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Zróżnicowanie i znaczenie wskaźnika palcowego (digit ratio) u wybranych przedstawicieli rzędu płazów bezogonowych (Anura).

Diversity and significance of the digit ratio in selected representatives of the order Anura.

Rozprawa doktorska w dziedzinie nauk ścisłych i przyrodniczych
w dyscyplinie nauki biologiczne
Doctoral dissertation in the field of natural sciences
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Spis treści

Wykaz stosowanych skrótów

EDCs – endocrine-disrupting compounds / związki endokrynnie czynne

BPA – bisfenol A

EE2 – etynyloestradiol

DR – digit ratio / wskaźnik palcowy

2D:4D – digit ratio / wskaźnik długości palca drugiego do czwartego

Streszczenie

Wskaźnik palcowy (z ang. digit ratio; DR) opisuje stosunek długości wybranych palców w kończynach kręgowców. Najlepiej poznanym wariantem jest stosunek długości palca drugiego do czwartego (2D:4D), który u wielu gatunków stanowi dymorficzną cechę płciową, powiązaną z działaniem hormonów w okresie rozwoju embrionalnego. U płazów bezogonowych pozostaje słabo poznany, a dotychczasowe badania w tym zakresie obarczone były istotnymi ograniczeniami metodologicznymi. Jak założono w niniejszej rozprawie doktorskiej, DR może być potencjalnie nieinwazyjnym wskaźnikiem płci oraz ekspozycji na rozpowszechnione w środowisku związki endokrynnie czynne (EDCs), na które płazy są szczególnie narażone. Z tego względu ważne jest lepsze zrozumienie, w jaki sposób DR kształtuje się u różnych taksonów płazów, oraz jakie jest jego powiązanie z płcią i innymi cechami. Celem niniejszej rozprawy było zebranie i podsumowanie wiedzy na temat wpływu EDCs na płazy, udoskonalenie metodyki analizy wskaźnika palcowego u płazów bezogonowych z wykorzystaniem zakonserwowanych okazów grzebiuszki ziemnej *Pelobates fuscus*, oraz przeprowadzenie eksperymentu w zakresie oceny oddziaływania dwóch wybranych EDCs: bisfenolu A (BPA) oraz 17α-etynyloestradiolu (EE2) na rozwój płciowy, budowę gonad, kondycję somatyczną i DR u żaby śmieszki *Pelophylax ridibundus*.

Na podstawie przeglądu literatury wykazano, że w badaniach rzadko wykorzystywane są niemodelowe gatunki płazów. Ponadto, niewiele badań dotyczy wpływu EDCs na zewnętrzne, dymorficzne cechy płciowe. W analizie materiału archiwalnego wykazano, że uwzględnienie możliwości allometrycznego wzrostu palców jest kluczowe dla prawidłowej interpretacji DR, oraz że jest to cecha związana z płcią u *P. fuscus*. W eksperymencie przeprowadzonym z wykorzystaniem *P. ridibundus* wykazano z kolei, że EE2 wywołał znaczące przesunięcia proporcji płci w populacji w kierunku samic, a także zmiany w budowie gonad wskazujące na feminizację. BPA nie wywoływał jednoznacznego efektu feminizującego, ale indukował zmiany w gonadach wskazujące na nieprawidłowy rozwój i procesy degeneracyjne. Oba związki chemiczne, w najwyższych stężeniach, negatywnie wpływały na kondycję osobników. Analiza DR wskazała, że nie jest on dymorficzną cechą płciową u *P. ridibundus*, ale może ulegać zmianie pod wpływem EDCs w sposób zależny od wielkości ciała i kończyny.

Uzyskane wyniki podkreślają złożoność oddziaływania EDCs na rozwój płazów, wskazując jednocześnie na potrzebę dalszych badań nad mechanizmami ich działania i możliwościami wykorzystania cech fenotypowych, takich jak DR, w biomonitoringu środowiska.

Abstract

The digit ratio (DR) describes the ratio between the lengths of selected digits in vertebrate limbs. The most commonly studied variant is the ratio of the second to the fourth digit (2D:4D), which in many vertebrate species is a sexually dimorphic trait, linked to the action of sex hormones during embryonal development. In anurans it remains poorly understood, and existing studies are of significant methodological limitations. As hypothesized in presented doctoral dissertation, DR may has a potential as a non-invasive indicator of sex and exposure to endocrine disrupting compounds (EDCs), widespread in the environment, to which amphibians are particularly vulnerable.

The aims of this dissertation were to summarize current knowledge on the effects of EDCs on amphibians, to improve the methodology of digit ratio analysis in amphibians using preserved specimens of the common spadefoot toad (*Pelobates fuscus*), and to experimentally assess the impact of two selected EDCs: bisphenol A (BPA) and 17α-ethinylestradiol (EE2), on sex differentiation, gonadal structure, somatic condition, and digit ratio in the marsh frog (*Pelophylax ridibundus*).

Literature reviews revealed the need of including non-model species of amphibians in research, as well as the insufficient number of studies on the effect of EDCs on external, sexually dimorphic traits in these animals. Analysis of the archival material demonstrated that accounting for allometric growth of digits is essential for the correct interpretation of digit ratio, and showed differences in DR between sexes in *P. fuscus*. Experimental studies showed the impact of EE2 in *P. ridibundus* on a female-biased sex ratio and the occurrence of gonadal alterations, indicating feminizing effect. BPA did not have a clear feminizing effect but induced other gonadal changes suggesting abnormal development and degenerative processes. Both compounds, at the highest concentrations, negatively affected somatic condition. DR was not a sexually dimorphic trait in *P. ridibundus*, however, it responded to exposure to both compounds in a limb and size-dependent manner.

The obtained results highlight the complexity of EDCs effects on amphibians, while also indicating the need for further research on underlying mechanisms and on the applicability of phenotypic traits, such as DR, in environmental biomonitoring.

