposure nights after stimulation with hCG (median and interquartile ranges). Animals that were exposed to bisphenol A (BPA), generally produced a higher amount of advertisement calls than control animals, however this effect was only significant regarding the higher concentrated BPA treatment (10^{-9} M; GLMM: $p = 0.027$).

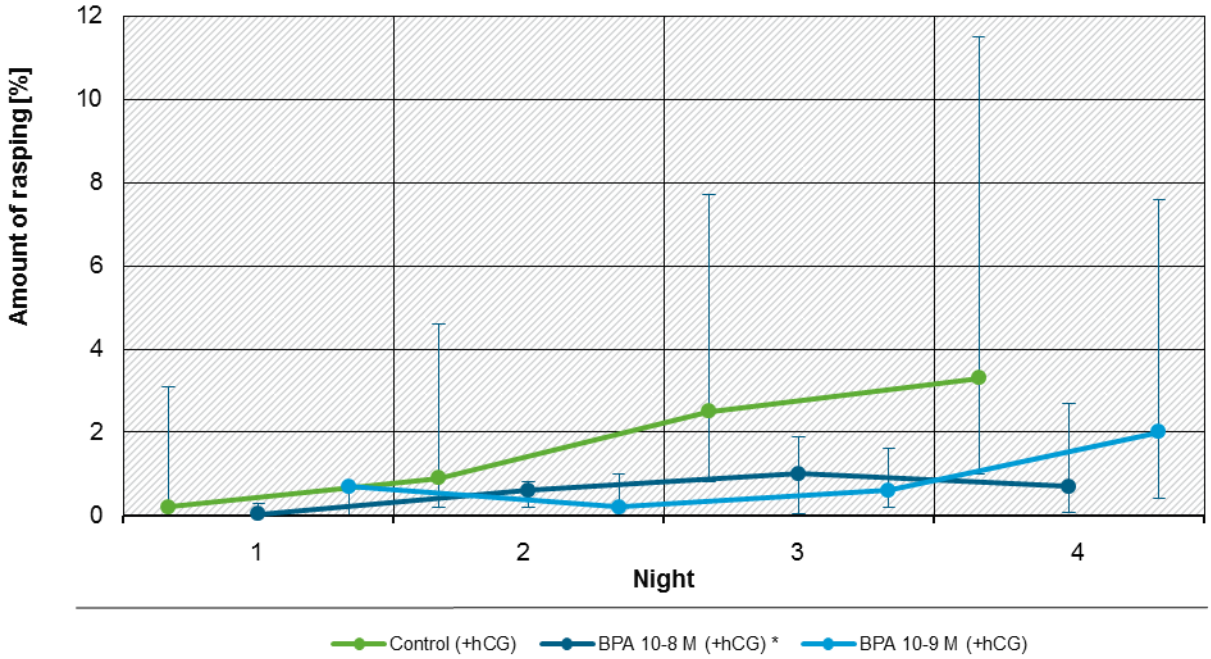

Figure 12 Line graph of the percentages of rasping uttered during the four exposure nights after stimulation with hCG (median and interquartile ranges). Animals that were exposed bisphenol A (BPA), generally produced a lower amount of rasping than control animals, however this effect was only significant regarding the higher concentrated BPA treatment (10^{-9} M; GLMM: p = 0.027).

B) BPA exposure in the semi-static system

BPA exposed frogs vocalized less than control frogs before hCG injection (p = 0.023; data not shown). Accordingly, the number of frogs that vocalized at all during the first four recorded nights, was lower in the BPA treatment group (p = 0.012; data not shown). Again, after hCG stimulation male frogs exposed to BPA uttered a significantly higher amount of advertisement calls compared to the control group (p = 0.027; Fig. 13), whereas the call type rasping was not affected by a BPA exposure (p > 0.05; Fig. 14). None of the other measured parameters, including temporal and spectral features of the advertisement calls, were affected by BPA.

Furthermore, the estrogenic biomarker VTG induction was not affected by BPA exposure (Fig. 15). The gene expression of gonadal steroidogenic enzymes was also not affected by BPA (p > 0.05; data not shown). However, LH gene expression was significantly reduced in frogs exposed to BPA at 10^{-8} M (p < 0.001; Fig. 16), while the expression of FSH was not affected by the EDC treatment (p > 0.05; data not shown). Sex steroid levels and ratios were also not impacted by an exposure to BPA (p > 0.05; data not shown).

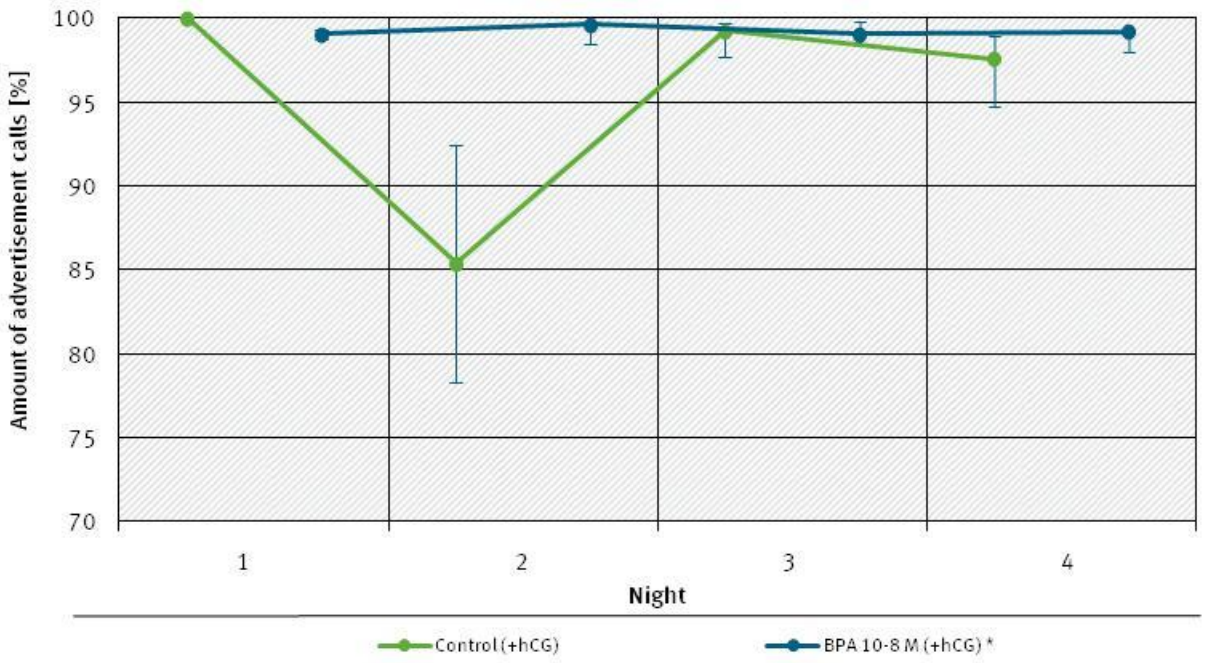

Figure 13: Line graph of the percentages of advertisement calls produced during the four exposure nights after stimulation with hCG (median and interquartile ranges). Animals that were exposed to bisphenol A (BPA) produced a higher amount of advertisement calls than control animals. This effect was significant (GLMM: $p = 0.026$).

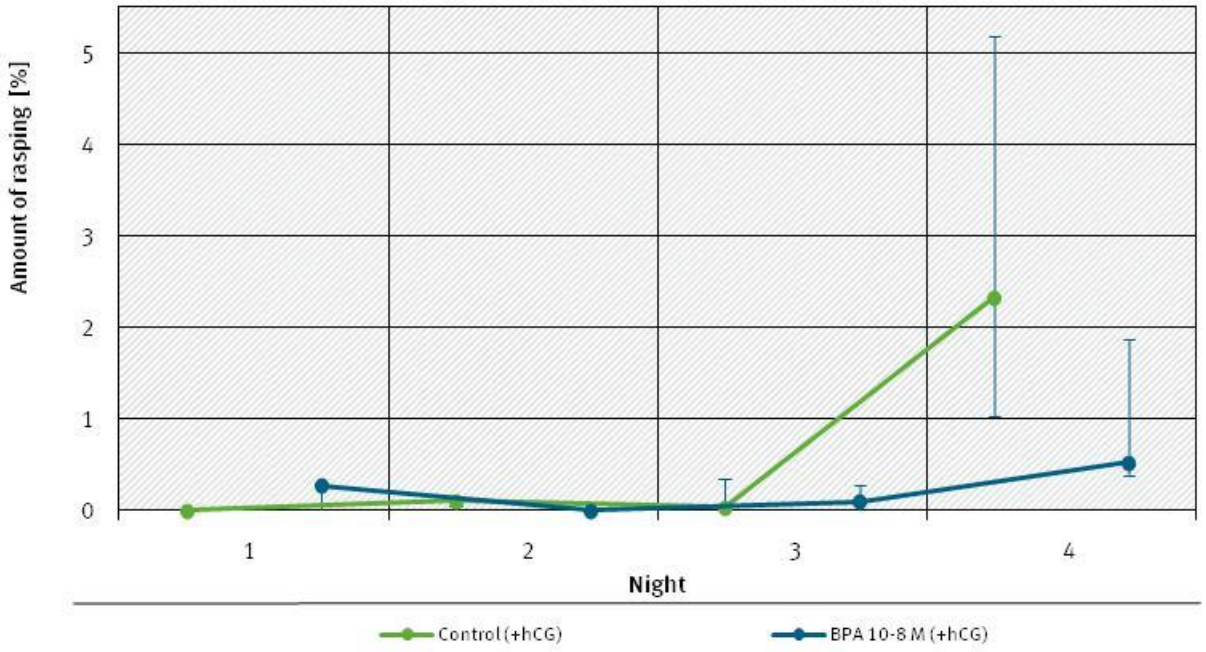

Figure 14: Line graph of the percentages of rasping uttered during the four exposure nights after stimulation with hCG (median and interquartile ranges). Animals that were exposed bisphenol A (BPA) produced a slightly lower amount of rasping than control animals, however this effect was not significant (GLMM: $p > 0.05$).

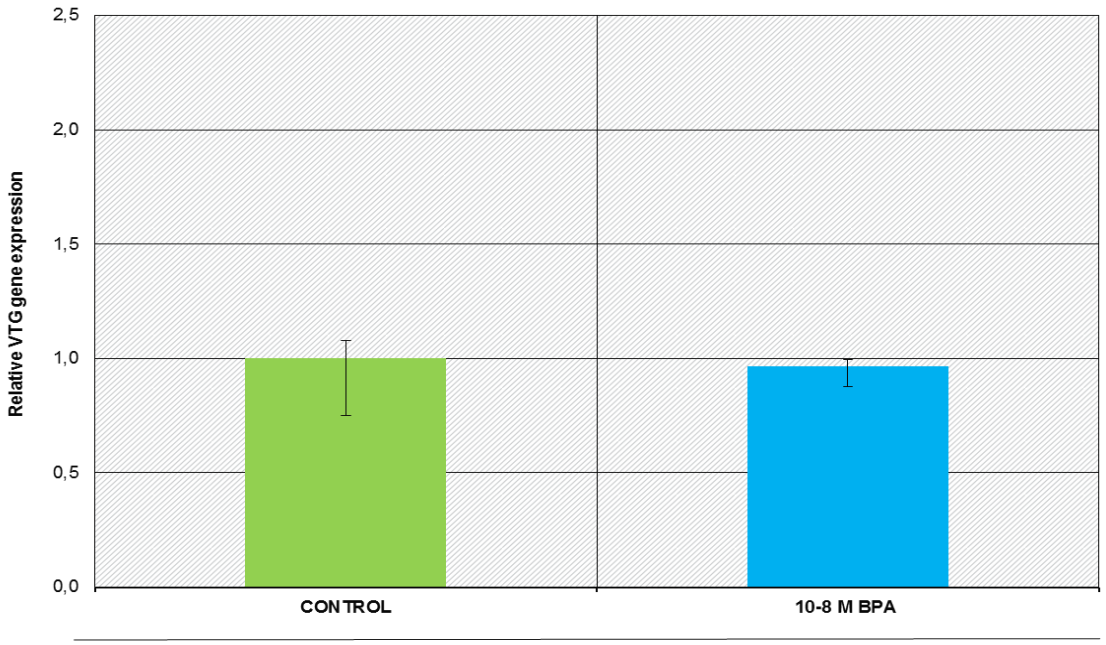

Figure 15: Medians and interquartile ranges of the relative gene expression of vitellogenin (VTG) in liver tissue of adult male *X. laevis* (n = 10) after an eight-day exposure to 10^{-8} M bisphenol A (BPA). Statistical differences were determined using General Linear Mixed models and Sidak post-hoc tests. Differences between treatments were not significant ($p > 0.05$).

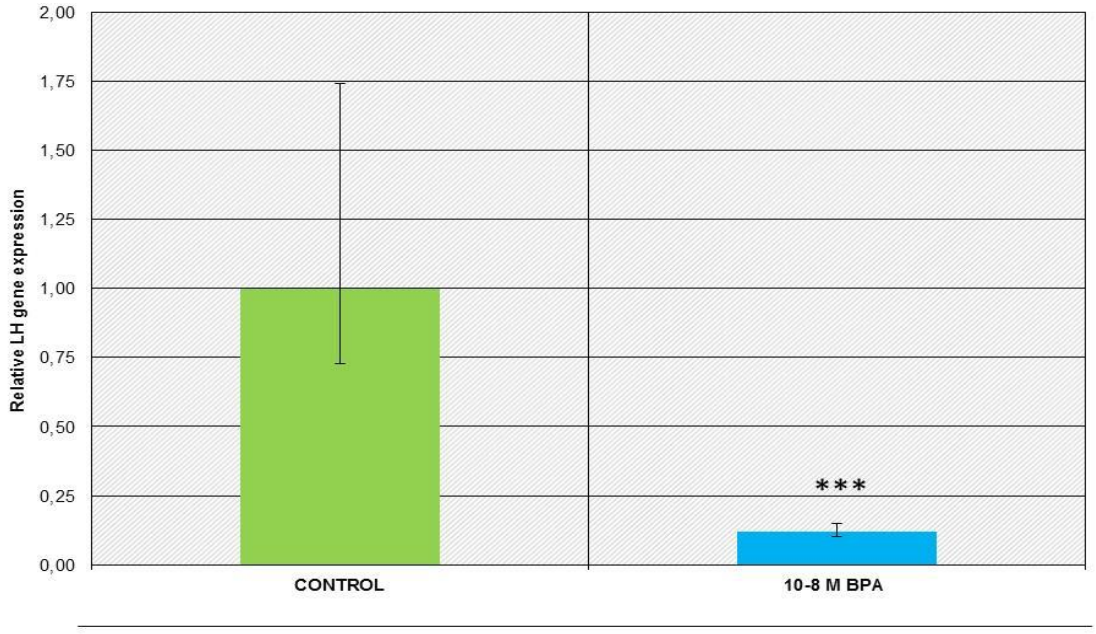

Figure 16: Medians and interquartile ranges of the relative gene expression of the gonadotropin luteinizing hormone (LH) in brain tissue of adult male *X. laevis* (n = 10) after an eight-day exposure to 10^{-8} M bisphenol A (BPA). Statistical differences were determined using General Linear Mixed models and Sidak post-hoc tests. Significant differences between treatments are marked by asterisk (*** $p < 0.001$).

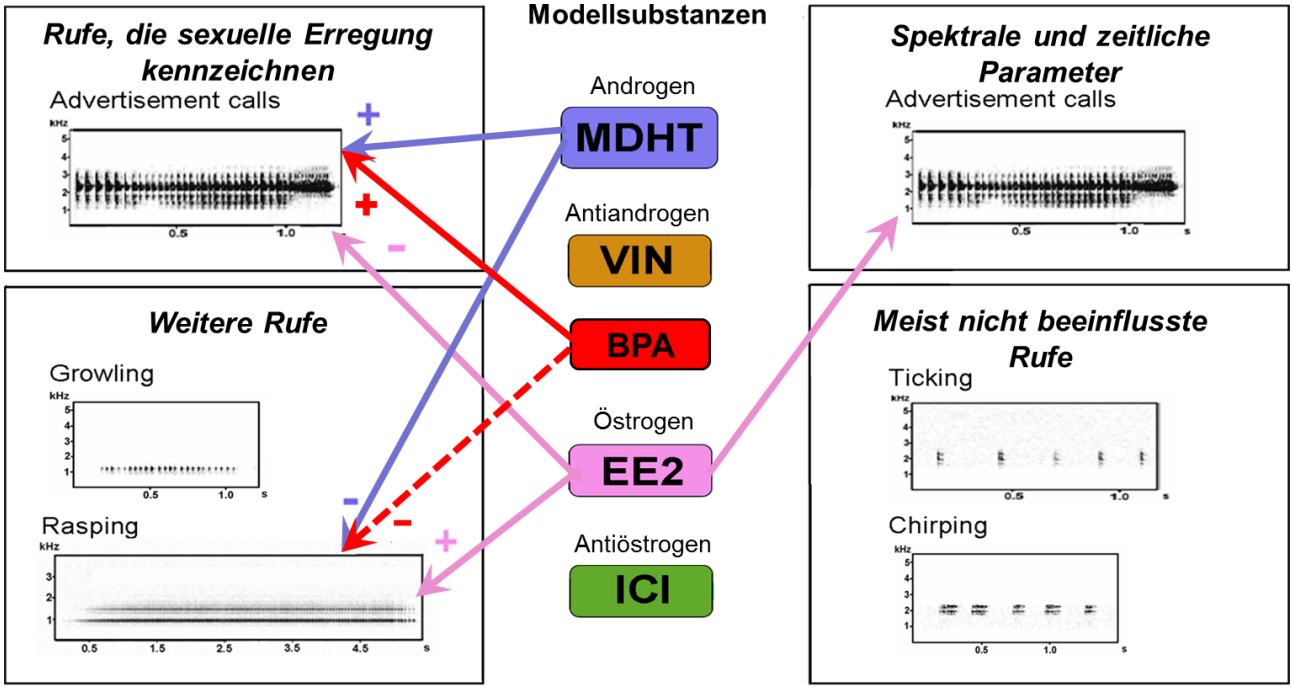

Figure 17 Schematic diagram of the effects of an exposure (96 h) to the supposedly estrogenic endocrine disrupting chemical (EDC) bisphenol A (BPA) in the flow-through (red arrows) and the androgenic methyldihydrotestosterone (MDHT; blue arrows) and the estrogenic ethinylestradiol (EE2; pink arrows) in the semi-static system. The estrogenic EE2 lowered the relative amount of advertisement calls and reduced the number of accentuated clicks and the click duration in the semi-static system (Hoffmann and Kloas 2012c). A semi-static EE2 exposure, however, did not affect growling but increased the amount of the call type rasping. Exposure to the androgenic MDHT resulted in an increased relative amount of advertisement calls and a lowered percentage of rasping in the semi-static system (Hoffmann and Kloas 2010, Hoffmann 2012). No temporal and spectral parameters of the advertisement calls were affected by MDHT. In the flow-through system, BPA caused the same effects than the androgenic MDHT, although BPA is considered an to elicit estrogenic MOA. BPA exposed animals produced higher amounts of advertisement calls and lower amounts of rasping. Spectral and temporal parameters were not affected by a BPA exposure.

5.3.2.4 Discussion

Surprisingly, both exposures to BPA resulted in similar or even identical effects in comparison to androgenic EDC (Hoffmann and Kloas 2010, Hoffmann 2012)(Fig. 17). It was previously shown that BPA elicits estrogenic MOA (Levy et al. 2004, Alonso-Magdalena et al. 2012). Hence, we expected similar impacts of BPA on the mate calling behavior of the frogs as seen in EE2 exposed male *X. laevis* (Hoffmann 2012, Hoffmann and Kloas 2012c). However, as in TREN and MDHT treated male frogs (Hoffmann 2012), BPA exposed males also uttered a higher percentage of advertisement calls and considerably less rasping compared to controls during both exposure experiments, indicating an increase in sexual arousal (Tobias et al. 1998, Hoffmann and Kloas 2010, Hoffmann 2012). Thus, it seems as if BPA does not elicit estrogenic MOA in adult male *X. laevis*. Accordingly, the established estrogenic biomarker VTG induction was not affected by the semi-static BPA exposure in this study. The results of further gene expression analyses and the sex steroid determination also correspond to that conclusion: the estrogen-characteristic elevated ARO gene expression and the increased levels of plasma E2 are missing in BPA exposed animals. Moreover, neither gonadal steroidogenic enzymes, nor FSH gene expression was altered due to a BPA exposure, while LH was reduced in BPA treated males. A reduction in LH expression can be the result of androgenic or estrogenic EDC treatment. Estrogenic EDC such as EE2, however, were shown to simultaneously affect sex steroid levels, resulting in decreased T and increased E2 levels, whereas an androgen exposure did not alter plasma sex steroid concentrations (Urbatzka et al. 2006). Since the estrogen biomarker VTG was not induced

by BPA, the inhibition of LH gene expression, thus, points towards a slight to moderate androgenic MOA of BPA. Accordingly, sex steroid levels and ratios were not affected by BPA exposure.

Previous studies suggested an estrogenic MOA for BPA in developing *X. laevis*, since a BPA exposure led to feminization (Levy et al. 2004). However, in that study different BPA concentrations lead to varying effects (hormesis effect)(Levy et al. 2004). Thus it might be possible, that an exposure of *X. laevis* to different concentrations of BPA results in distinct effects at different developmental stages, e.g. in the androgen-characteristic effects on the calling behavior in adult males. Furthermore, it cannot be ruled out that different endpoints are affected differently by an exposure to BPA, for instance via different pathways and mechanisms. Consistently, other studies demonstrated that BPA does not elicit any estrogenicity, e.g. in *Daphnia magna* (Caspers 1998) or only at extremely high concentrations, as demonstrated in a reporter gene assay (Tarumi et al. 2000). Future studies should focus on this phenomenon and systematically screen for potential estrogenic or androgenic effects resulting from an exposure of various aquatic vertebrate species to different environmentally relevant concentrations of BPA.

Concerning the applicability of XENOCALL, further substances, which clearly elicit direct estrogenic effects in adult *X. laevis*, such as E2 or nonylphenol (Lutz et al. 2005), should be tested to ascertain that known estrogenic EDC generally elicit the estrogen-specific alterations in the mate calling behavior of the frogs. BPA, moreover, should not generally be considered as a pure estrogenic EDC.

5.3.3 Exposure to the androgenic 17beta-trenbolone (TREN)

5.3.3.1 Introduction

17α-trenbolone, as well as 17β-trenbolone (TREN) are the metabolites of the synthetic androgen trenbolone acetate, which is tremendously used as a promoter for a more effective meat production in beef feedlots in the US and China (Wei et al. 2011, Bartelt-Hunt et al. 2012). Both metabolites, which also elicit androgenic MOA have already been found in the environment at concentrations in the two-digit ng/L-range (Soto et al. 2004, Durhan et al. 2006, Wei et al. 2011, Khan and Lee 2012). Alarmingly, TREN has a long half-life up to 260 days (Schiffer et al. 2001). Thus, TREN effects on wildlife, especially on the reproductive physiology and development of aquatic vertebrates are detected and assessed frequently (Ankley et al. 2003, Holbech et al. 2006, Örn et al. 2006, Seki et al. 2006, Larsen and Baatrup 2010, Morthorst et al. 2010, Baumann et al. 2014a). TREN exposure during larval development, for instance, led to an androgen induced masculinization of in fish (Ankley et al. 2003, Larsen and Baatrup 2010, Morthorst et al. 2010, Holbech et al. 2012), which was shown to be irreversible(Morthorst et al. 2010, Baumann et al. 2014a). It was also demonstrated that TREN can affect the reproductive behavior of fish (Saaristo et al. 2013): in mosquitofish (*Gambusia holbrooki*) TREN exposure caused a reduced mating behavior of female fish, whereas TREN did not affect any male fish behaviors. Nevertheless, to date it is unknown whether TREN can also affect the courtship behavior of male aquatic vertebrates, such as *X. laevis*. Moreover, it needs to be examined, whether potential TREN effects on the calling behavior of male frogs resemble the effects of the androgenic MDHT(Hoffmann 2012, Hoffmann and Kloas 2012b), and thus can be considered androgen-specific.

5.3.3.2 Material and methods

30 male *X. laevis* (5 years of age; weight: 68.9 g ± 18.9 g; length: 9.0 cm ± 1.1 cm) were exposed to two different TREN concentrations (10^{-9} M and 10^{-11} M) and a solvent control, respectively. Experiments were carried out in the flow-through system and the calling behavior of the experimental frogs was recorded and analyzed as described previously (Hoffmann 2012). Temporal and spectral parameters of the advertisement calls were determined using software developed by Dr. Henning Thielemann. For this purpose, accurately

classified audio files of the single frogs were gathered manually and then fed to the software, which analyzed the following parameters of those calls: the duration of the whole call, as well as the duration and the mean frequency of the slow trill and the fast trill part, the mean number of clicks, as well as accentuated clicks, the click rate (number of clicks per second in the slow trill part of the advertisement calls), the duration of clicks and pauses between clicks (ICI) and the bandwidth within the slow trill part, and the mean frequency and bandwidth of the fast trill part of the calls. Frogs were injected with 100 units hCG and then exposed to TREN for 96 h. Exposure chemicals were dissolved in DMSO and stock solutions were prepared once at the beginning of the experiment. All chemicals were obtained from Sigma Aldrich (Steinheim, Germany). During the experiment, frogs were fed a commercial fish diet (Metabolica, Aller Aqua, Golßen, Germany; 2 pellets) every second day and water temperature was measured daily (21.6 ± 0.2 °C). The light:dark cycle was 12:12 h, with a light period starting at 7 am. At the end of the exposure period, all frogs were returned to the animal husbandry of the institute and transferred to different 60 L tanks, according to their assigned treatment.

As previously described, General Linear Mixed Model analyses (GLMM) were conducted (Hoffmann and Kloas 2010, Hoffmann 2012, Hoffmann and Kloas 2012b, c, a, d, Efosa et al. 2016, submitted) and subsequent Sidak post-hoc tests (SPSS 20, IBM, Ehningen, Germany) were performed to identify statistical differences between treatments. Normal distribution of data or residuals was ensured with Kolmogorov-Smirnov tests.

For verifying the desired test concentrations, water samples of each tank were taken and analyzed at the beginning and at the end of the experiment UHPLC-MS-MS (table 1).

5.3.3.3 Results

Male frogs exposed to TREN at both concentrations vocalized a higher amount of advertisement calls compared to the controls ($p = 0.040$; Fig. 18), however this effect was only statistically significant regarding the higher concentrated TREN treatment (10^{-9} M; $p = 0.040$) and not significant in the 10^{-11} M TREN treatment group ($p = 0.081$). TREN treated frogs also tended to utter less ticking (data not shown) and a significantly lower proportion of rasping (Fig. 19). However, again this effect was only statistically significant regarding the 10^{-9} M TREN treatment ($p = 0.032$) and not in the 10^{-11} M TREN treatment group ($p = 0.078$).

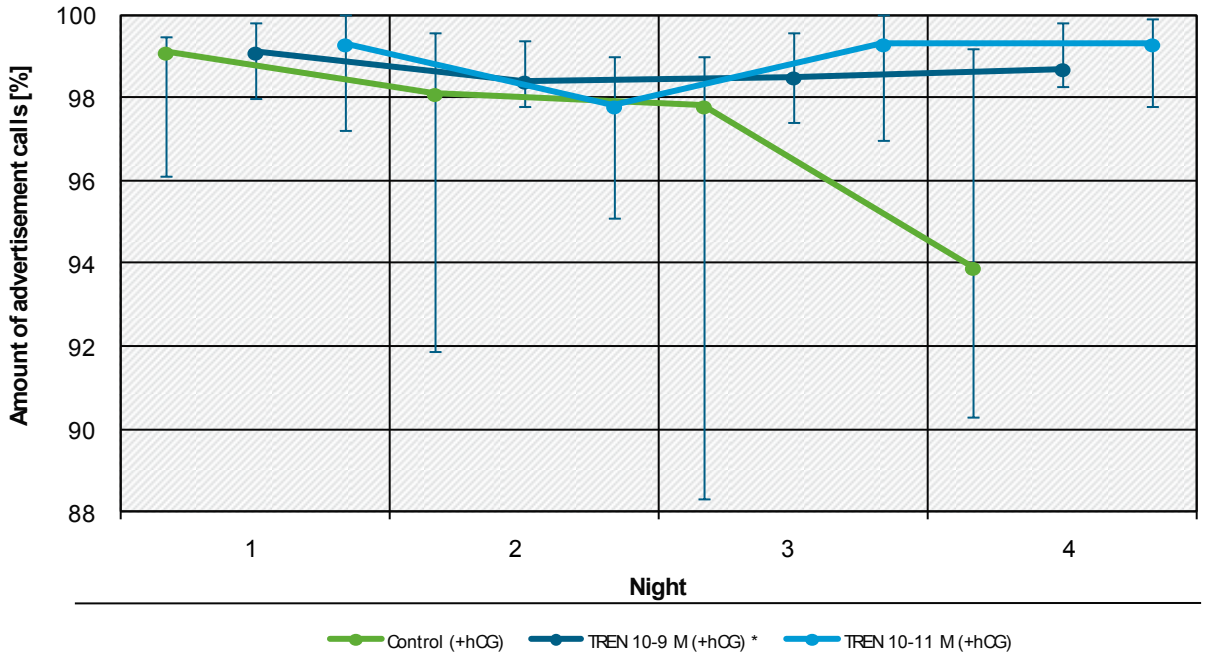

Figure 18 Line graph of the percentages of advertisement calls uttered during the four exposure nights after stimulation with hCG (median and interquartile ranges). Animals that were exposed TREN at both concentrations uttered a higher amount of advertisement calls compared to the control. However, this effect was only significant regarding the 10^{-9} M TREN treatment (GLMM: $p = 0.040$) and slightly missed a significant level in the 10^{-11} M TREN treatment group ($p = 0.081$).

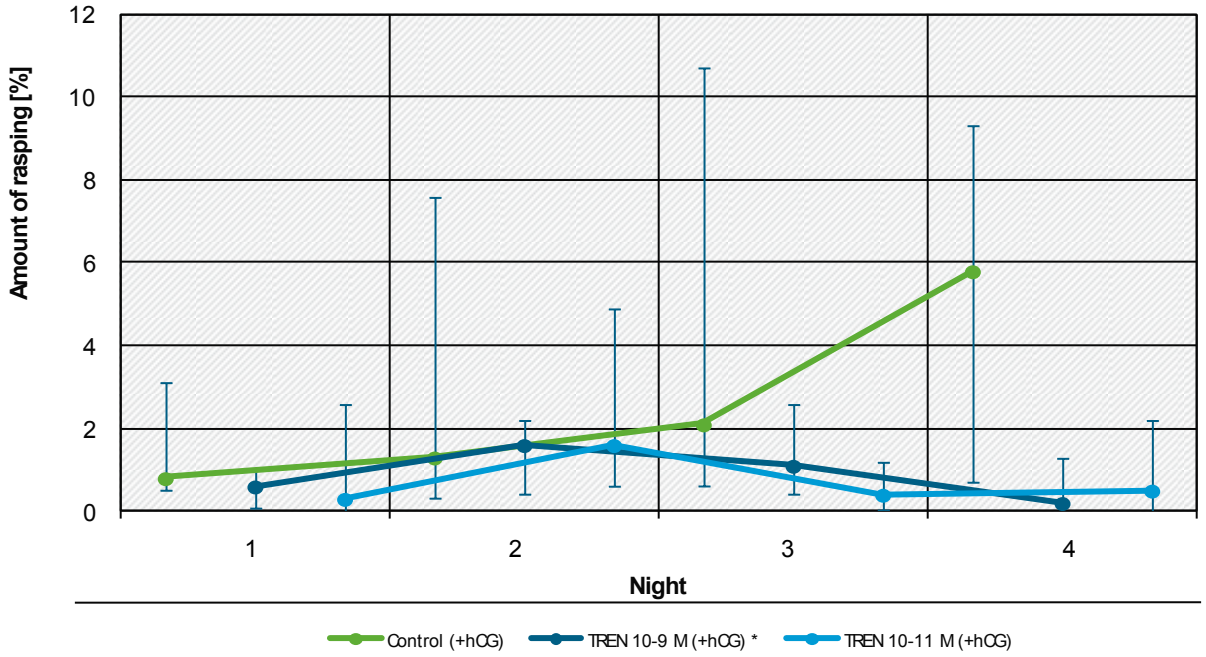

Figure 19 Line graph of the percentages of rasping uttered during the four exposure nights after stimulation with hCG (median and interquartile ranges). Animals that were exposed TREN at both concentrations uttered a lower amount of rasping compared to the control. However, this effect was only significant regarding the 10^{-9} M TREN treatment (GLMM: $p = 0.032$) and slightly missed a significant level in the 10^{-11} M TREN treatment group ($p = 0.078$).