Wstęp

Wskaźnik palcowy

Wskaźnik palcowy (z ang. digit ratio; DR) opisuje stosunek długości palców w kończynach kręgowców. Najlepiej poznanym i najczęściej analizowanym wariantem jest stosunek długości palca drugiego do czwartego (2D:4D). U wielu gatunków kręgowców jest dymorficzną cechą płciową, powiązaną ze stężeniem hormonów w okresie rozwoju embrionalnego, przede wszystkim androgenów i estrogenów (Lofeu i in., 2017; Manning i in., 1998; Manning i in., 2002). Z tego powodu może być powiązany z licznymi cechami anatomicznymi, fizjologicznymi i behawioralnymi (Lofeu i in., 2017; Lofeu i in., 2020; Pasanen i in., 2022; Ribeiro i in., 2016) i postrzegany jest jako potencjalnie ważny biomarker procesów ontogenetycznych oraz cech biologicznie istotnych z punktu widzenia ewolucji gatunków (Lofeu i in., 2020). Płazy bezogonowe, pomimo swojej wyjątkowej pozycji systematycznej i znaczenia w badaniach ekologicznych i ewolucyjnych, pozostają grupą słabo poznaną w kontekście DR oraz jego powiązań z płcią i innymi cechami biologicznymi (Kaczmarski i in., 2021). Analizy wskaźnika palcowego, w okresie poprzedzającym opublikowanie prac składających się na niniejszą rozprawę doktorską, przeprowadzono tylko dla 11 gatunków płazów bezogonowych (Alinezhadi i in., 2020; Beaty i in., 2016; Chang, 2008; Direnzo i Stynoski, 2012; Germano i in., 2011; Kaczmarski i in., 2021; Lofeu i in., 2017; Rajabi i Javanbakht, 2019).

Co więcej, jak zwrócili uwagę Kaczmarski i in. (2021), większość badań w tym temacie opierała się na niepewnych założeniach metodologicznych: nieprawidłowej numeracji palców w kończynach przednich, co w znacznym stopniu podważyło wiarygodność uzyskanych wyników. Płazy bezogonowe w kończynach przednich posiadają cztery palce, a we wcześniejszych badaniach zakładano, że w toku ewolucji doszło do redukcji palca piątego. Dane z zakresu rozwoju embrionalnego wskazują jednak, że redukcji uległ palec pierwszy, co zasadniczo zmienia sposób pomiaru wskaźnika palcowego u tej grupy zwierząt i wymaga rewizji dotychczas stosowanych metod badawczych (Kaczmarski i in., 2021).

Pojawiły się również istotne wątpliwości dotyczące wpływu allometrii na wyniki analiz wskaźnika palcowego (Lolli i in., 2017). Poszczególne struktury ciała mogą rosnąć względem siebie w różnym tempie wraz ze wzrostem całego organizmu, co w przypadku palców, zwłaszcza analizowanych u młodych osobników, może wpływać na wartości

wskaźnika palcowego niezależnie od działania czynników hormonalnych. W wielu badaniach nad płazami efekt allometrii nie był uwzględniany, mimo że wykazano, iż wartości DR mogą zależeć od wzajemnych relacji skalowania długości poszczególnych palców, a nie wyłącznie od płci (Beaty i in., 2016; Lolli i in., 2017). W konsekwencji może to prowadzić zarówno do przeszacowania, jak i niedoszacowania rzeczywistego dymorfizmu płciowego. Jak założono w niniejszej rozprawie doktorskiej, uwzględnienie wpływu allometrii może pozwolić na uzyskanie bardziej wiarygodnych wyników oraz dokładniejszą interpretację subtelnych różnic morfologicznych między samcami a samicami.

W rezultacie dotychczasowe badania nad wskaźnikiem palcowym u płazów bezogonowych nie pozwalają na sformułowanie jednoznacznych wniosków. Jednym z celów niniejszej rozprawy doktorskiej było podkreślenie ograniczeń metodologicznych oraz udoskonalenie procedury mierzenia DR oraz jego analizy statystycznej poprzez uwzględnienie wpływu allometrii, jak również dostarczenie nowych danych dotyczących zależności między DR a płcią u dwóch wybranych przedstawicieli Anura: grzebiuszki ziemnej *Pelobates fuscus,* w oparciu o okazy znajdujące się w zbiorach Katedry Zoologii Uniwersytetu Przyrodniczego w Poznaniu (Frątczak i in., 2025a) oraz żaby śmieszki *Pelophylax ridibundus*, na podstawie badań osobników odchowanych w warunkach eksperymentalnych (Frątczak i in., 2026).

Związki endokrynnie czynne

Ponieważ DR odzwierciedla wpływ hormonów działających w kluczowych etapach rozwoju organizmu, coraz częściej rozważa się jego zastosowanie nie tylko jako wskaźnika płci, ale także jako narzędzia wykrywania zaburzeń gospodarki hormonalnej oraz bioindykatora zanieczyszczeń środowiska (Auger i in., 2013; Chang i in., 2008). Jak założono w niniejszej rozprawie, może on się okazać przydatny w badaniach nad wpływem środowiskowych związków endokrynnie czynnych (ang. *endocrine disrupting compounds*, EDCs) u płazów.

Termin EDCs został wprowadzony w latach 90. XX wieku i obejmuje substancje egzogenne zdolne do naśladowania działania hormonów endogennych (Komisja Europejska, 1996). W większości są to związki pochodzenia antropogenicznego, należące do różnych grup chemicznych. Do środowiska trafiają jako pochodne m.in. pestycydów, plastyfikatorów, surfaktantów, kosmetyków i leków (Gore i in., 2015; Kloas i in., 2024; La Merrill i in., 2020). Ich obecność odnotowuje się w różnego typu wodach

powierzchniowych, osadach dennych, glebie, atmosferze, jak również w miejskiej wodzie pitnej na całym świecie (Annamalai i Namasivayam, 2015; Frątczak i in., 2025b; Puri i in., 2023; Ślósarczyk i in., 2021; Wilk i in., 2019).

EDCs, przez naśladowanie lub blokowanie działania hormonów endogennych, mogą ingerować w kluczowe szlaki regulacyjne: estrogenowe, androgenowe czy hormonów tarczycy (La Merrill i in., 2020). Wykazano, że mogą niekorzystnie wpływać na rozwój płci i cechy z nią związane u przedstawicieli wszystkich grup kręgowców (Komisja Europejska, 1996; Ghosh i in., 2022; Kloas i Lutz, 2006). Poprzez wpływ na działanie hormonów tarczycy mogą również zakłócać rozwój somatyczny zwierząt (Iwamuro i in., 2006). Coraz częściej podkreśla się, że zakres działania EDCs na rozwój i funkcjonowanie organizmów może być znacznie szerszy, a rzeczywiste konsekwencje ich rozprzestrzenia w środowisku dla ochrony przyrody i zdrowia publicznego nie zostały jeszcze w pełni poznane (Ghosh i in., 2022; Gore i in., 2015; Schönfelder i in., 2002).

Zagadnienie zagrożeń związanych z EDCs zyskało na znaczeniu w środowisku naukowym i w debacie publicznej dopiero w 1994 roku, po opublikowaniu pionierskich badań, które wykazały feminizację samców pstrąga tęczowego *Oncorhynchus mykiss* po ekspozycji na ścieki zanieczyszczone związkami endokrynnie czynnymi (Purdom i in., 1994). Wielu naukowców kontynuowało badania w tym kierunku, koncentrując się na wpływie EDCs na biologię rozrodu ryb i sugerując ich potencjalną rolę jako bioindykatorów zanieczyszczenia wody (Dang i Kienzler, 2019; Zhou i in., 2019). W porównaniu do dobrze udokumentowanego wpływu EDCs na ryby, ich oddziaływanie na płazy pozostaje stosunkowo słabo poznane (Frątczak i in., 2025c).

Płazy jako bioindykatory

Działanie EDCs jest szczególnie niebezpieczne w okresie wczesnego rozwoju organizmów, kiedy układ endokrynny kształtuje rozwój gonad oraz drugorzędowe i trzeciorzędowe cechy płciowe. Zaburzenia na tym etapie mogą prowadzić do trwałych zmian morfologicznych i fizjologicznych, które ujawniają się na późniejszych etapach życia (Gore i in., 2015; McLachlan, 2001). Szczególnie wrażliwe na te efekty mogą być płazy bezogonowe, u których występuje złożony cykl życiowy uzależniony od środowiska wodnego. Zarodki rozwijają się w jajach pozbawionych ochronnych skorupek, co naraża je na kontakt ze związkami chemicznymi rozpuszczonymi w wodzie. Dodatkowo, larwy płazów posiadają cienką i łatwo przepuszczalną skórę, przez co również mogą być wrażliwe na ekspozycję w kluczowych etapach rozwoju (Kloas i Lutz,

2006). Sugeruje się, że EDCs przyczyniają się do drastycznego spadku populacji płazów na całym świecie, co podkreśla pilną potrzebę dalszych badań w tym zakresie (Kloas i in., 2024; Luedtke i in., 2023).

Dotychczasowe prace nad wpływem EDCs na płazy opierają się przede wszystkim na gatunkach modelowych z rodzaju *Xenopus*, wykorzystywanych rutynowo w badaniach endokrynologicznych i analizach toksykologicznych w laboratoriach na całym świecie. Choć gatunki te odegrały kluczową rolę w rozwoju metod badawczych, istnieje ryzyko, że nie odzwierciedlają w pełni zróżnicowania genetycznego, fizjologicznego i ekologicznego płazów (Frątczak i in., 2025a; Schiesari i in., 2007). W konsekwencji coraz częściej podkreśla się konieczność prowadzenia badań na gatunkach niemodelowych (Piprek et al., 2012; Tamschick i in., 2016 a-c) oraz w warunkach zbliżonych do naturalnych, uwzględniających dodatkowe czynniki środowiska (Fuentes i in., 2014; LaFiandra i in., 2008; Paetowi in., 2023; Tang i in., 2020), co pozwoli na bardziej wiarygodną ocenę wpływu EDCs na różne grupy płazów. W niniejszej rozprawie doktorskiej przeprowadzono szczegółowy przegląd literatury mający na celu uwidocznienie dysproporcji gatunkowych w badaniach nad wpływem EDCs na płazy oraz identyfikację najczęstszych scenariuszy ekspozycji na te związki chemiczne (Frątczak i in., 2025c).

DR jako wskaźnik zaburzeń hormonalnych

Stosunkowo niewiele wiadomo o wpływie EDCs na zewnętrzne, morfologiczne cechy zależne od hormonów płciowych, które mogą być cennymi bioindykatorami płci oraz wskaźnikami zaburzeń endokrynnych. Równocześnie rośnie potrzeba opracowania metod umożliwiających wczesne i nieinwazyjne wykrywanie zaburzeń hormonalnych, które mogłyby być stosowane zarówno w badaniach eksperymentalnych, jak i terenowych (Orton i in., 2023). Należy podkreślić, że u większości gatunków płazów wciąż nie jest możliwe określenie płci na podstawie analizy morfologii chromosomów lub markerów genetycznych (Bullejos i in., 2024), co utrudnia analizę subtelnych zmian wywołanych przez EDCs. W związku z tym w ramach niniejszej rozprawy doktorskiej przeprowadzono również dodatkowy przegląd literatury ukierunkowany na ocenę aktualnego stanu wiedzy dotyczącego wpływu EDCs na morfologiczne cechy związane z płcią u płazów oraz zachowania rozrodcze, stanowiące potencjalne bioindykatory (Frątczak i in., 2025d).

W poszukiwaniu nieinwazyjnych wskaźników zaburzeń hormonalnych szczególną uwagę zwraca DR. Wskaźnik palcowy, który odzwierciedla poziomy hormonów w czasie rozwoju embrionalnego, może potencjalnie stanowić stosunkowo proste narzędzie do oceny płci oraz subtelnych zaburzeń endokrynnych (Auger i in., 2013). Wcześniejsze badania eksperymentalne wykazały, że DR u płazów jest cechą podatną na hormonalną manipulację. Kijanki żaby z gatunku *Leptodactylus fuscus* eksponowane w okresie larwalnym na testosteron w środowisku wodnym wykazywały istotne zmiany w proporcji długości palców w kończynach tylnych, przekładające się na niższy DR, interpretowany jako jego maskulinizacja (Lofeu i in., 2017).

W świetle tych wyników kluczowym etapem badań w ramach niniejszej rozprawy doktorskiej było eksperymentalne zweryfikowanie wpływu EDCs na wskaźnik palcowy u płazów bezogonowych. W tym celu przeprowadzono kontrolowaną ekspozycję na EDCs wybranego przedstawiciela tej grupy, żaby śmieszki *Pelophylax ridibundus*. Do eksperymentu wybrano dwa szeroko rozpowszechnione w środowisku, estrogenowe związki endokrynnie czynne: bisfenol A (BPA) oraz, jako kontrolę pozytywną, 17α-etynyloestradiol (EE2) (Frątczak i in., 2026).

Wybrane związki endokrynnie czynne

Bisfenol A (BPA) jest syntetycznym związkiem chemicznym o szerokim zastosowaniu przemysłowym, wykorzystywanym głównie do produkcji tworzyw poliwęglanowych oraz żywic epoksydowych, którego globalna produkcja sięga około trzech milionów ton rocznie (Czarczyńska-Goślińska i in., 2017). Ze względu na powszechne wykorzystanie, m.in. w plastikowych opakowaniach żywności, BPA jest szeroko rozpowszechnionym zanieczyszczeniem środowiska. Pod względem chemicznym stanowi analog estrogenów i może wiązać się z receptorami estrogenowymi, wykazując działanie agonistyczne lub antagonistyczne, w zależności od dawki i podtypu receptora (Li i in., 2012), może również mieć wpływ na działanie androgenów i hormonów tarczycy (Heimeier i in., 2009; Teng i in., 2013).