The slow trills of the advertisement calls of frogs exposed to TREN at 10^{-11} M contained significantly less clicks than in the control group ($p = 0.036$), while this effect was not detectable in the 10^{-9} M TREN treatment (data not shown). Furthermore, the frequency of the slow and fast trills of the advertisement calls produced by male *X. laevis* of the two EDC treatments was significantly higher (slow trill: $p_{10^{-9}\text{ M TREN}} = 0.002$; $p_{10^{-11}\text{ M TREN}} = 0.028$; Fig. 20; fast trill: $p_{10\text{-}9\text{ M TREN}} < 0.001$; $p_{10\text{-}11\text{ M TREN}} = 0.049$; Fig. 21).

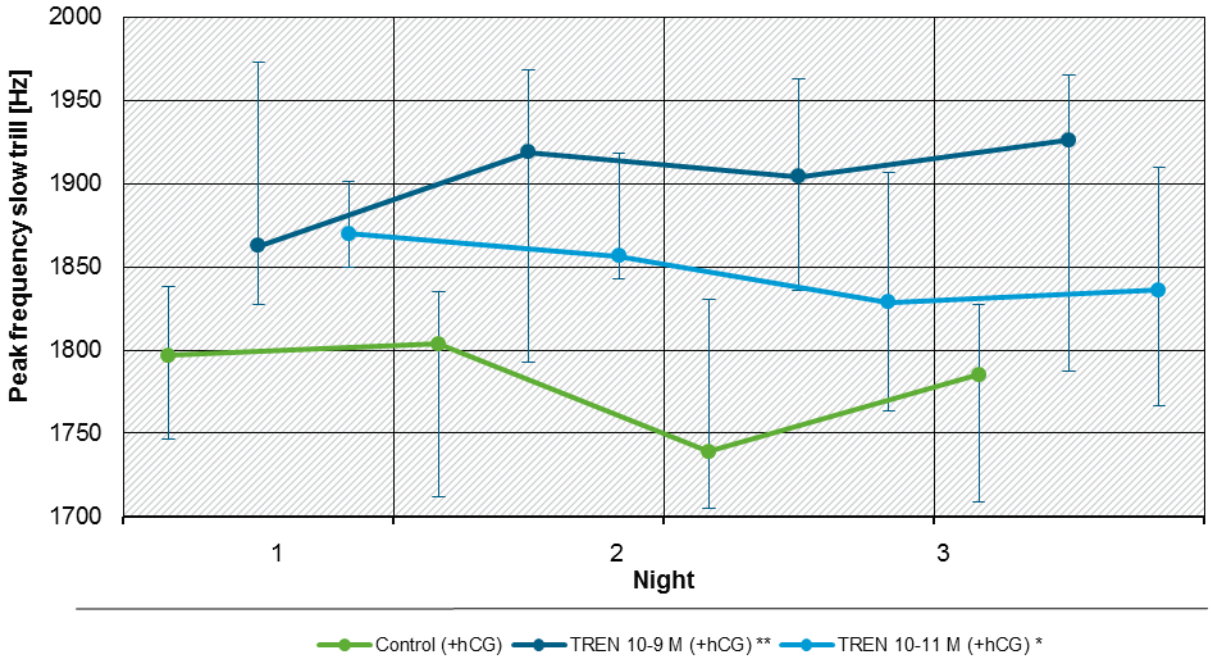

Figure 20: Line graph of the peak frequency (Hz) of the slow trill part of the advertisement calls uttered during the four exposure nights after stimulation with hCG (median and interquartile ranges). Animals that were exposed TREN at both concentrations produced higher slow trills within their advertisement calls compared to the controls (GLMM: $p_{10\text{-}9\text{ M TREN}} = 0.002$; $p_{10\text{-}11\text{ M TREN}} = 0.028$).

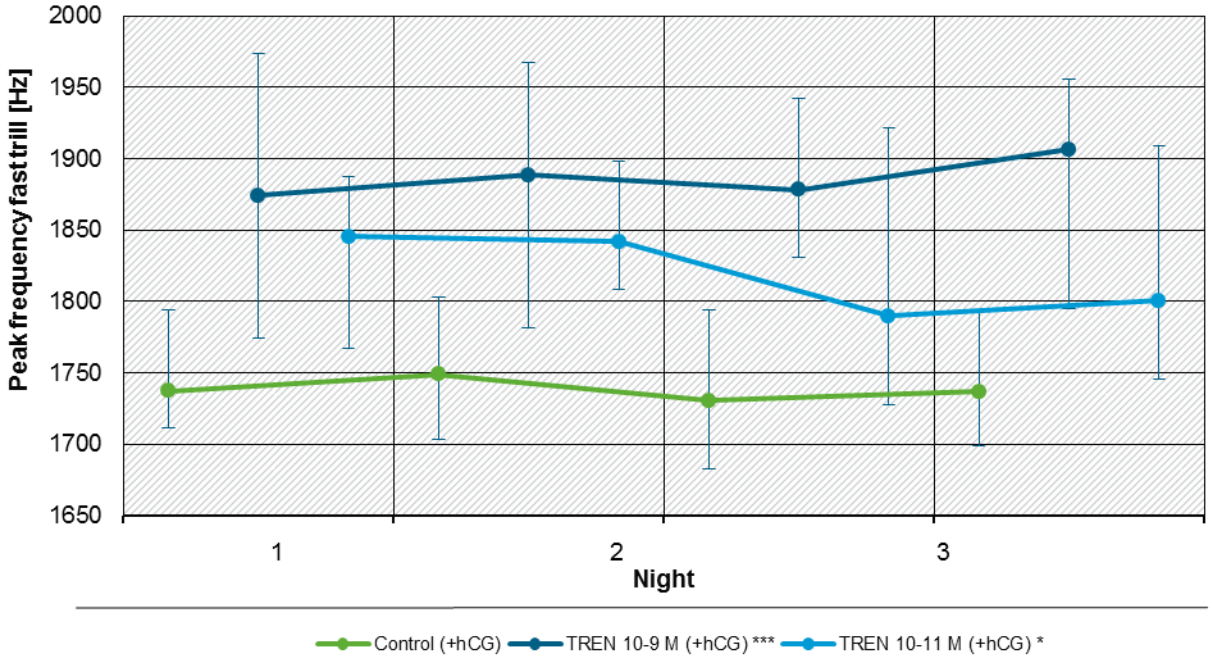

Figure 21: Line graph of the peak frequency (Hz) of the fast trill part of the advertisement calls uttered during the four exposure nights after stimulation with hCG (median and interquartile ranges). Animals that were exposed TREN at both concentrations produced higher fast trills within their advertisement calls compared to the controls (GLMM: $p_{10\text{-}9\ M\ TREN} < 0.001$; $p_{10\text{-}11\ M\ TREN} = 0.049$).

5.3.3.4 Discussion

Compared to the effects of the androgenic MDHT on the calling behavior of male *X. laevis* in a semi-static system (Hoffmann 2012, Hoffmann and Kloas 2012b), TREN had exactly the same effects (Fig. 22). Both EDC led to an increase in advertisement calling and lower percentages of the call type rasping, suggesting an enhanced sexual arousal of the exposed male frogs. This is probably due to direct androgen action on the male larynx and the vocal pathway within the central nervous system of male *X. laevis*, respectively (Kelley et al. 1975, Kelley 1980, 1981, Sassoon et al. 1986, Gorlick and Kelley 1987, Sassoon et al. 1987, Segil et al. 1987, Pérez et al. 1996, Kelley and Tobias 1999). Furthermore, as in MDHT>EE2 treated frogs (5.1.2), the slow trills of the advertisement calls of frogs exposed to TREN at 10^{-11} M contained significantly less clicks and the frequency of the slow and fast trills of the advertisement calls produced by male frogs of both EDC treatments was significantly higher. Hence, these effects might be androgen-specific. During a semi-static exposure to the androgenic MDHT, these effects on temporal and spectral parameters could not be detected (Hoffmann and Kloas 2012b), although similar effects were noticeable in frogs simultaneously exposed to MDHT and EE2 in the flow-through system (5.1.2). One reason for these differences in temporal and spectral parameters of advertisement calls between tests and test systems might have been the different methods of data exploration and measurement. While only few calls per frog could be analyzed manually in the semi-static experiment, many more advertisement calls could be analyzed using analysis software developed by Dr. Henning Thielemann. The analysis of more calls, hence, can result in more accurate data. Moreover, slight differences in parameter determination between the two software programs (semi-static: Avisoft, flow-through: Thielemann-software) might also be the reason for the slightly deviating results. Nevertheless, since spectral features of *X. laevis* calls seem to be relevant for the attractiveness of the respective calls (Vignal and Kelley 2007), deviations of those stereotype parameters might result in a lower or even higher mating success of TREN exposed frogs. Further experiments using a female choice setup should investigate these potential outcomes.

Furthermore, in the semi-static system, as well as in the flow-through system similar concentrations (10^{-10} M versus 10^{-9} M) resulted in significant results. Lower concentrations (10^{-11} M TREN) also resulted in obvious effects, which however, were mostly not significant. The utilization of more test animals or a prolonged exposure period, hence, might increase the sensitivity of this detection method for androgenic EDC. We could show that the XENOCALL method can be also used as biomarker for androgenic EDC, as an exposure to these substances results in identical effects.

Previous studies in fish indicated that reproductive behaviors of male aquatic vertebrates are not affected by TREN exposure, while female behaviors are (Saaristo et al. 2013). In a prior study, we could show as well that androgenic EDC can affect female amphibian mating behavior (Hoffmann 2012, Hoffmann and Kloas 2012b). Supraphysiological concentrations of the androgen MDHT, for example, decreased the phonotactic behavior of females, while physiological concentrations of the same substance resulted in an increase in phonotactic behavior (Hoffmann und Kloas 2012b). However, we also found androgenic effects on the calling behavior of male *X. laevis* even at very low, environmentally relevant concentrations (Hoffmann and Kloas 2010, Hoffmann 2012). The androgenic effects in females were less prominent and occurred only at higher concentrations (Hoffmann and Kloas 2012b). In this study, we could confirm that that the mate calling behavior of male *X. laevis* can be affected by androgenic EDC, and an exposure to the androgen TREN causes identical effects on the calling behavior as previously shown for MDHT (Hoffmann and Kloas 2010, Hoffmann 2012).

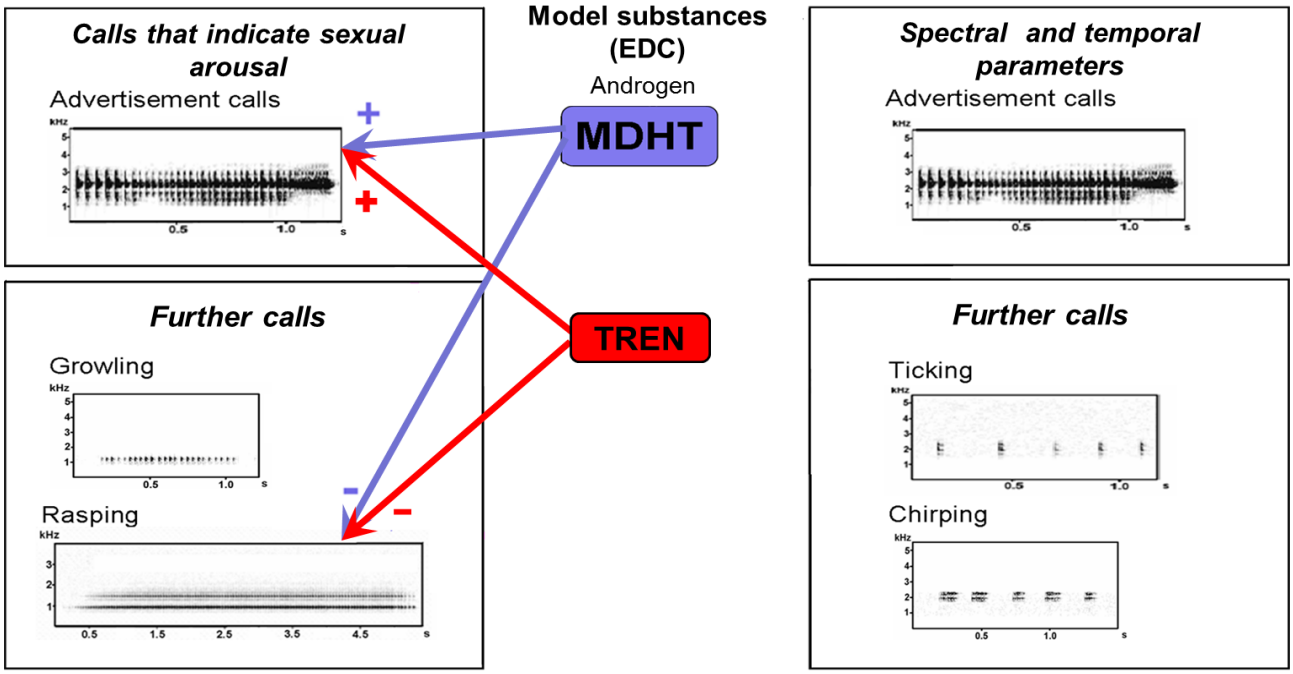

Figure 22 Schematic diagram of the effects of an exposure (96 h) to the androgenic endocrine disrupting chemical (EDC) trenbolone (TREN) in the flow-through (red arrows) and methyldihydrotestosterone (MDHT) in the semi-static (blue arrows) system. Exposure to both androgenic EDC resulted in identical results: an increased relative amount of advertisement calls and a lowered percentage of rasping (Hoffmann and Kloas 2010, Hoffmann 2012). The slow trills of the advertisement calls of frogs exposed to TREN at 10-11 M contained significantly less clicks than in the control group, while this effect was not detectable in the 10-9 M TREN treatment. Furthermore, the frequency of the slow and fast trills of the advertisement calls produced by male *X. laevis* of the two TREN treatments was significantly higher

5.3.4 Exposure to the antiandrogenic vinclozolin (VIN)

5.3.4.1 Introduction

The fungicide VIN has been shown to elicit antiandrogenic MOA (Kang et al. 2004). In 2001 the use of VIN was forbidden in Germany, however, down to the present day, VIN is still commonly used against fungi on fruits, vegetables, and wine grapes across the United States of America and Europe, illicitly including Germany (Spencer 1982, Laws et al. 1996, EPA 2000, Greenpeace 2004). Its consumption is considered to be several tons per year (Readman et al. 1997, Steeger and Garber 2009a). VIN and its metabolites M1 and M2 (Kelce et al. 1994, Kelce et al. 1997) have the capability to relocate from treatment sites by runoff and leaching (Steeger and Garber 2009b). In surface waters, VIN has been detected at concentrations in the one-digit µg/L-range (Gülden et al. 1997, Readman et al. 1997, El-Shahat et al. 2003) and even in drinking water, its maximum detected concentration was 0.1 µg/L (Iwan 1988). Besides its use in agriculture, VIN and its metabolites are also used as model substances for antiandrogenic modes of action (Ottinger et al. 2001, Kubota et al. 2003, Loutchanwoot et al. 2008). They competitively inhibit androgen binding to the AR by fitting into the hormone binding domain (Kelce and Wilson 1997) and inhibiting the expression of AR-dependent genes (Kelce et al. 1994, Wong et al. 1995, Kelce and Wilson 1997). Studies in rats and birds, as well as our previous study demonstrate that VIN treatment can alter hormonally regulated behaviors, including amphibian mate calling (Hotchkiss et al. 2003, Satre et al. 2009, Hoffmann and Kloas 2010).

5.3.4.2 Material and methods

Adult male *X. laevis* (3 years of age; weight: 74.1 g ± 8.1 g; length: 9.4 cm ± 0.7 cm) were exposed to three different VIN concentrations (10^{-10} M, 10^{-11} M and 10^{-12} M; n = 8) in the flow-through system and their calling behavior was recorded and analyzed as described previously (5.1.2.1). Temporal and spectral parameters of the advertisement calls were determined using software developed by Dr. Henning Thielemann. For this purpose, accurately classified audio files of the single frogs were gathered manually and then fed to the software, which analyzed the following parameters of those calls: the duration of the whole call, as well as the duration and the mean frequency of the slow trill and the fast trill part, the mean number of clicks, as well as accentuated clicks, the click rate (number of clicks per second in the slow trill part of the advertisement calls), the duration of clicks and pauses between clicks (ICI) and the bandwidth within the slow trill part, and the mean frequency and bandwidth of the fast trill part of the calls. VIN, similar to EE2, can reduce the sexual arousal of male *X. laevis* after an hCG-stimulation (Hoffmann and Kloas 2010, Hoffmann 2012). Thus, in this experiment, male frogs were likewise injected with 100 units hCG before exposing them to VIN for four consecutive nights. DMSO (> 99%) was used as solvent to prepare stock solutions and all chemicals were obtained from Sigma Aldrich (Steinheim, Germany). During the experiment, the light:dark cycle was 12:12 h and frogs were fed a commercial fish diet (Metabolica, Aller Aqua, Golßen, Germany; 2 pellets) every second day. Water temperature in the test tanks was 21.6 ± 0.2 °C throughout the whole experiment. At the end of the exposure period, all frogs were returned to the animal husbandry of the institute and transferred different 60 L tanks, according to their assigned treatment. Animals were tested again after a basic recovery time without EDC exposure (5.4).

As previously mentioned, GLMM were conducted for identifying statistical differences between treatments as described previously (Hoffmann and Kloas 2010, Hoffmann 2012, Hoffmann and Kloas 2012b, c, a, d, Efosa et al. 2016, submitted) with subsequent Sidak post-hoc tests (SPSS 20, IBM, Ehningen, Germany). Normal distribution of data or residuals was ensured with Kolmogorov-Smirnov tests.

For verifying the desired test concentrations, water samples of each tank were taken and analyzed at the beginning and the end of the experiment using gas-chromatography (GC; also see paragraph 4.2). Water samples were concentrated as previously explained in (Garmshausen et al. 2015) (appendix 3) and table 1.

5.3.4.3 Results

Analytical water analyses

None of the supply tanks of the control group contained measurable traces of VIN. Due to the low sensitivity of the testing method, only water samples from the supply tank of the highest VIN treatment (10^{-10} M) could be analyzed in the GC. Water samples from the supply tanks of this treatment group (desired concentration: 10^{-8} M = 2610 ng/L VIN) contained 2603.5 ng/L (2058.6 ng/L–2863.9 ng/L VIN) (median (interquartile range). The solutions in the supply tanks were directly used to produce the supply solutions for the other treatments (≈ 10^{-9} M and 10^{-10} M VIN), as well as the exposure solutions in the test tanks (≈ 10^{-10} M, 10^{-11} M and 10^{-12} M VIN; 200-fold dilution) and the amount of dilution water was measured daily. Thus, it is reasonable to assume that the exposure concentrations in those tanks were in acceptable ranges as well (around 80%). For simplification purposes, the nominal test concentrations are used in the denotation of the particular treatments in the following sections.

Calling behavior

Frogs exposed to VIN at 10^{-10} M produced a lower proportion of advertisement calls than control animals, but implementing all three VIN treatments, this effect was not significant ($p = 0.181$; Fig. 23). However, considering only the 10^{-10} M VIN treatment, this effect resulted in a statistically significant value ($p = 0.022$). Furthermore, exposure to VIN at 10^{-10} M resulted in a significantly higher amount of ticking ($p < 0.001$; Fig. 24), while VIN at lower concentrations did not affect these parameters.

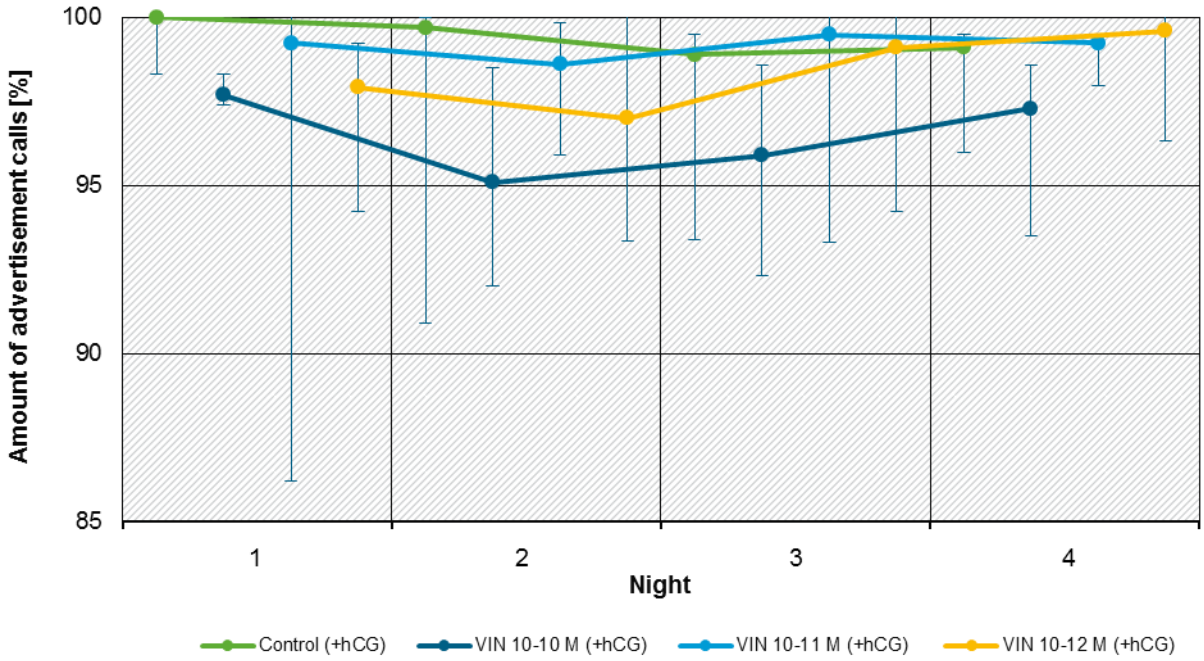

Figure 23 Line graph of the percentages of advertisement calls uttered during the four exposure nights after stimulation with hCG (median and interquartile ranges). Animals that were exposed VIN at 10^{-10} M produced a lower amount of advertisement calls than control animals, however this effect slightly missed statistical significance (GLMM: $p > 0.05$).

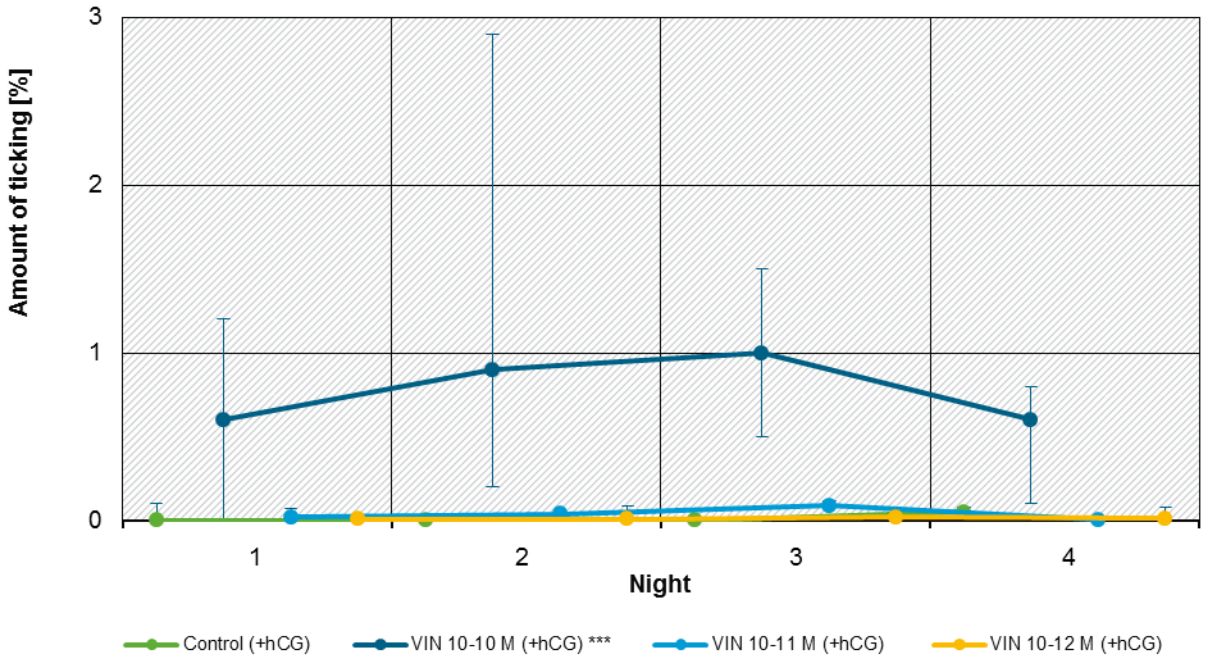

Figure 24 Line graph of the percentages of ticking uttered during the four exposure nights after stimulation with hCG (median and interquartile ranges). Animals that were exposed VIN at 10^{-10} M produced a significantly higher amount of ticking than control animals (GLMM: $p < 0.001$), while exposure to lower VIN concentrations did not affect this parameter.

The inter click interval (ICI), defined as the duration of the pauses between single clicks within the slow trill part of the advertisement calls, was higher in advertisement calls of VIN 10^{-10} M exposed frogs (Fig. 25). Moreover, the click rate of the advertisement calls (number of clicks per second in the slow trill part of the advertisement calls) of VIN 10^{-10} M exposed frogs was lower than within advertisement calls of control frogs (Fig. 26). However, again these effects were only significant when solely the 10^{-10} M treatment was considered in the statistical analysis ($p_{10^{-10}\text{ M - ICI}} < 0.001$; $p_{\text{all treatments-ICI}} = 0.057$; $p_{10^{-10}\text{ M - click rate}} = 0.019$; $p_{\text{all treatments - click rate}} = 0.092$). Furthermore, the duration of the fast trill part of the advertisement calls was lower in VIN 10^{-10} M treated animals, but again this effect was not significant when all treatments were implemented in the statistical analysis ($p_{10^{-10}\text{ M}} = 0.046$; $p_{\text{all treatments}} = 0.072$; data not shown).

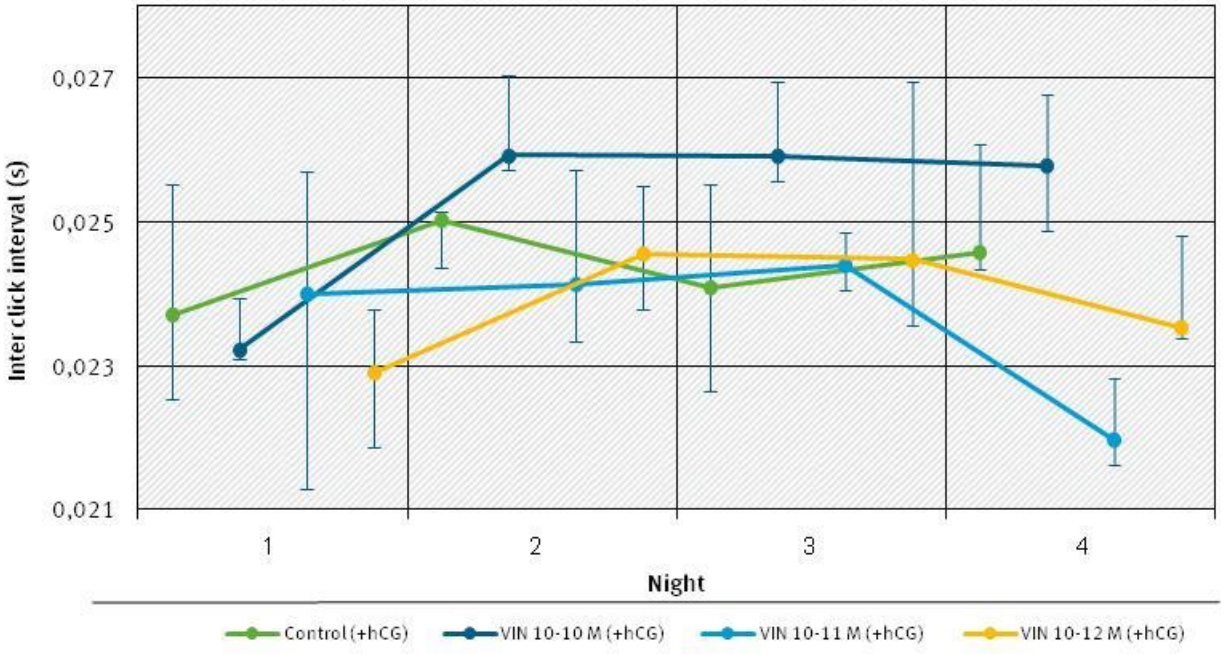

Figure 25 Line graph of the duration of inter click intervals (ICI) within the slow trill part of advertisement calls uttered during the four exposure nights after stimulation with hCG (median and interquartile ranges). Animals that were exposed VIN at 10^{-10} M produced advertisement calls with a higher ICI duration, however this effect slightly missed statistical significance when implementing all treatment groups (GLMM: $p > 0.05$).