U płazów bezogonowych wykazano, że BPA może silnie zaburzać rozwój fenotypowej płci i powodować widoczną na poziomie morfologicznym i histologicznym feminizację w gonadach, m.in. u gatunków takich, jak żaba szponiasta *Xenopus laevis*, rzekotka drzewna *Hyla arborea* oraz ropucha zielona *Bufotes viridis* (Tamschick i in., 2016a), jak również prowadzić do przesunięcia proporcji płci w populacji w kierunku samic u *X. laevis* (Kloas i in., 1999; Levy i in., 2004; Mosconi i in., 2002). Ponadto

wykazano jego negatywny wpływ rozwój somatyczny płazów, przez wpływ na szlaki regulowane przez hormony tarczycy (Heimeier i in., 2009; Iwamuro i in., 2003; Yang i in., 2005).

Mimo dostępnych wyników badań, wpływ BPA na różnicowanie płci oraz rozwój układu rozrodczego płazów pozostaje niejednoznaczny (Tamschick i in., 2016a). Dostępne dane wskazują na wyraźne różnice w wrażliwości między gatunkami, a podobne schematy eksperymentalne i stężenia BPA czasami prowadziły do rozbieżnych rezultatów (Levy i in., 2004). W przypadku wielu gatunków mechanizmy oddziaływania BPA na rozwój płciowy pozostają niewyjaśnione, co uzasadnia potrzebę dalszych badań obejmujących przedstawicieli różnych linii ewolucyjnych płazów.

W kontekście badań nad wskaźnikiem palcowym, BPA wydaje się szczególnie interesujący, ponieważ jego wpływ na długość palca drugiego i czwartego potwierdzono w badaniach na modelowym gatunku ssaka, jakim jest szczur (*Rattus norvegicus domestica*). U samców szczurów szczepu Wistar Han eksponowanych na BPA stwierdzono wyższy DR, wskazujący na jego feminizację. Co ciekawe, obserwowane zmiany były dziedziczne i wystąpiły także u nieeksponowanego na BPA potomstwa (Auger i in., 2013). Autorzy badań podsumowali, że wskaźnik palcowy u dojrzałych osobników może stanowić czuły biomarker wczesnej ekspozycji na niskie dawki związków endokrynnie czynnych.

17α-etynyloestradiol (EE2) jest syntetycznym estrogenem stosowanym w środkach antykoncepcyjnych oraz hormonalnej terapii zastępczej. W badaniach wykazano jego silne działanie feminizujące u płazów, przejawiające się m.in. przesunięciem proporcji płci w populacjach w kierunku samic u *X. laevis* (Pettersson i Berg, 2007), żaby z gatunku *X. tropicalis* (Gyllenhammar i in., 2009; Pettersson i Berg, 2007), żaby trawnej *Rana temporaria* (Pettersson i Berg, 2007), żaby lamparciej *Lithobates pipiens* (Mackenzie i in., 2003), żaby leśnej *Lithobates sylvaticus* (Tompsett i in., 2013) oraz ropuchy szarej *Bufo bufo* (Ujhegyi i Bókony, 2020). Fenotypowe odwrócenie płci u samców potwierdzono analizami genetycznymi w badaniach na *X. tropicalis* (Hirakawa i in., 2013; Tamschick i in. 2016b), *X. laevis* oraz *B. viridis* (Tamschick i in., 2016b). Wykazano również zdolność EE2 do indukowania zmian patomorfologicznych w gonadach wskazujących na ich feminizację. Efekty takie opisano u *X. laevis* (Cevasco i in., 2008; Tompsett i in., 2012, 2013), *X. tropicalis* (Hirakawa i in., 2013), *R. temporaria* (Pettersson i Berg, 2007), *Lithobates pipiens* (Hogan i in., 2008) oraz *Rana septentrionalis* (Park i Kidd, 2005).

Do przeprowadzenia badań eksperymentalnych wybrano stężenia BPA i EE2 odpowiadające poziomom obserwowanym w wodach powierzchniowych regionu Polski (Czarczyńska-Goślińska i in., 2017; Czerwonka i in., 2014; Ślósarczyk i in., 2021; Wilk i in., 2019).

Hipotezy badawcze

Praca na okazach ze zbiorów Katedry Zoologii – Publikacja III:

H1: Uwzględnienie allometrii w analizie wskaźnika palcowego ujawnia różnice płciowe niewidoczne bez jej zastosowania.

H2: Wskaźnik palcowy jest dymorficzną cechą płciową u grzebiuszki ziemnej *Pelobates fuscus*.

Praca eksperymentalna – Publikacja IV:

H1: Bisfenol A (BPA) i etynyloestradiol (EE2) mają wpływ na rozwój płciowy żaby śmieszki *Pelophylax ridibundus*.

H2: Wskaźnik palcowy jest dymorficzną cechą płciową u *P. ridibundus*.

H3: BPA i EE2 mają wpływ na kondycję somatyczną u *P. ridibundus*.

H4: BPA i EE2 mają wpływ na wartości wskaźnika palcowego u *P. ridibundus*.

H4: Skutki oddziaływania BPA i EE2 na płeć, kondycję somatyczną i wskaźnik palcowy u *P. ridibundus* są zależne od stężenia związków.

Cele i zakres rozprawy

Podsumowując, głównymi celami badań składających się na niniejszą rozprawę były:

1. Podsumowanie aktualnego stanu wiedzy dotyczącej wpływu związków endokrynnie czynnych (EDCs) na płazy oraz wskazanie głównych luk badawczych.

2. Udoskonalenie metodyki pomiaru oraz analizy wskaźnika palcowego poprzez ocenę jego korelacji z płcią i wielkością ciała u dwóch przedstawicieli płazów bezogonowych: grzebiuszki ziemnej *Pelobates fuscus* oraz żaby śmieszki *Pelophylax ridibundus*.

3. Eksperymentalna weryfikacja wpływu wybranych EDCs (bisfenolu A i etynyloestradiolu) na rozwój płciowy oraz cechy morfometryczne, w tym wskaźnik palcowy, u przedstawiciela płazów bezogonowych (*P. ridibundus*).

4. Określenie przydatności wskaźnika palcowego jako indykatora zaburzeń hormonalnych u przedstawiciela płazów bezogonowych (*P. ridibundus*).