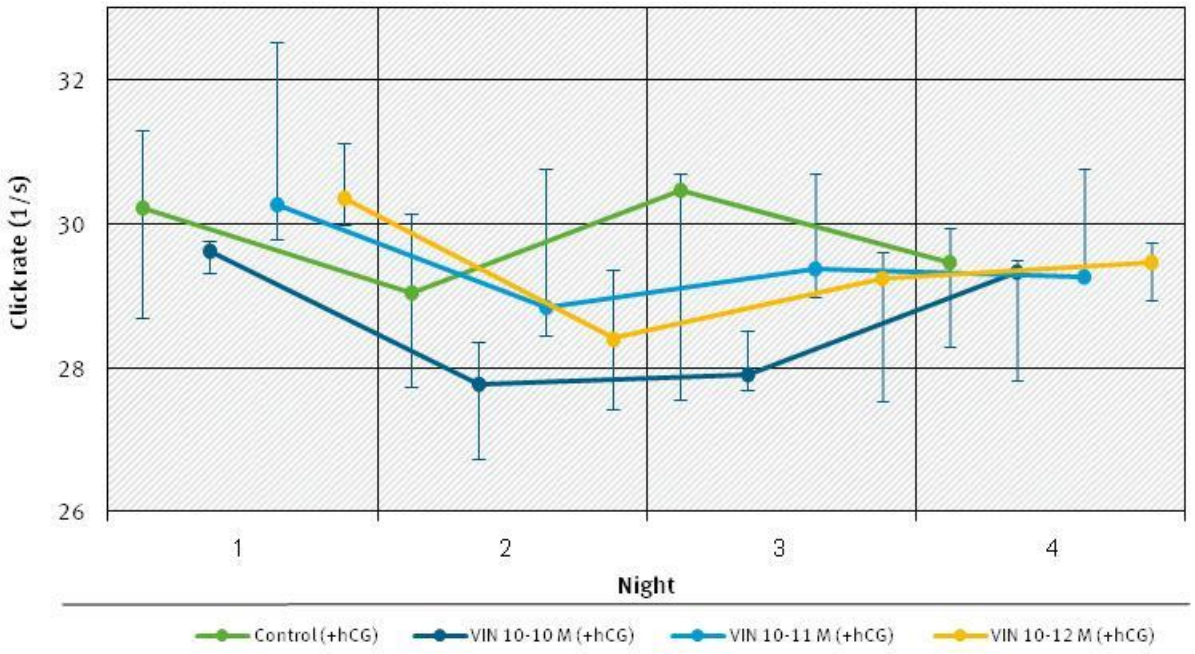

Figure 26 Line graph of the click rate (1/s) within the slow trill part of advertisement calls uttered during the four exposure nights after stimulation with hCG (median and interquartile ranges). Animals that were exposed VIN at 10^{-10} M produced advertisement calls with a lower click rate, however this effect slightly missed statistical significance when implementing all treatment groups (GLMM: $p > 0.05$).

5.3.4.4 Discussion

Animals exposed to VIN in the flow-through system basically showed the same alterations in their calling behavior as male *X. laevis* exposed to VIN in the semi-static system (Hoffmann and Kloas 2010, Hoffmann 2012)(Fig. 27). In both systems, animals produced a lower percentage of advertisement calls and a higher amount of ticking, indicating a lower sexual arousal of exposed males (Hoffmann and Kloas 2010, Hoffmann 2012)(Fig. 27). In the semi-static system, these effects were highly significant at a concentration of 10^{-10} M VIN. In the flow-through system a statistical calculation implementing only the 10^{-10} M treatment resulted in significant effects, suggesting that a statistical approach including all conducted treatments might result in non significant results, similarly as it could be seen in EE2 treated male frogs. This lower sensitivity of XENOCALL in the flow-through system again might be a consequence of the lower sample size (n = 8 versus n = 10), but also of the increased background noise and the constantly running water in the flow-through system accompanied by periodical water movements in the exposure tanks, which leads to abrupt interruptions in the calling behavior of the males (Fig. 7). These interferences made more difficult to detect possible alterations of the calling behavior. To still be able to detect environmentally relevant concentrations of antiandrogenic EDC in the flow-through system, probably more test animals would have to be exposed to the respective testing chemical ($n \geq 10$) (Hoffmann and Kloas 2010, Hoffmann 2012) and the flow-through should be adjusted to a minimum tolerable water exchange rate.

In the flow-through system the call parameter amount of growling was not affected by a VIN exposure, although previous, semi-static results demonstrated that VIN can affect this call parameter (Hoffmann and Kloas 2010, Hoffmann 2012). The fact that EE2 can also affect this parameter (5.3.1.3) further strengthens the suggestion that this call type can be used to indicate a low sexual arousal in general not only in amphibians exposed to antiandrogenic EDC. However, the amount of ticking was also increased in 10^{-10} M VIN treated frogs, semi-statically and in the flow-through system (10^{-10} M), but never in EE2 treated frogs. Thus, this call type might be an indicator of antiandrogenic activity broadcasting a low sexual arousal of the exposed frogs. The temporal and spectral parameters which were affected by a VIN exposure in the semi-static system (number of accentuated clicks and the duration of clicks within the slow trill part of the advertisement calls) also tended to be affected in the flow-through system, but differences between treatments were not significant. However, VIN had further impacts on temporal and spectral parameters of the advertisement calls when experiments were carried out in the flow-through system. The ICI was higher, while the click rate was lower in 10^{-10} M VIN treated males. Furthermore, the duration of the fast trill part of the advertisement calls was higher in 10^{-10} M VIN exposed animals. One reason why these effects could not be detected in the semi-static system might be the method of data exploration and measurement. While only few calls per frog could be analyzed in the manual fashion in the semi-static tests, many more advertisement calls could be analyzed using analysis software newly developed by Dr. Henning Thielemann. The analysis of more calls might have resulted in more accurate data. Moreover, slight differences in parameter determination between the two software programs (semi-static: Avisoft, flow-through: Thielemann-software) might also be the reason for the slightly deviating results. Nevertheless, since especially the temporal characteristics of those calls are important for species and individual recognition (Loftus-Hill 1971, Picker 1983, Klump and Gerhardt 1992, Schwartz 1994, Bibikov and Nizamov 1996, Gerhardt et al. 1996, Gerhardt and Schul 1999, Gerhardt et al. 2000, Gerhardt 2001, Schwartz et al. 2001, Bush et al. 2002, Schul and Bush 2002, Beckers and Schul 2004, Deily and Schul 2004, Gerhardt 2005b, Vignal and Kelley 2007, Bush et al. 2009, Deily and Schul 2009, Gerhardt and Brooks 2009, Gordon and Gerhardt 2009, Klump and Gerhardt 2013), deviations of those stereotype parameters might result in a lower mating success of VIN exposed frogs. Lower concentrations of VIN (10^{-11} M and10^{-12} M VIN), on the other hand, did not affect the calling behavior of the frogs. Thus, the lowest observed effect concentration (LOEC) of VIN in the flow-through system was 10^{-10} M VIN, while the no observed effect concentration (NOEC) of VIN was 10^{-11} M.

Regarding antiandrogenic EDC, a testing method applying a completely or at least partly semi-static EDC exposure might be more sensitive and thus, more suitable than the XENOCALL method in the flow-through system.

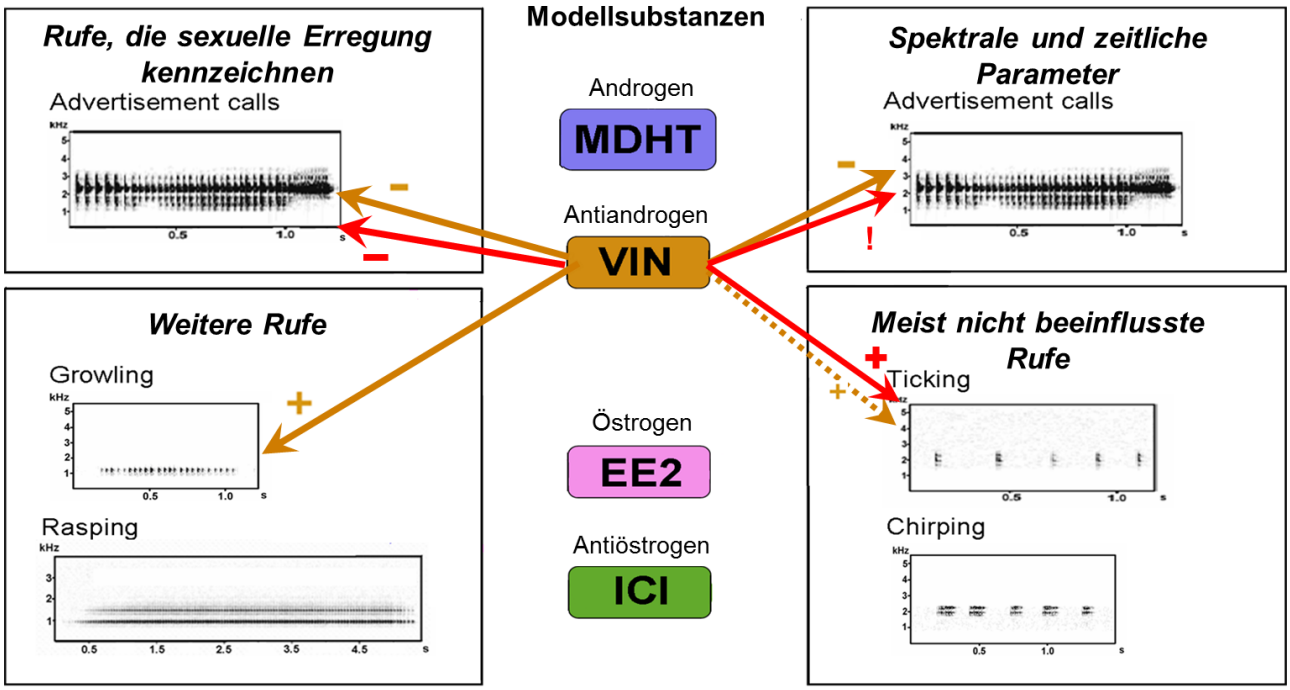

Figure 27 Schematic diagram of the effects of an exposure (96 h) to the endocrine disrupting chemical (EDC) vinclozolin (VIN) in the semi-static (orange arrows) and the flow-through (red arrows) system. In both systems, the antiandrogenic VIN lowered the relative amount of advertisement calls and enhanced the percentages of ticking (Hoffmann and Kloas 2010, Hoffmann 2012). A semi-static VIN exposure, however, did also increased the amount of growling (Hoffmann and Kloas 2010, Hoffmann 2012). In the flow-through system, VIN did not affect this call type. Semi-statically, VIN exposure reduced the number of accentuated clicks and the click duration of the advertisement calls, in the flow-through system these effects were also visible but effects were not statistically significant. However, further significant temporal and spectral alterations of the advertisement calls were found: advertisement calls of VIN exposed frogs had higher ICIs and a lower click rate, as well as longer fast trills than control advertisement calls. The other two treatments (10^{-11} M and 10^{-12} M) did not affect the calling behavior of the frogs.

5.3.5 Exposure to the antiandrogenic dichlorodiphenyldichloroethylene (DDE)

5.3.5.1 Introduction

P,p'-dichlordiphenyldichloroethylene (DDE) is a metabolite of the massively used insecticide DDT (dichlorodiphenyltrichloroethane). In the early 1970's adverse impacts of those substances were revealed (Markowitz 1998) and from then on, the use of DDT was forbidden in most of the world's countries. Nevertheless, due to its long half-life, DDE can still be found in the (aquatic) environment (Stevens and Neilson 1989, Guillette Jr et al. 1994, Semenza et al. 1997, Mayer et al. 2000, Wurl and Obbard 2004). DDE can elicit toxic (Bowerman et al. 2000, Kumar et al. 2002) and other adverse effects, e.g. on the reproductive physiology of vertebrates (Guillette Jr et al. 1994, Semenza et al. 1997, Bayley et al. 2002, Clotfelter et al. 2004, Zala and Penn 2004, Yang et al. 2005, Kristensen et al. 2006, Benachour et al. 2007, Hotchkiss et al. 2008, Quinn et al. 2008). It was shown to elicit estrogenic and antiandrogenic MOA in various species (Guillette Jr et al. 2005). However, evidence that DDE can alter the mate calling behavior of male

amphibians, especially of *X. laevis*, and particularly in a distinctive antiandrogen-specific way, is still lacking.

Details of this study can be obtained from the respective manuscript "P,p'-dichlordiphenyldichloroethylene (p,p'-DDE) can exhibit antiandrogenic and estrogenic modes of action in an amphibian (*Xenopus laevis*)", which is submitted for publication in *Physiology and Behavior* and which can be found in appendix 6.

In short, animals exposed to DDE (10^{-9} M and 10^{-11} M) experienced similar alterations in their calling behavior than male *X. laevis* exposed to the antiandrogenic VIN in the semi-static (Hoffmann and Kloas 2010, Hoffmann 2012) and the flow-through system (5.3.3.3), respectively. The typical effects evoked by antiandrogenic EDC like VIN in adult male *X. laevis* such as a lower amount of advertisement calls and a higher proportion of ticking (Hoffmann and Kloas 2010) were also caused by DDE exposure (Fig. 28). Surprisingly an increased amount of rasping was detected as well. This effect is typically elicited when male *X. laevis* are exposed to estrogenic EDC (Hoffmann 2012, Hoffmann and Kloas 2012c)(Fig. 28). A reason for those "mixed effects" might be a partially estrogenic activity of DDE besides its antiandrogenic MOA. It was previously suggested that DDE can elicit both MOA (estrogenic and antiandrogenic), but few studies actually tested this hypothesis in a straightforward way (Vonier et al. 1996, Clark et al. 1998, Willingham and Crews 1999, Frigo et al. 2002, Willingham 2004). Hence, the XENOCALL method can be used as biomarker for estrogenic and antiandrogenic EDC, since an exposure to such chemicals results in MOA-specific effects distinguishing between those two MOA, even if a tested chemical triggers both MOA.

Taken together, the results demonstrate that DDE, which is still environmentally relevant (Stevens and Neilson 1989, Guillette Jr et al. 1994, Semenza et al. 1997, Mayer et al. 2000, Wurl and Obbard 2004), indeed alters the reproductive behavior of male *X. laevis* potentially, resulting in a lowered sexual arousal of exposed males. This is indicated by the decreased production advertisement calls and higher amounts of calls that indicate a sexually unaroused state of the male (Tobias et al. 1998, Hoffmann and Kloas 2010). Similarly it was previously shown for several species, that DDE can alter reproductive physiology, especially in male aquatic vertebrates (Guillette Jr et al. 1994, Semenza et al. 1997, Yang et al. 2005, Kristensen et al. 2006, Quinn et al. 2008). Our results further indicate that DDE can display both, estrogenic and antiandrogenic MOA, either of which can have adverse effects on reproductive physiology and behavior (Urbatzka et al. 2007, Cevasco et al. 2008, Hoffmann and Kloas 2010, Massari et al. 2010, Hoffmann 2012, Hoffmann and Kloas 2012c). The disruption of mating behavioral traits, which are crucial for a successful procreation, might result in a reduced reproductive success of DDE exposed animals, as it was shown for other aquatic species, such as alligators, turtles and fish (Guillette Jr et al. 1994, Semenza et al. 1997, Zala and Penn 2004, Yang et al. 2005, Kristensen et al. 2006, Quinn et al. 2008).