Materiały i Metody

Prace przeglądowe

Publikacja I

Przeprowadzono szeroki przegląd literatury naukowej opublikowanej do końca 2023 roku dotyczącej wpływu związków endokrynnie czynnych (EDCs) na płeć oraz cechy z nią związane u płazów. Uwzględniono w nim prace opisujące wpływ EDCs na gonady oraz drugorzędowe i trzeciorzędowe cechy płciowe (Sever & Staub, 2011). Do analizy włączono również badania dotyczące wpływu EDCs na sygnalizację hormonalną, ekspresję genów związanych z rozrodem, zachowania płciowe, płodność i sukces rozrodczy płazów.

Wyszukiwanie publikacji przeprowadzono w dwóch bazach danych: Web of Science Core Collection® oraz Google Scholar, wykorzystując zdefiniowany zestaw słów kluczowych. Lista fraz była aktualizowana w trakcie przeglądu wraz z rozszerzaniem przeglądu na kolejne grupy związków o potencjalnym działaniu endokrynnym. Do analizy włączono prace, które spełniały następujące kryteria: (a) publikacja była w języku angielskim, (b) praca miała charakter eksperymentalny, (c) praca przedstawiała dane dotyczące wpływu ekspozycji płazów lub ich tkanek na przynajmniej jedną ze zdefiniowanych cech związanych z płcią. Nie uwzględniano niepublikowanych w recenzowanych czasopismach wyników prac magisterskich i doktorskich.

Publikacja II

Pierwszy przegląd literatury pozwolił zidentyfikować istotne luki badawcze. Jednym z najsłabiej poznanych obszarów okazał się wpływ związków endokrynnie czynnych (EDCs) na zewnętrzne, dymorficzne cechy płciowe oraz zachowania rozrodcze płazów, w tym komunikację akustyczną, chemiczną i wizualną. W odpowiedzi przeprowadzono drugi, uzupełniający przegląd literatury, skoncentrowany na tych zagadnieniach. Wyszukiwanie obejmowało publikacje dostępne do 1 stycznia 2025 roku i opierało się na podobnym schemacie wyszukiwania jak w pierwszym przeglądzie.

Do analizy włączono prace eksperymentalne, które spełniały następujące kryteria: (a) zostały opublikowane w języku angielskim, (b) obejmowały kontrolowaną ekspozycję płazów na związki endokrynnie czynne, oraz (c) prezentowały dane dotyczące cech związanych z komunikacją płciową (m.in. zachowania godowe, wokalizacje, produkcję

feromonów) lub zewnętrznych cech morfologicznych, potencjalnie pełniących funkcję sygnalizacyjną (m.in. rozwój rezonatorów głosowych, ubarwienie ciała).

Praca na okazach ze zbiorów: Publikacja III

Badania mające na celu udoskonalenie metodyki analizy DR u płazów bezogonowych oparto na materiale obejmującym 68 okazów *P. fuscus* przechowywanych w 70% etanolu w zbiorach Katedry Zoologii Uniwersytetu Przyrodniczego w Poznaniu. Choć pomiary wykonywane na żywych osobnikach uznaje się za rozwiązanie optymalne, do analiz mogą być również wykorzystywane preparaty utrwalone, zgodnie z założeniami przedstawionymi w literaturze (Balogová i in, 2015; Kaczmarski i in., 2015).

Płeć osobników określono podczas sekcji, na podstawie makroskopowej budowy gonad. Długość ciała (SVL) oraz szerokość głowy (HW), standardowe pomiary morfometryczne wykorzystywane w badaniach batrachologicznych, wykonano z dokładnością do 0,01 mm przez dwóch niezależnych obserwatorów, a zgodność pomiarów oceniono przy użyciu współczynnika korelacji wewnątrzklasowej (ICC). Następnie wykonano zdjęcia wszystkich kończyn przy użyciu aparatu Pentax Optio WG-5 z funkcją mikroskopową i stałym oświetleniem LED. Długości poszczególnych palców zmierzono przez dwóch niezależnych obserwatorów, z wykorzystaniem punktów homologicznych (landmarków) oznaczonych na zdjęciach przy użyciu programu Tmorph Gen 6, zgodnie z metodyką opisaną przez Kaczmarskiego i in. (2015, 2021).

Analizy statystyczne przeprowadzono w dwóch etapach. W pierwszym, do wyznaczenia DR wykorzystano stosunek długości palców bez dodatkowej korekcji allometrycznej. Wyznaczono klasyczny DR: 2D:4D, oraz, w celu dalszej eksploracji danych, stosunki pozostałych palców: 2D:3D, 2D:5D, 3D:4D oraz 3D:5D. W analizie wykorzystano modele liniowe, które pozwoliły sprawdzić, czy wskaźnik palcowy (DR) różni się między samcami i samicami oraz czy jest zależny od wielkości ciała. Do opisu wielkości ciała zastosowano analizę głównych składowych (PCA), która pozwoliła połączyć w jeden wyznacznik dwie silnie skorelowane cechy: długość ciała i szerokość głowy. W modelach sprawdzano zarówno prosty wpływ płci i rozmiaru, jak i ich możliwą interakcję. Normalność rozkładów i jednorodność wariancji oceniano na podstawie histogramów, wykresów Q–Q oraz testu Levene'a.

Rycina 1. Lokalizacja punktów homologicznych (landmarków) na palcach kończyny lewej przedniej (1), prawej przedniej (2), lewej tylnej (3) oraz prawej tylnej (4) u przykładowego osobnika grzebiuszki ziemnej *Pelobates fuscus*. Punkty wyznaczono przy użyciu oprogramowania TpsDig2w64 w celu obliczenia długości palców oraz wskaźnika palcowego, zgodnie z metodyką opracowaną przez Kaczmarskiego i in. (2015).

Następnie przy pomocy testów *t* dla prób sparowanych porównano wartości wskaźnika palcowego między kończynami lewej i prawej strony ciała, a siłę różnic określono za pomocą współczynnika *d* Cohena. Dla każdego palca obliczono również indeks dymorfizmu płciowego (SDI), który pokazuje kierunek i skalę różnic między płciami (Kaczmarski i in. 2021).

W drugim etapie analizy powtórzono, ale tym razem dla DR obliczonych z uwzględnieniem efektu allometrii, czyli skalowania długości palców względem siebie. W tym celu obliczono zależności między długościami poszczególnych palców przy pomocy korelacji Pearsona. W przypadku stwierdzenia istotnej zależności ($p < 0,05$) dopasowywano dwa modele opisujące relację między długościami palców: model liniowy (2D~4D) oraz model potęgowy oparty na transformacji logarytmicznej (log(2D) ~ log(4D)). Następnie porównywano dopasowanie modeli przy użyciu kryterium informacyjnego Akaikego (AIC).

Na podstawie modelu o niższej wartości AIC obliczono wartości resztowe, które odzwierciedlają odchylenie obserwowanej długości palca drugiego (2D) od wartości oczekiwanej na podstawie długości palca czwartego (4D). Reszty te wykorzystano do wyznaczenia skorygowanej długości drugiego palca poprzez dodanie ich wartości do średniej. Następnie obliczono skorygowany wskaźnik palcowy (z ang. *adjusted digit ratio*) jako stosunek skorygowanej długości palca drugiego do długości palca czwartego. Tak uzyskane wartości wskaźnika DR były niezależne od ogólnych proporcji palców osobnika, co umożliwiło bardziej precyzyjną ocenę dymorfizmu płciowego. Wszystkie obliczenia wykonano w środowisku R (v. 4.3.3).

Praca eksperymentalna: Publikacja IV

Układ eksperymentalny i zebranie danych

W celu przeprowadzenia eksperymentu, od licencjonowanego hodowcy komercyjnego (Frogs Po-Land) pozyskano jaja *P. ridibundus* od trzech par rodzicielskich. Zarodki płazów, a następnie larwy przetrzymywano od kwietnia do sierpnia 2024 r. w półotwartym układzie eksperymentalnym w mezokosmach, położonym na terenie należącym do Ogrodu Dendrologicznego w Poznaniu (Ryc. 2). Do eksperymentu wykorzystano 300 kijanek, które w momencie osiągnięcia stadium Gosnera 21 (Gosner, 1960) losowo przydzielono do pięciu grup: kontrolnej lub jednej z czterech doświadczalnych: eksponowanych na BPA (w stężeniach: 10^{-7} M, 10^{-8} M, 10^{-9} M) lub EE2 (w stężeniu 10^{-9} M), który pełnił funkcję pozytywnej grupy kontrolnej. Każda grupa obejmowała trzy zbiorniki replikacyjne, do których przydzielono po 20 osobników.

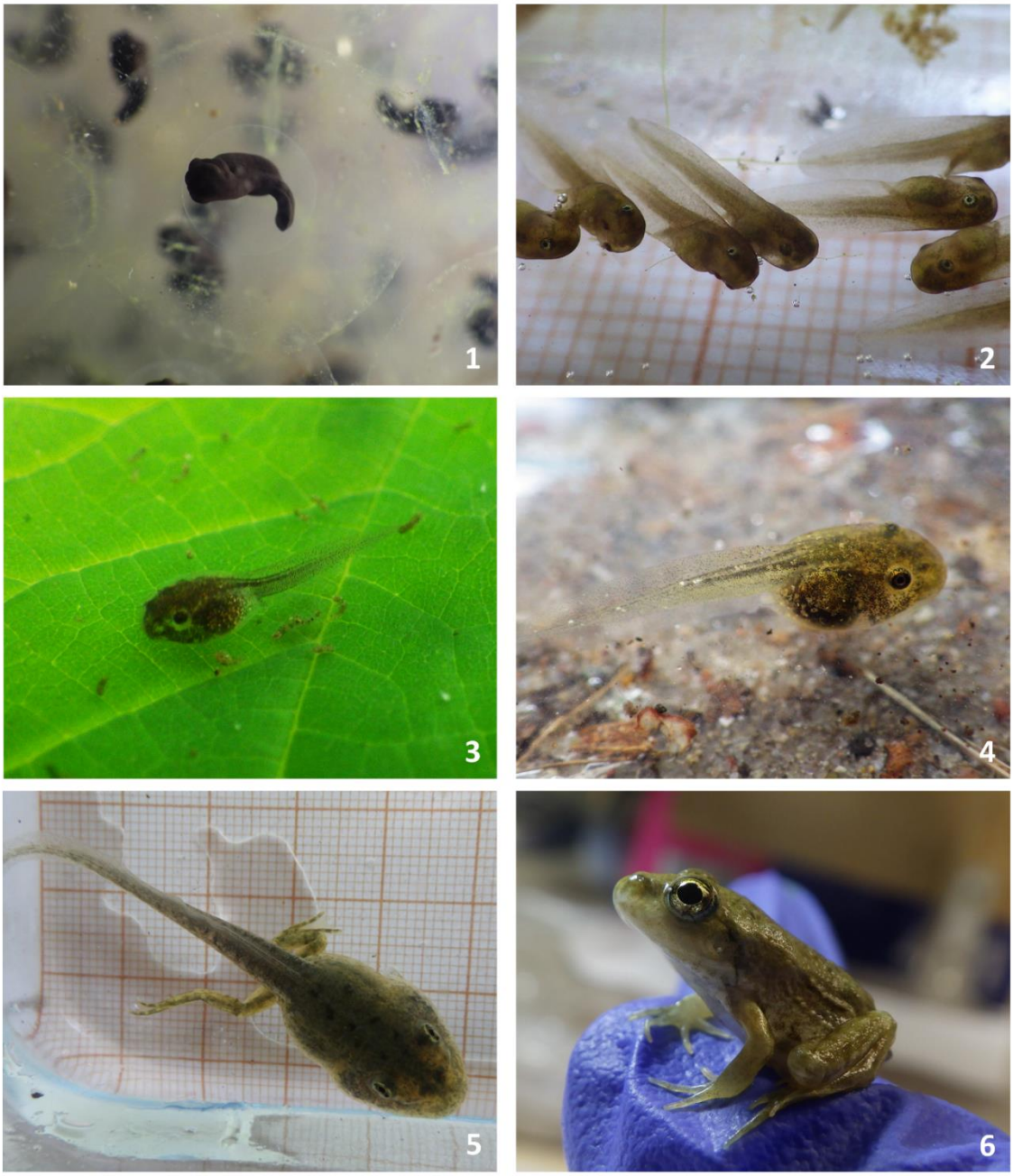
Rycina 2. Zbiorniki wykorzystane do odchowu i ekspozycji kijanek.

Ekspozycję prowadzono w zbiornikach wykonanych z chemicznie neutralnego, wysokiej gęstości polietylenu (HDPE), napełnionych 150 l wody (Ryc. 2), tj. do poziomu 30 cm. Raz w tygodniu kijanki odławiano, a całą objętość wody wymieniano na świeżą oraz grupach doświadczalnych przygotowywano nowe roztwory BPA lub EE2. Stężenia BPA i EE2 kontrolowano po wymianie wody w losowo wybranych zbiornikach, metodą LC-MS/MS w komercyjnym laboratorium. Cotygodniowo kontrolowano również parametry fizykochemiczne wody (temperatura, pH, przewodność, zasolenie, TDS) i prowadzono pomiar ciągły temperatury za pomocą data loggerów (Thermochron iButton, model DS1921G). Ekspozycja objęła okres od stadium Gosnera 21, poprzedzającego kształtowanie się gonad u płazów bezogonowych (Ogielska, 2009), do stadium Gosnera 43, po czym przenoszono je do izolatorów lądowych aż do momentu ukończenia metamorfozy, ok stadium Gosnera 46 (Ryc. 3).