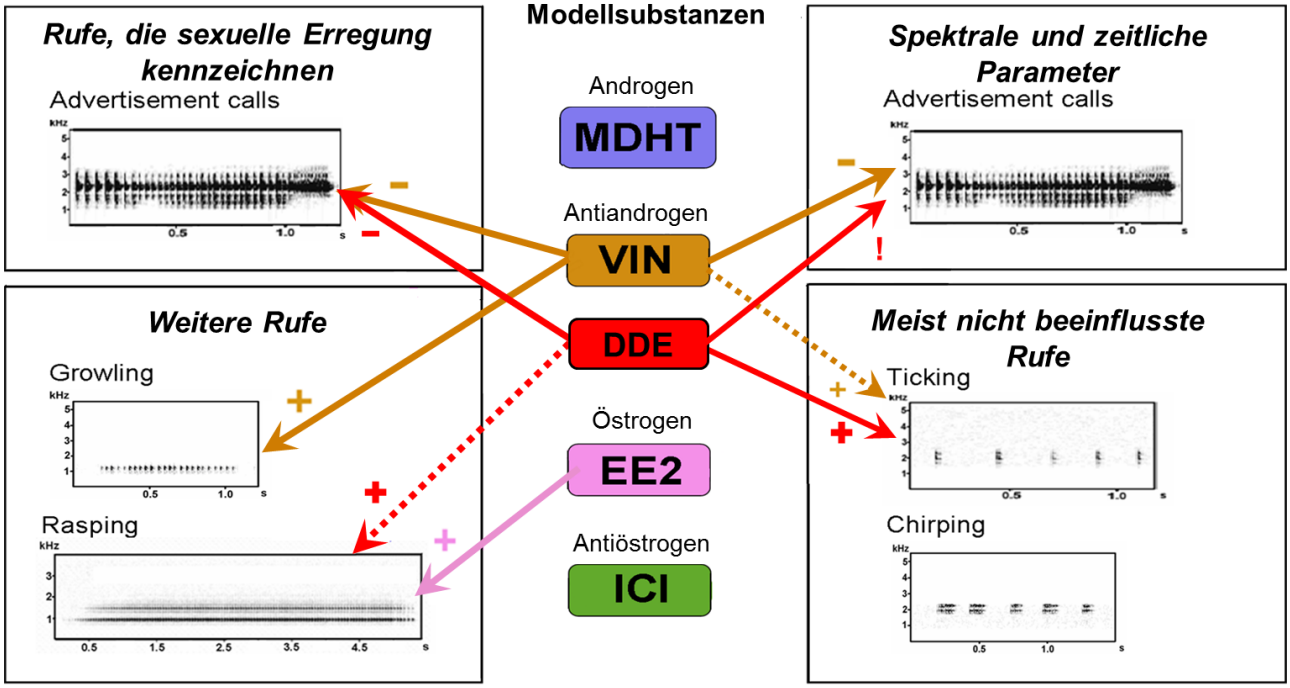

Figure 28 Schematic diagram of the effects of an exposure (96 h) to the antiandrogenic endocrine disrupting chemical (EDC) vinclozolin (VIN, orange arrows) and the estrogenic ethinylestradiol (EE2, pink arrows) in the semi-static system and the supposedly antiandrogenic P,p'-dichlordiphenyldichloro-ethylene (DDE) in flow-through system (red arrows). VIN lowered the relative amount of advertisement calls and enhanced the percentages of ticking and growling (Hoffmann and Kloas 2010, Hoffmann 2012). A semi-static EE2 exposure, on the other hand led to a lower relative amount of advertisement calls and an increased amount of rasping (Hoffmann 2012, Hoffmann and Kloas 2012c). A DDE exposure in the flow-through system also led to reduced percentages of advertisement calls, however, DDE also elevated the amount of ticking and growling, which are antiandrogen-specific effects, as well as the proportions of rasping, a typical estrogen-specific effect. These accumulated results suggest that DDE might trigger both, estrogenic and antiandrogenic effects, as it was previously assumed in different species (Vonier et al. 1996, Clark et al. 1998, Willingham and Crews 1999, Frigo et al. 2002, Willingham 2004).

5.4 Re-utilization of experimental animals

5.4.1 Ethinylestradiol (EE2)

We could previously show, that the EE2 induced alterations of the calling behavior persists during four weeks under control conditions but reached control levels again after six to eight weeks without EDC exposure (Hoffmann 2012, Hoffmann and Kloas 2012c). Similarly, it was previously demonstrated that feminizing EE2 effects on sexual development can be reversed (Baumann et al. 2014b). Because these findings suggest that it might be possible to test experimental animals in more than one test trial, we exposed male frogs to EE2 in the flow-through system (see 5.3.1), and afterwards kept the frogs under control conditions for approximately 8 weeks. We then tested the calling behavior of the frogs again in the flow-through system, this time without EDC exposure. The aim was to examine whether males recover from the EE2 effects or whether those effects persist. If the estrogen-specific effects vanished, we would re-expose the frogs to EE2 and analyze their calling behavior once more, to see whether the typical EE2 effects return.

In paragraph 5.3.1 we demonstrated that an exposure to environmentally relevant concentrations in the flow-through system results in the estrogen-specific alterations in the mate calling behavior of male frogs. In the first recovery trial without EE2 exposure, the previous estrogen-specific impacts (5.3.1) could not be detected in the 10^{-10} M and 10^{-13} M EE2 treatment. However regarding the 10^{-12} M EE2 treatment, the

estrogen-specific effects that were visible but statistically insignificant during the EE2 exposure (5.3.1), were now even more pronounced. Without having EE2 in the surrounding exposure water, these estrogenic effects (lower amount of advertisement calls and higher percentages of rasping) were significantly different from the controls (Fig. 29 and 30). Temporal and spectral parameters of the advertisement calls of the frogs, on the other hand, did not differ between the treatments and the control calls anymore.

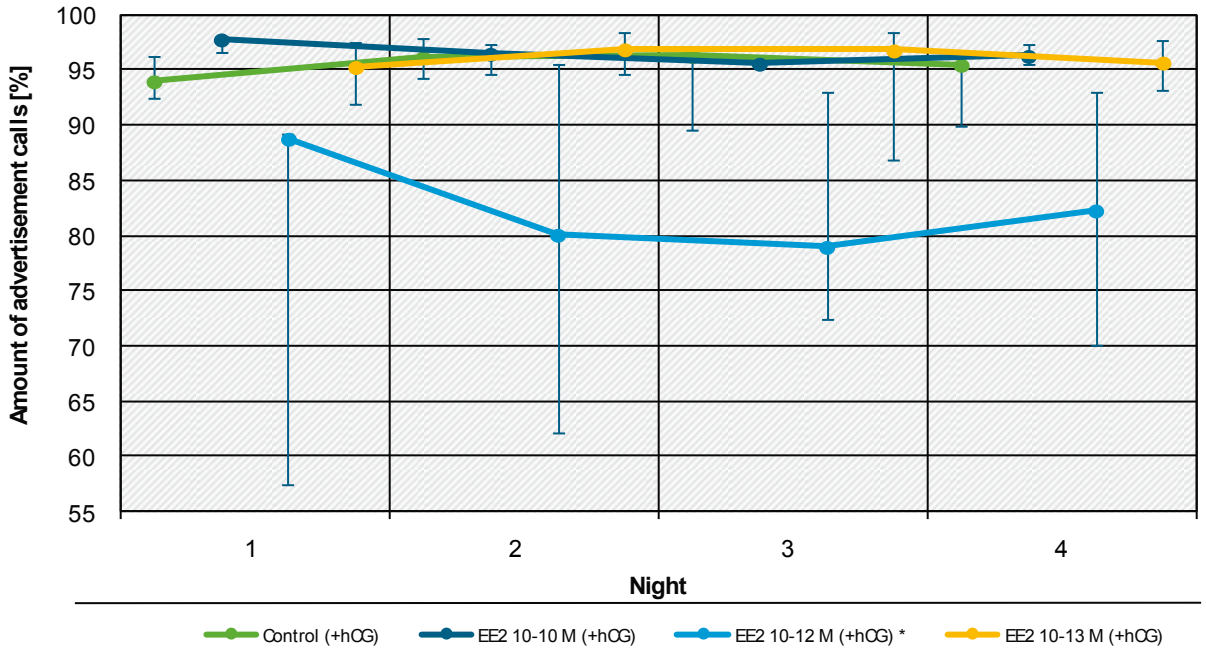

Figure 29 Line graph of the percentages of advertisement calls uttered during the four exposure nights after stimulation with hCG (median and interquartile ranges). Calling behavior of animals that were previously exposed to EE2 at 10^{-10} M and 10^{-13} M did not differ from the controls in this EDC-free experiment. 10^{-12} M EE2 treated males, however, elicited the typical estrogen-specific effects (5.3.1, lower amount of advertisement calls) even in a more pronounced and statistically significant manner ($p = 0.028$).

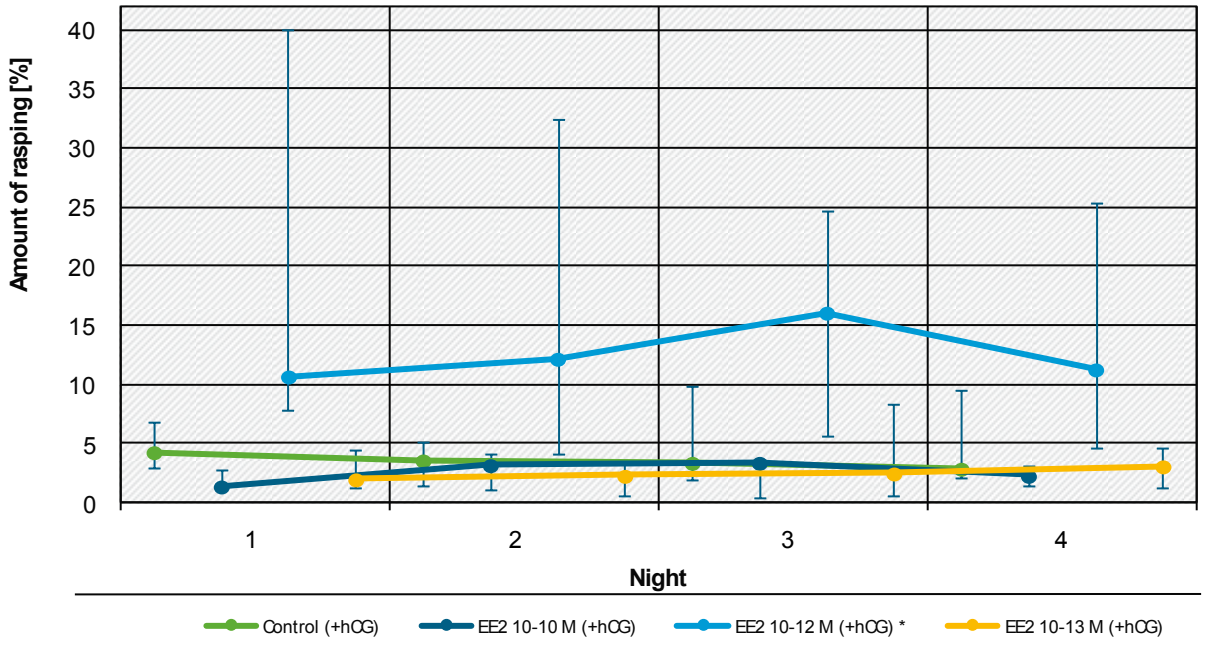

Figure 30 Line graph of the percentages of rasping uttered during the four exposure nights after stimulation with hCG (median and interquartile ranges). Calling behavior of animals that were exposed to EE2 at 10^{-10} M and 10^{-13} M did not differ from the controls. 10^{-12} M EE2 treated males, however, experienced the typical estrogen-specific effects (lower amounts of advertisement calls and higher amounts of rasping) in pronounced and statistically significant manner (GLMM: $p = 0.038$).

In a re-trial after another 8 weeks, where individuals were exposed to the test substance EE2 for 96 h once more, animals of the treatment groups (10^{-10} M EE2 und 10^{-12} M EE2) showed the estrogen-typical effects on the calling behavior again. They called a lower proportion of advertisement calls ($p < 0.001$; Fig. 31) and a higher amount of rasping ($p < 0.001$; Fig. 32). Those effects were even more pronounced than in the first EE2 exposure trial. A reason for this increased effectiveness of EE2 on the calling parameters might have been a potentiation effect due to the repeated exposure regime, a phenomenon which was previously demonstrated in other species (Li et al. 1989, Diamond et al. 1994, Taylor and Jentsch 2001). Furthermore, animals of both treatments uttered a higher proportion of growling and ticking, respectively. Accordingly, those call types that were additionally produced increasingly in the EE2 re-exposure might also be a result of the potentiation.

During the second recovery trial, none of the parameters of the calling behavior of the frogs of the two EE2 treatment groups differed from the control treatment (data not shown). Spectral and temporal analyses of the advertisement calls during the EE2 re-trial and the second recovery test, however, could not be performed during the project period, due to the lack of automated analysis software. Analyses had to be performed manually. Correspondingly, analyses lasted a significantly increased amount of time (~4 weeks per test trial compared to ~48 h using automatic software). Nevertheless, it seems as if the temporal and spectral parameters recover and attain control values after the applied recovery time, in which frogs were not exposed to any EDC.

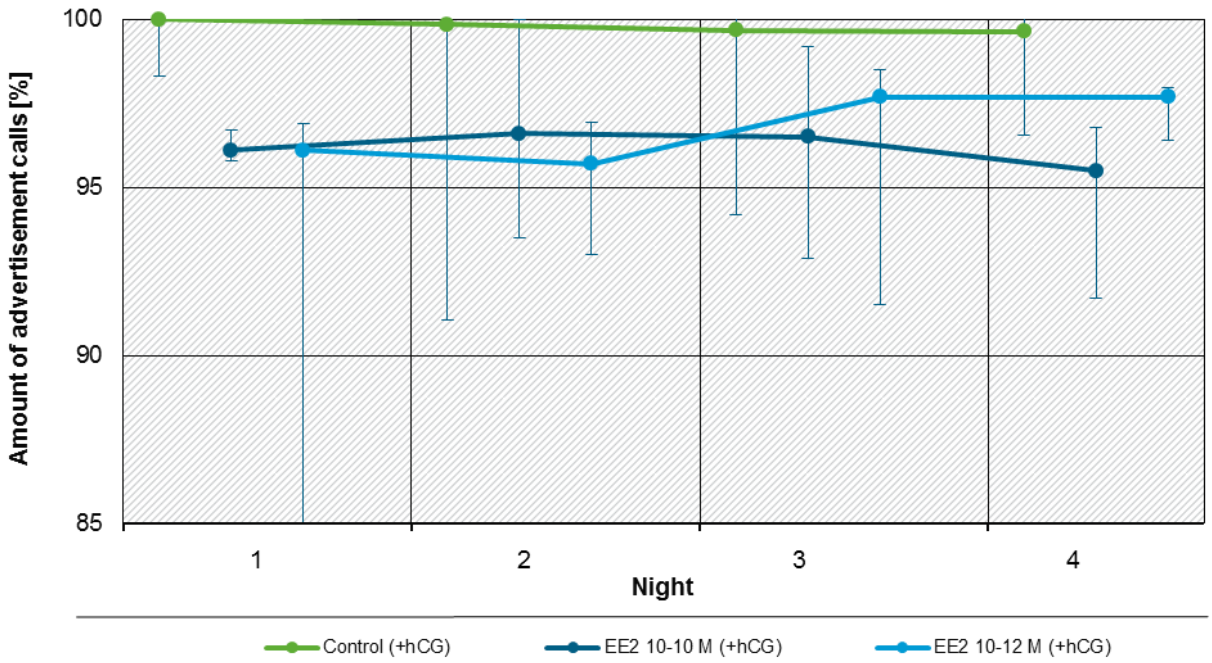

Figure 31: Line graph of the percentages of advertisement calls uttered during the four exposure nights of the EE2 re-trial after stimulation with hCG (median and interquartile ranges). Animals that were exposed to EE2 at 10^{-10} M and 10^{-12} M uttered a significantly lower amount of advertisement calls (GLMM: $p < 0.001$).

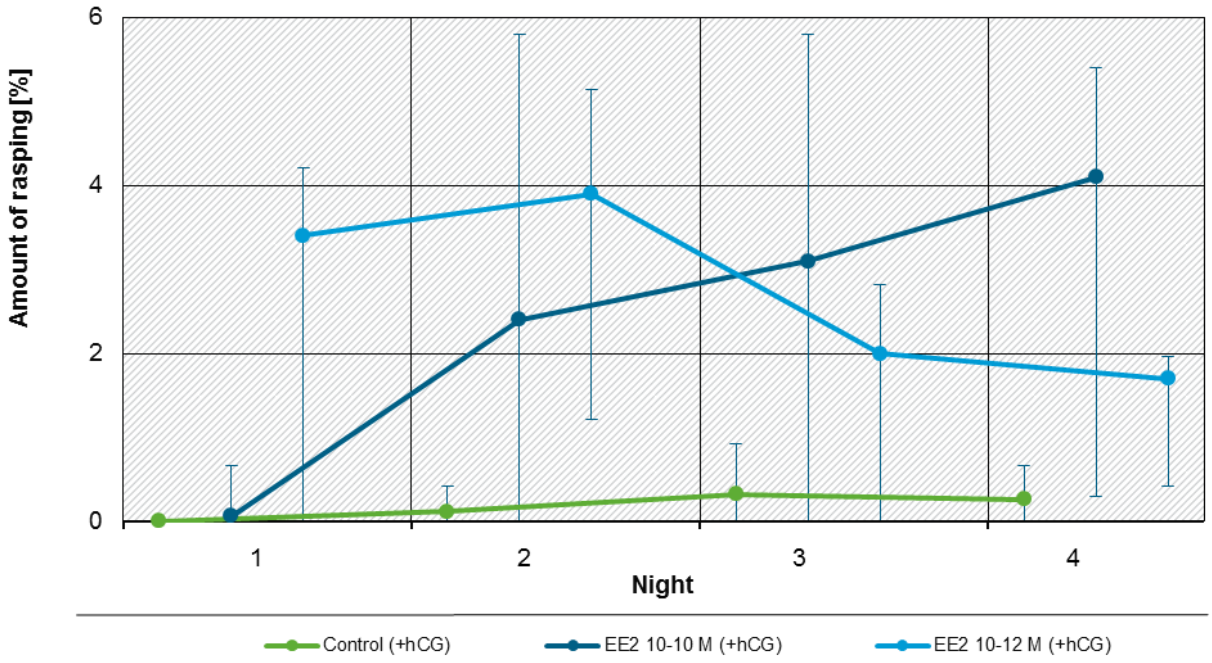

Figure 32: Line graph of the percentages of rasping uttered during the four exposure nights of the EE2 re-trial after stimulation with hCG (median and interquartile ranges). Animals that were exposed to EE2 at 10^{-10} M and 10^{-12} M produced a significantly lower proportion of rasping compared to the control (GLMM: $p < 0.001$).

Considering these continuous and persistent but not dose-dependent EE2 effects after keeping the experimental animals under control conditions, we have to recognize that EDC exposed male frogs cannot necessarily be reused in several test trials. A prolonged exposure-free period might resolve this problem, but animals either way have to be tested for an altered calling behavior prior to every test trial to ensure that they have recovered from potential enduring EDC effects.

5.5 Testing of "negative substances" (3 chemicals)

So called "negative substances" are chemicals, which do not elicit (anti)androgenic and (anti)estrogenic MOA but which still might be able to affect the calling behavior of the male *X. laevis*. To ensure that chemicals without (anti)androgenic and (anti)estrogenic MOAs do not affect this behavior at all or at least not in the demonstrated MOA-specific ways, we tested 3 negative substances (diclofenac [DCF], triiodothyronine [T3] and metoprolol [METO]) and evaluated their impacts on the male amphibian mate calling behavior.

5.5.1 Exposure to diclofenac (DCF)

In this experiment, we exposed male *X. laevis* to two different concentrations of DCF for 2 x 96 h (10^{-8} M and 10^{-10} M). DCF is a non-steroidal anti-inflammatory drug, which elicits its medical purpose by inhibiting inflammation-inducing cyclooxygenases. For the first 96 h animals were not stimulated with hCG, whereas they were injected with hCG prior to the second 96 h exposure. During the exposure we recorded and analyzed the mate calling behavior of the frogs. Afterwards we sacrificed the frogs and examined gene expression profiles of various genes involved in the HPG-axis, as well as plasma sex steroid levels. Details of this study can be obtained from the respective manuscript "Diclofenac exhibits direct estrogenic modes of action in male *Xenopus laevis*, and causes further side effects disturbing the hypothalamus-pituitary-gonad axis and mating vocalizations" (Efosa et al. 2016, submitted) which is submitted for publication in *Environmental Science and Technology* and which can be found in appendix 4.

In short, we could show that DCF elicits slight to moderate estrogenic MOA. Exposure to DCF resulted in the estrogen-characteristic alterations of the calling behavior of male *X. laevis*: exposed frogs uttered a lower percentage of advertisement calls and a higher amount of rasping compared to the controls before being sexually stimulated by hCG. The stimulating potency of the applied hCG treatment, which is known to promote increases in sex steroid production (Forest et al. 1979, Rasar and Hammes 2006) might have masked the decrease in sexual arousal of DCF exposed males. Moreover, exposure to DCF led to a dose-dependent induction of VTG gene expression, an estrogenic biomarker. Furthermore, we could demonstrate that DCF can elicit further pharmacological impacts on gonadal steroidogenesis, imbalancing sex steroid ratios. Since DCF can be found in considerable amounts in surface waters (Ternes 1998, Sacher et al. 2001, Heberer 2002), the estrogen-typical alterations of the calling behavior of male *X. laevis* due to an exposure to DCF might result in a decreased mating and reproductive success, although the estrogenic MOA of DCF was only slight to moderate.

Thus, DCF should not be considered a negative substance for the assessment of the applicability of XENOCALL, but rather as an estrogenic EDC. XENOCALL, nevertheless, was able to detect the slight to moderate estrogenicity of DCF, since DCF exposed frogs exhibited similar estrogen-characteristic alterations of their calling behavior: they uttered a lower amount of advertisement calls and a higher proportion of rasping before the hCG stimulation.

5.5.2 Exposure to triiodothyronine (T3)

In this experiment, we exposed male *X. laevis* to two different concentrations of T3 for 2 x 96 h (10^{-8} M and 10^{-10}). T3 is one of the main thyroid hormones which interfere with the hypothalamus-pituitary-thyroid system. Thyroid hormones play an important role in vertebrate development, including ontogeny or amphibian metamorphosis (Brown and Cai 2007, Forhead and Fowden 2014). Synthetic thyroid hormones or antithyroidal drugs are commonly used in the production of medical compounds against several thyroid diseases. Therefore a wide range of (anti)thyroidal pharmaceuticals can be found in effluents and consequently also in surface waters (Svanfelt et al. 2010, Pérez-Fernández et al. 2014).

5.5.2.1 Material and methods

In this experiment, adult male *X. laevis* (3 years of age; weight: 64.2 g ± 6.4 g; length: 9.2 cm ± 0.5 cm) were exposed to two different T3 concentrations (10^{-8} M and 10^{-10} M) in a flow-through system (n = 10) and their calling behavior was recorded and analyzed as described previously (5.1.2.1). Temporal and spectral parameters of the advertisement calls were determined using software developed by Dr. Henning Thielemann. For this purpose, accurately classified audio files of the single frogs were gathered manually and then fed to the software, which analyzed the following parameters of those calls: the duration of the whole call, as well as the duration and the mean frequency of the slow trill and the fast trill part, the mean number of clicks, as well as accentuated clicks, the click rate (number of clicks per second in the slow trill part of the advertisement calls), the duration of clicks and pauses between clicks (ICI) and the bandwidth within the slow trill part, and the mean frequency and bandwidth of the fast trill part of the calls.

To ensure that T3 is neither enhancing nor reducing the mate calling behavior of the frogs, we exposed the males for 96 h without prior stimulation with hCG (Russell 1960, Hoffmann and Kloas 2012b). We then injected each male with 100 units hCG (dissolved in 50 µL distilled water) in the dorsal lymph sack to stimulate a basic mate calling behavior and exposed them again for another 96 h. Exposure chemicals were dissolved in dimethyl sulfoxide (> 99%; DMSO) to prepare the stock solutions (0.00001 %). All chemicals were obtained from Sigma Aldrich (Steinheim, Germany). During the experiment, frogs were fed a commercial fish diet (Metabolica, Aller Aqua, Golßen, Germany; 2 pellets) every other day. Water temperature in the test tanks was 22.0 ± 0.1 °C throughout the whole experiment and the light:dark cycle was 12:12 h, with a light period starting at 7 am.

At the end of the exposure period, all frogs were anesthetized using MS 222 (0.01%), sacrificed and tissue samples were taken and processed (RNA extraction, RT, qPCR) as described in paragraph 5.1.2. The gene expression of the gonadotropins LH and FSH was measured in brain samples, while gene expression profiles of RED 1 and 2 and ARO were examined in gonad samples. VTG expression was investigated in liver samples. Primer pair sequences can be found in Efosa et al. (2016, submitted) and Garmshausen et al. (2015) (appendix 3). Primer pair efficiencies ranged between 1.98 and 2.01 (Pfaffl 2001, Urbatzka et al. 2010). Data was analyzed applying the $\Delta\Delta C_T$ method (Pfaffl 2001, Efosa et al. 2016, submitted) and the elongation factor 1 α (EF) was used as normalizing housekeeping gene.

GLMM (Hoffmann and Kloas 2010, Efosa et al. 2016, submitted) with subsequent Sidak post-hoc tests (SPSS 20, IBM, Ehningen, Germany) were conducted to identify statistical differences between treatments. Normal distribution of data or residuals was ensured with Kolmogorov-Smirnov tests.

To verify the desired test concentrations, water samples of each tank were taken and analyzed at the beginning and the end of the experiment using UHPLC-MS-MS (table 1).

5.5.2.2 Results

Analytical water analyses

None of the control tanks contained traces of T3. T3 concentrations in the supply tanks of the flow-through system (desired concentration: 659 µg/L and 6.59 µg/L), were adequate with a recovery rate of 81 % (71.1 – 83.4 %) (Median (interquartile range), but T3 concentrations in the test tanks were only about 10 – 30% of the expected concentration (Tab. 1).

Calling behavior and gene expression analyses

T3 slightly but statistically insignificant reduced the total vocal output of exposed male frogs, especially when their mating behavior was stimulated with hCG beforehand (data not shown). No other call parameters were affected by a T3 exposure. LH, FSH, ARO, RED 1 and 2 levels did not differ between the treatments. T3 treated animals did not express the thyroid stimulating hormone (TSH) gene at all (data not shown), but showed a dose-dependent elevation in thyroid hormone receptor beta (TRβ) gene expression (Fig. 33).

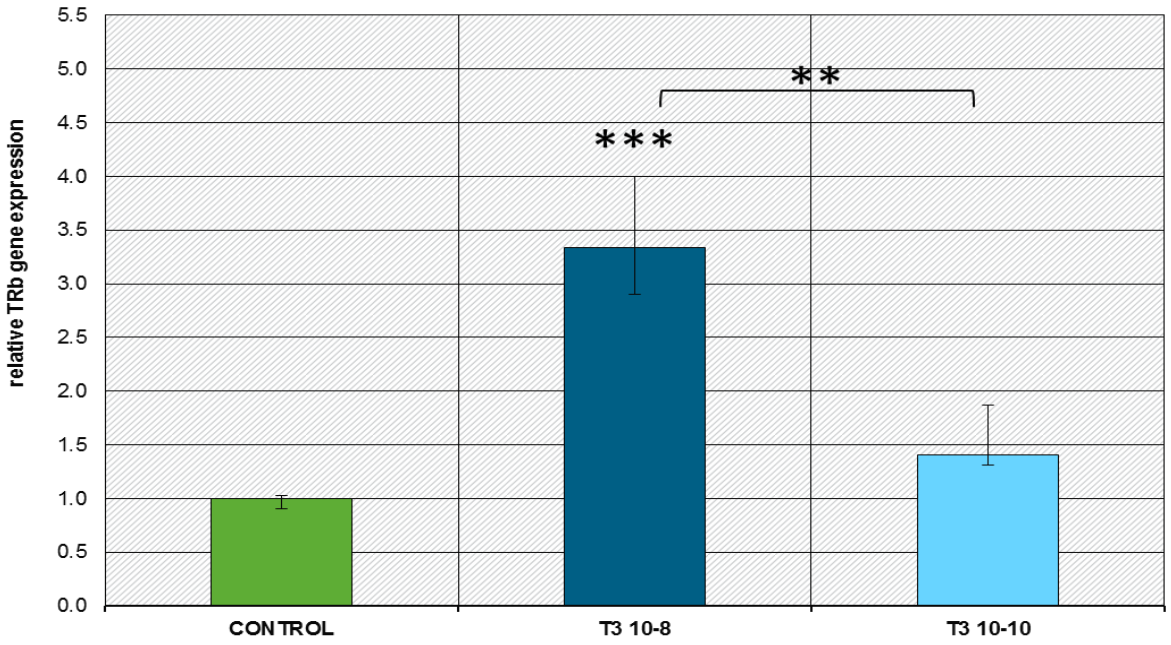

Figure 33: Medians and interquartile ranges of the relative gene expression of the thyroid receptor beta (TRb) in (brain tissue of adult male *X. laevis* (n = 10) after an eight-day exposure to 10-10 M (29.6 ng/L) and 10-8 M (2961.4 ng/L) triiodothyronine (T3). Statistical differences were determined using General Linear Mixed models and Sidak post-hoc tests. Significant differences are marked by asterisks (** $p \leq 0.01$; *** $p \leq 0.001$).

5.5.2.3 Discussion

Frogs exposed to T3 uttered a slightly lower amount of vocalizations, in general. This reduction in behavioral motivation might be the result of an elevated stress response to the administered T3, since stress was previously shown to be closely linked to the thyroid metabolism in different species (Bianco et al. 1987, Mason et al. 1994). No other calling parameters were affected by a T3 exposure. Moreover, neither the gene expression of the gonadotropins LH and FSH, nor of the gonadal steroidogenic enzymes was affected by T3.

Furthermore, T3 treated animals did not express the thyroid stimulating hormone (TSH) gene at all, which was expected, since TSH stimulates T3 production, which in turn has a negative feedback on the hypothalamus (Kloas et al. 2009). Thus, high concentrations of T3 inhibit the production of TSH. Furthermore, T3 exposed frogs showed a dose-dependent elevation in thyroid hormone receptor beta (TRβ) gene expression, confirming the fast bioaccumulation and metabolization of T3 by the male *X. laevis* (Kloas et al. 2009). As a result T3 concentrations in the test tanks might have been low (~25 %), although the amount of T3 in the supplying stock solutions were adequate.

Regarding the applicability of the XENOCALL test, T3 did not affect the calling behavior of the frogs in any significant way. Hence, neither the typical estrogen-specific alterations of the calling behavior (decreased amounts of advertisement calls and an increased rasping behavior), nor the typical androgen-specific (increased percentages of advertisement calls and lower amounts of rasping) or antiandrogen-characteristic (decreased amounts of advertisement calling and lower proportions of ticking) alteration were elicited by a T3 exposure. Therefore, XENOCALL seems to be an applicable and specific biomarker for the detection and assessment of (anti)androgenic and (anti)estrogenic EDC, respectively.

5.5.3 Exposure to metoprolol (METO)

To further ensure the general applicability and validity of the XENOCALL testing method, we exposed male *X. laevis* to one more negative substance, namely the $beta_1$-receptor blocker metoprolol (METO), which is commonly used as medication to treat high blood pressure and diverse heart conditions (Psaty et al. 1989) and analyzed the mate calling behavior before and after hCG injection (2 x 96 h).

5.5.3.1 Material and methods

In this experiment, adult male *X. laevis* (3 years of age; weight: 64.2 g ± 6.4 g; length: 9.2 cm ± 0.5 cm) were exposed to two different METO concentrations (10^{-8} M and 10^{-10} M) in the flow-through system (n = 10) and their calling behavior was recorded and analyzed as described previously (5.1.2.1). Temporal and spectral parameters of the advertisement calls were determined using software developed by Dr. Henning Thielemann. For this purpose, accurately classified audio files of the single frogs were gathered manually and then fed to the software, which analyzed the following parameters of those calls: the duration of the whole call, as well as the duration and the mean frequency of the slow trill and the fast trill part, the mean number of clicks, as well as accentuated clicks, the click rate (number of clicks per second in the slow trill part of the advertisement calls), the duration of clicks and pauses between clicks (ICI) and the bandwidth within the slow trill part, and the mean frequency and bandwidth of the fast trill part of the calls.

To ensure that METO is neither enhancing nor reducing the mate calling behavior of the frogs, we exposed the males for 96 h without prior stimulation with hCG (Russell 1960, Hoffmann and Kloas 2012b). Afterwards we injected each male with 100 units hCG (dissolved in 50 µL distilled water) in the dorsal lymph sack to stimulate a basic mate calling behavior. Exposure chemicals were dissolved in dimethyl sulfoxide (> 99%; DMSO) to prepare the stock solution and were obtained from Sigma Aldrich (Steinheim, Germany). During the experiment, frogs were fed a commercial fish diet (Metabolica, Aller Aqua, Golßen, Germany; 2 pellets) every other day. Water temperature in the test tanks was 20.2 ± 0.1 °C throughout the whole experiment and the light:dark cycle was 12:12 h, with a light period starting at 7 am.