Po zakończeniu metamorfozy osobniki znieczulono i uśmiercono przy użyciu 0,5% roztworu tricaine mesylate (MS-222) zgodnie z obowiązującymi protokołami etycznymi (Haczkiewicz i Ogielska, 2013). Następnie, zgodnie z metodyką opisaną dla Publikacji III, osobniki zważono, wykonano pomiary morfometryczne ciała (HW, SVL) oraz mikrofotografie kończyn. W kolejnym kroku wykonano sekcję osobników pod stereomikroskopem (Olympus SZX7). Oceniono makroskopową budowę gonad, a następnie pobrano je i utrwalano w płynie Bouin'a, według metod opisanych przez Haczkiewicz i Ogielską, 2013.

Do dalszych analiz histologicznych wybrano łącznie 105 osobników. Losowo wyselekcjonowano po 20 osobników z każdej grupy eksperymentalnej (10 samców i 10 samic), z wyjątkiem grupy EE2, gdzie, ze względu na silne przesunięcie stosunku płci, wybrano 20 osobników fenotypowo żeńskich i 4 męskich. Preparaty histologiczne (zatopienie w parafinie, przygotowanie skrawków tkanek o grubości 7 µm przy użyciu mikrotomu Leica RM 2255, zabarwienie tkanek metodą Mallory'ego) zostały przygotowane we współpracy z laboratorium Zakładu Biologii Ewolucyjnej i Ochrony Kręgowców Uniwersytetu Wrocławskiego, według metod opisanych przez Haczkiewicz i Ogielską, 2013. Gotowe preparaty gonad zostały zanalizowane przy użyciu mikroskopu Zeiss Axioskop 20 oraz dokumentowane fotograficznie z wykorzystaniem kamery CCD Carl Zeiss Axio-Cam HRc. Na ich podstawie oceniono płeć fenotypową, stadium rozwoju gonad oraz ewentualne zmiany patologiczne (Haczkiewicz i Ogielska, 2013; Ogielska i Kotusz, 2004).

Wszystkie procedury na żywych zwierzętach przeprowadzono za zgodą Lokalnej Komisji Etycznej ds. Doświadczeń na Zwierzętach w Poznaniu (nr decyzji 2/2024/P1, data wydania decyzji: 27 lutego 2024 r.).



Rycina 3. Wybrane stadia rozwojowe żaby śmieszki (*Pelophylax ridibundus*): embrion w ok. 18 stadium wg. Gosnera, 1960 (1), osobniki po wykluciu, ok. 21 stadium Gosnera (2), kijanka na liściu pokrzywy, ok. 25 stadium Gosnera (3), kijanka w szklanym zbiorniku ustawionym na podłożu imitującym naturalny sedyment, ok. 26 stadium Gosnera (4), kijanka z dobrze widocznymi tylnymi kończynami, ok. 39 stadium Gosnera (5), osobnik po ukończeniu metamorfozy, ok. 46 stadium Gosnera (6).

Opracowanie danych

Długości palców zmierzono na podstawie mikrofotografii kończyn, zgodnie z metodyką opisaną dla Publikacji III. Następnie, na podstawie uzyskanych pomiarów wyznaczono DR. Skupiono się na wskaźniku palcowym 2D:4D, opisującym stosunek długości drugiego palca do długości czwartego palca dla każdej kończyn. Uzyskane wartości skorygowano allometrycznie, zgodnie z wcześniej opracowaną metodyką (Frątczak i in., 2025a).

Na podstawie danych o masie ciała i SVL osobników określono ich kondycję somatyczną. W tym celu zastosowano wskaźnik *scaled mass index* (SMI), który pozwala porównywać kondycję osobników niezależnie od ich wielkości i w przypadku płazów jest skuteczniejszym wyznacznikiem kondycji niż tradycyjnie stosowany u wielu zwierząt *body mass index* (BMI; Hinderer i in., 2025). SMI obliczano oddzielnie dla samców i samic, aby uwzględnić dymorfizm płciowy w kondycji ciała. Dla każdej płci dopasowano model liniowy, w którym logarytm masy ciała stanowił zmienną zależną, a logarytm długości ciała (SVL) zmienną objaśniającą. Na podstawie parametrów tego modelu obliczono współczynnik skalowania (bsma), uwzględniający zależność między masą a długością ciała. Następnie dla każdego osobnika wyznaczono wartość SMI według wzoru: SMI = masa × (L_0 / SVL)^bsma, gdzie L_0 oznacza średnią długość ciała dla danej płci (Peig i Green, 2009).

W celu uniknięcia uwzględnienia w modelach statystycznych silnie skorelowanych zmiennych objaśniających oceniono współliniowość między analizowanymi parametrami. Współliniowość między zmiennymi objaśniającymi oceniono na podstawie współczynników inflacji wariancji (VIF) dla długości ciała (SVL), szerokości głowy (HW), masy ciała oraz wskaźnika kondycji (SMI). Ze względu na silną korelację między SVL, HW i masą ciała oraz wysokie wartości VIF dla tych zmiennych, w dalszych analizach jako wskaźnik wielkości ciała wykorzystano wyłącznie SVL.

Analiza danych

W kolejnym kroku sprawdzono wpływ grupy eksperymentalnej na SVL oraz kondycję somatyczną (logarytm SMI) przy użyciu modeli liniowych, w których jako zmienne objaśniające uwzględniono wariant eksperymentalny (EXP), płeć (SEX) oraz ich interakcję (EXP×SEX). Uproszczenie modeli przeprowadzono poprzez stopniowe

usuwanie nieistotnych interakcji i efektów głównych z wykorzystaniem testów F (funkcja *drop1*), pozostawiając jedynie zmienne istotne statystycznie ($p < 0,05$). Istotne efekty wizualizowano na podstawie wartości predykowanych oraz porównań post-hoc z korektą Tukeya.

Proporcję płci w grupach analizowano przy użyciu uogólnionego modelu liniowego (GLM) z rozkładem dwumianowym i funkcją łączącą logit. Model oceniał udział samic w poszczególnych grupach eksperymentalnych względem grupy kontrolnej. Porównania między grupami wykonano z zastosowaniem testów post-hoc z korektą Tukeya.