At the end of the exposure period, all frogs were anesthetized using MS 222 (0.01%), sacrificed and tissue samples were taken and processed (RNA extraction, RT, qPCR) as described in paragraph 5.1.2.1. The gene expression of the gonadotropins LH and FSH was measured in brain samples, while gene expression profiles of RED 1 and 2 and ARO was examined in gonad samples. VTG expression was investigated in liver samples. Primer pair sequences can be found in Efosa et al. (2016, submitted) and Garmshausen et al. (2015)

(appendix 3). Primer pair efficiencies ranged between 1.98 and 2.01 (Pfaffl 2001, Urbatzka et al. 2010). Data was analyzed applying the $\Delta\Delta C_T$ method (Pfaffl 2001, Efosa et al. 2016, submitted) and the elongation factor 1 α (EF) was used as normalizing housekeeping gene.

GLMM were conducted (Hoffmann and Kloas 2010, Efosa et al. 2016, submitted) with subsequent Sidak post-hoc tests (SPSS 20, IBM, Ehningen, Germany) to identify statistical differences between treatments. Normal distribution of data or residuals was ensured with Kolmogorov-Smirnov tests.

To verify the desired test concentrations, water samples of each tank were taken and analyzed at the beginning and the end of the experiment using UHPLC-MS-MS (table 1).

5.5.3.2 Results

Analytical water analyses

None of the control tanks contained traces of METO. Water samples from the tanks of the METO 10^{-10} M treatment group (desired concentration: 26.7 ng/L), contained 24.1 ng/L (21.2 ng/L – 30.8 ng/L) METO (median (interquartile range)) and water samples out of the tanks of the METO 10^{-8} M treatment (desired concentration: 2673.6 ng/L) contained 2564.8 ng/L (2190.0 ng/L – 3152.9 ng/L) METO. Nominal concentrations are used in the following for simplification purposes.

Calling behavior

METO treatment only affected the calling behavior of male *X. laevis* that where in a sexually stimulated condition (+ hCG). In this trial, METO exposed males (both concentrations) uttered considerably more calls compared to the controls (Fig. 34). However, these differences were not statistically different ($p = 0.066$). When injected with hCG, exposed frogs tended to utter a higher amount of advertisement calls and a lower amount of rasping (data not shown). No other parameters were affected by METO exposure.

Gene expression analyses

METO neither affected LH and FSH gene expression in brain, nor RED1, RED 2 and ARO in gonads. VTG gene expression in liver samples was also not affected by any METO treatment.

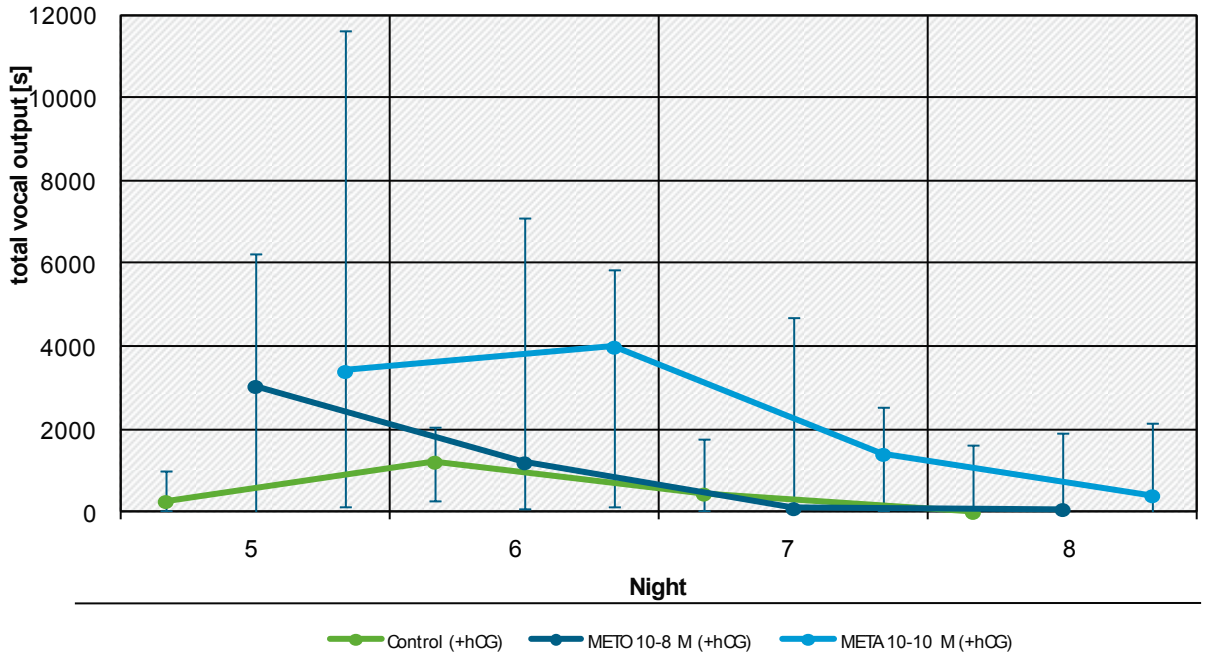

Figure 34 Line graph of the total vocal output of the test animals during the four exposure nights after stimulation with hCG (median and interquartile ranges). Animals that were exposed to 10^{-8} M and 10^{-10} M metoprolol (METO), respectively produced more calls than control animals, in general, however these effects slightly missed a statistically significant level (GLMM: p = 0.066).

5.5.3.3 Discussion

The typical effects that (anti)estrogenic and (anti)androgenic EDC evoke in exposed adult male *X. laevis* (Hoffmann and Kloas 2010, Hoffmann 2012, Hoffmann and Kloas 2012b, c, a) are missing in METO treated frogs. Similar findings, namely the lack of (adverse) effects of METO on sexual behavior, were presented in a previous study with male rats (Smith et al. 1996). The slightly increased total vocal output, including the slightly enhanced advertisement calling, points to a small increase in sexual arousal of hCG-stimulated METO exposed males (Hoffmann and Kloas 2010, 2012d). Neither gonadotropin nor gonadal enzyme gene expression was affected by METO, which coincides with findings of a previous study (Tornatzky and Miczek 1994). The increase in sexual arousal might therefore be attributable to an elevated behavioral motivation, e.g. due to the strong anxiety-inhibiting and slight stress-reducing activity of β-blockers, such as METO (Bonn and Turner 1971, Lader and Tyrer 1972, Tornatzky and Miczek 1994, Bezchlibnyk-Butler et al. 2013).

Regarding the applicability of the XENOCALL test, the measured behavioral effects of METO exposure did not coincide with the established (anti)androgenic and (anti)estrogenic EDC effects, namely an reduced or elevated advertisement calling or rasping and ticking, respectively, confirming the practicability of the newly developed method.

6 General discussion and conclusions

Taken together all results obtained in this study, it is reasonable to say that the basic XENOCALL method can be successfully conducted in a flow-through system. Tested EDC with (anti)androgenic and estrogenic MOA resulted in mode-specific effects, which were previously also detected semi-statically (Hoffmann and Kloas 2010, Hoffmann 2012, Hoffmann and Kloas 2012b, c). Androgenic EDC resulted in an enhanced sexual arousal, indicated by the production of higher percentages of advertisement calls, while both, estrogenic and antiandrogenic EDC lowered this percentage and thus the sexual arousal of the exposed males. However, the previously detected differences in calling behavior of estrogen and antiandrogen exposed male *X. laevis* (Hoffmann 2012, Hoffmann and Kloas 2012b, c) were also detectable in the flow-through system: exposure to estrogenic EDC lead to an enhanced production of rasping, while antiandrogens elicited a higher amount of ticking compared to controls.

One exception to this rule was the supposedly estrogenic BPA (Levy et al. 2004). Surprisingly, an exposure to BPA did not cause the typical estrogen-specific alterations of the calling behavior but rather typical androgen-specific effects, namely a higher proportion of advertisement calls and a slightly lower amount of rasping. These effects were detected during both exposure experiments, in the semi-static and the flow-through system. Moreover, a semi-static exposure to BPA did also not induce the estrogenic biomarker VTG gene expression, indicating that BPA does not elicit estrogenic MOA in adult male *X. laevis*, at all. The results of the further gene expression analyses and the sex steroid determination also correspond to that conclusion: the estrogen-characteristic elevated ARO gene expression and the increased levels of plasma E2 are missing in BPA exposed animals. Moreover, neither gonadal steroidogenic enzymes, nor FSH gene expression was altered due to a BPA exposure, while LH expression was reduced in BPA treated males. A reduction in LH expression can be the result of androgenic or estrogenic EDC treatment. Estrogenic EDC such as EE2, however, were shown to simultaneously affect sex steroid levels, resulting in decreased T and increased E2 levels, whereas an androgen exposure did not alter plasma sex steroid concentrations (Urbatzka et al. 2006). Since the estrogen biomarker VTG was not induced by BPA, the inhibition of LH gene expression, thus, points towards a slight to moderate androgenic MOA of BPA. Accordingly, sex steroid levels and ratios were not affected by this substance. BPA should, hence, not be generally considered as an estrogenic EDC, but rather as a pollutant, which might be able to trigger further endocrine actions, such as androgenic MOA.

Moreover, according to the obtained results the supposed negative substance DCF should in fact not be considered as negative substance, but rather as an EDC with slight to moderate estrogenic MOA. XENOCALL, nevertheless, was able to detect the slight to moderate estrogenicity of DCF, since DCF exposed frogs exhibited similar estrogen-characteristic alterations of their calling behavior: they uttered a lower amount of advertisement calls and a higher proportion of rasping before hCG stimulation. Furthermore, none of the two actual negative substances (T3 and METO) affected the calling behavior of the male *X. laevis* at all or in a similar way to the (anti)androgenic and (anti)estrogenic EDC. Hence, no EDC-typical effects like an altered amount of advertisement calls (increase: androgen-characteristic; decrease: antiandrogen- and estrogen-characteristic), ticking (increase: antiandrogen-typical) or rasping (increase: estrogen-characteristic), respectively, could be detected.

Taken together, the XENOCALL method performed in the flow-through can be used as a reliable detection method for (anti)androgenic and estrogenic EDC, being capable to differentiate between the different MOA and no such MOA. However, antiestrogenic EDC, which should cancel out estrogenic impacts on the male calling behavior (Hoffmann 2012, Hoffmann and Kloas 2012a), still need to be tested in the flow-through system in future experiments.

Compared to the semi-static system the XENOCALL method seems to be less sensitive when performed in a flow-through system. Exposure concentrations which caused significant effects semi-statically still resulted

in visible and MOA-specific alterations of the calling behavior, but those alterations were often not statistically significant. The reason for this lower sensitivity is probably the elevated background noise and especially the water drifts due to the running exposure water of the flow-through system, which both seemed to disturb the male experimental frogs, especially the newly purchased male *X. laevis* from NASCO (USA). When confronted with one of those or both trigger events, male frogs abruptly interrupted their calling behavior. These interruptions were often followed by longer periods of silence, which led to lower amounts of vocalizations in general and, thus, made it harder to detect possible alterations of the calling behavior. Performing the experiments semi-statically or at least with an extremely low flow-rate or a combination of both, flow-through during the day and static during the recording nights, might therefore be more effective when testing for (anti)androgenic and (anti)estrogenic EDC using XENOCALL. Furthermore, differences in origin or ancestry might have been the reason for the obvious differences between the frogs (IGB versus NASCO) in coping with stress. Hence, it might be helpful to determine the exact strain of the experimental frogs to be able to recommend the most suitable and stress-resistant frog strain for the XENOCALL experiments. Stricter validity criteria, such as a minimum amount of time the individuals spent calling during each of the recorded night, might also help to acquire a higher sensitivity of the XENOCALL method. To date the XENOCALL test counted as valid, if the individual was uttering any vocalization during at least one of the recorded nights. Nevertheless, because it is not known beforehand whether a frog will be calling for a sufficient amount of time, the implementation of such stricter validity criteria might be difficult.

Tests concerning the reusability of test animals revealed that male frogs cannot necessarily be reused. A prolonged exposure-free period might resolve that problem, but animals definitely have to be tested for an altered calling behavior prior to every test trial to ensure that test animals recovered from potentially enduring EDC effects. However, the applicability and practicability of such an “in-between testing” needs to be carefully considered. First, the question arises what to do with the frogs during the period of time when the animals cannot be used in any test trial. Ecotoxicological laboratories would need to have enough space and resources for keeping the frogs during those times, which might turn out to be time consuming and costly. And second, the “in-between testing” also needs to be performed in the test system under supervision and exposure as well as call analyses take time. Thus, it might be difficult, tedious and expensive to implement the reuse of test animals under practical conditions. Nevertheless, the potential of the XENOCALL method, utilizing already tested animals, which would corroborate with the internationally demanded 3R-strategy for the replacement, reduction and refinement of animal testing, should not be discarded entirely. Instead, it might be possible to determine a decisive amount of time after which the EDC-triggered effects are vanished for good, so that an additional “in-between testing” might not be necessary. However, the fact that the experimental animals have to be kept, fed and adequately supplied during the recovery phase cannot be neglected and might constitute a limiting factor.

As expected, frogs exposed to a mixture of EDC with different MOA (androgenic and estrogenic), did not show additive behavioral effects. However, the arousal-enhancing androgenic effects did also not cancel out the estrogenic effects, even when a higher androgen concentration was applied. Nevertheless, the estrogenic effects were slightly decreased by androgenic EDC in a dose-dependent manner. Furthermore, an exposure of male *X. laevis*, to EDC, such as EE2 or VIN, resulted in strong adverse effects not only on the calling behavior as demonstrated here and previously (Hoffmann and Kloas 2010, Hoffmann 2012, Hoffmann and Kloas 2012b, c), but (in the case of EE2) also on the clasping behavior and the reproductive success of males. In this case, the presence of a competitive party seems to play an important role. Frogs exposed to a higher EE2 concentration clasped the female less than unexposed males or frogs which were exposed to the lower tested EE2 concentration. When no competitor was present, all males, except the ones previously exposed to the highest EE2 concentration (10^{-8} M), which almost never clasped at all, clasped their female for a fairly long time. The clasping duration, as well as the amount of advertisement calls uttered during the exposure period, correlated positively with the hatching and survival rate of the offspring, resulting from EE2 exposed fathers and control fathers. The more the frogs vocalized and clasped, the more tadpoles

hatched and survived until the end of the experiment. Since vocalization and clasping were significantly and adversely affected by EE2, especially at higher concentrations, the number and survival of descendants of exposed male frogs were declined, too. The results suggest the possibility that EE2 exposure can reduce the reproductive success of animals and these effects might contribute to the global problem of amphibian decline.

7 Literature

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8 Appendixes

Appendix 1

"Chronic 17α-ethinylestradiol exposure of male *Xenopus laevis* can impair amphibian mating success" (Garmshausen et al. 2016, ready for submission).

Appendix 2

"Co-exposure to the estrogen ethinylestradiol and the androgen methyldihydrotestosterone causes antagonistic, independent and synergistic impacts on male mate calling behavior, vitellogenin induction and heme metabolism of *Xenopus laevis*, respectively" (Hoffmann and Kloas 2016, ready for submission).

Appendix 3

"17α-Ethinylestradiol can disrupt hemoglobin catabolism in amphibians." (Garmshausen et al. 2015, Comparative Biochemistry and Physiology, Part C 171, 34 – 40.

Appendix 4

"Diclofenac exhibits direct estrogenic modes of action in male Xenopus laevis, and causes further side effects disturbing the hypothalamus-pituitary-gonad axis and mating vocalizations." (Efosa et al. 2016, submitted to Environmental Science and Technology).

Appendix 5

"Classification of sounds of *Xenopus laevis*." Software and Manual by Dr. Henning Thielemann.

Appendix 6

"P,p'-dichlordiphenyldichloroethylene (p,p'-DDE) can exhibit antiandrogenic and estrogenic modes of action in an amphibian (*Xenopus laevis*)." (Hoffmann and Kloas 2016; ready for submission).