Do analizy skorygowanych allometrycznie wartości wskaźnika palcowego zastosowano modele liniowe, osobno dla każdej kończyny. W modelach początkowych jako zmienne objaśniające uwzględniono wariant eksperymentalny (EXP), wskaźnik kondycji somatycznej (SMI), płeć (SEX), długość ciała (SVL) oraz ich interakcje (EXP×SMI, EXP×SVL, EXP×SEX). Następnie modele upraszczano poprzez usuwanie nieistotnych składników na podstawie testów F ($p < 0,05$). Na tej podstawie wybrano ostateczne modele dla każdej kończyny. Wszystkie analizy statystyczne przeprowadzono w środowisku R (v. 4.1.3).

Najważniejsze wyniki

Prace przeglądowe

Publikacja I

W trakcie przygotowywania pierwszego przeglądu literatury, początkowo zidentyfikowano 3450 publikacji, z których na podstawie analizy tytułów i streszczeń wyselekcjonowano 757 potencjalnie istotnych. Po szczegółowej analizie pełnych tekstów do dalszego opracowania zakwalifikowano ostatecznie 241 badań dotyczących wpływu EDCs na płeć i cechy z nią związane u płazów, z czego 214 stanowiły eksperymenty *in vivo*.

Zdecydowana większość badań prowadzona była na wodnych formach rozwojowych płazów, z ekspozycją polegającą na przetrzymywaniu zwierząt w roztworze związku chemicznego. Jedynie dwa badania obejmowały ekspozycję poprzez spożycie pokarmu (Tang i in., 2020; Ziková i in., 2013). W części innych prac stosowano ekspozycję poprzez iniekcje lub implanty dootrzewnowe, które stanowiły 9,81% wszystkich badań *in vivo* i miały ograniczone znaczenie środowiskowe.

Łącznie w badaniach *in vivo* analizowano 46 gatunków, co stanowi jedynie 0,52% wszystkich obecnie znanych gatunków płazów (stan na 31.12.2024). Dwa najczęściej wykorzystywane gatunki, należące do rodzaju *Xenopus*, stanowiły niemal połowę (49,78%) wszystkich analizowanych przypadków.

Publikacja II

W trakcie wykonywania drugiego przeglądu literatury, początkowo zidentyfikowano ponad 3000 rekordów, z których większość dotyczyła wpływu EDCs na rozwój gonad, poziomy hormonów lub ekspresję genów u płazów. Spośród nich jedynie 27 publikacji spełniało kryteria włączenia do przeglądu. W tym podzbiorze, 21 badań analizowało wpływ EDCs na komunikację godową i zachowania rozrodcze, natomiast zaledwie 6 dotyczyło zmian w ubarwieniu ciała.

Ponadto kilka prac opisywało wpływ EDCs na inne, morfologiczne cechy powiązane z płcią, które mogą odgrywać rolę w komunikacji między osobnikami w okresie rozrodu. Zmiany obejmowały morfologię modzeli godowych (Hayes i in., 2010) oraz drugorzędowe cechy płciowe: kształt ogona i błony pławne u traszki helweckiej *Lissotriton helveticus* (Secondi i in., 2013; Secondi i in., 2009). Choć dane te

dostarczają cennych wskazówek, liczba badań koncentrujących się na morfologicznych wskaźnikach hormonalnych u płazów pozostaje nadal bardzo ograniczona.

Praca na okazach ze zbiorów: Publikacja III

W analizie wskaźników palcowych (DR) bez uwzględnienia korekty allometrycznej nie stwierdzono istotnych różnic między samcami a samicami u *P. fuscus*. Ani płeć, ani wielkość ciała nie miały odrębnie istotnego wpływu na wartości DR. Jedynie w przypadku palców kończyn przednich, zwłaszcza dla proporcji 3D:5D i 4D:5D u samców, zauważono tendencję wzrostu wartości wskaźnika wraz ze wzrostem długości ciała, jednak zależność ta była statystycznie nieistotna.

Po uwzględnieniu korekty allometrycznej uzyskano inne wyniki: analiza regresji wykazała istotny wpływ zarówno płci, jak i długości ciała. W tym ujęciu samce charakteryzowały się wyższymi wartościami niektórych wskaźników, zwłaszcza 2D:4D i 3D:4D we wszystkich kończynach. Dymorfizm płciowy ujawnił się również w przypadku wskaźnika 2D:3D w lewej kończynie przedniej. Ponadto stwierdzono istotną, ujemną zależność między długością ciała a wartościami większości wskaźników palcowych: większe osobniki miały relatywnie krótsze przednie palce w stosunku do pozostałych. Zależność ta była wysoce istotna statystycznie ($p \leq 0{,}001$). Wskazuje to na obecność systematycznych zmian proporcji długości palców wraz ze wzrostem osobników.

Praca eksperymentalna: Publikacja IV

Kondycja ciała i rozwój płciowy

Analiza wyników uzyskanych z badań wykazała, że ekspozycja na EDCs negatywnie wpłynęła na kondycję somatyczną osobników. W porównaniu z grupą kontrolną wartości SMI były istotnie niższe w grupie eksponowanej na najwyższe stężenie BPA (10^{-7} M) oraz w grupie eksponowanej na EE2, podczas gdy dla grup eksponowanych na niższe stężenia BPA (10^{-8} M i 10^{-9} M) nie odnotowano istotnych różnic.

W przypadku proporcji płci w grupach, zaobserwowano istotny wpływ ekspozycji na EE2. W grupie eksponowanej na EE2 stwierdzono wyraźne przesunięcie stosunku płci w kierunku samic (około 1:9). Szansa wystąpienia osobnika o fenotypie żeńskim była w tej grupie około 5,2-krotnie wyższa niż w grupie kontrolnej.

Wpływ ekspozycji na badane EDCs był widoczny również w budowie histologicznej gonad, która wykazała wyraźne różnice między grupą kontrolną a grupami eksperymentalnymi. W grupie kontrolnej rozwój gonad u obu płci przebiegał zgodnie z oczekiwanym wzorcem. U samic dominowały jajniki w stadiach rozwojowych VII–VIII (Ogielska i Kotusz, 2004) z prawidłową dla tych etapów organizacją tkanki. W stadium VII obserwowano duży udział komórek płciowych młodszych klas: oogoniów pierwotnych oraz mejocytów, zlokalizowanych w korze gonady, przy jednoczesnej obecności pojedynczych, starszych komórek linii płciowej: oocytów diplotenowych w części centralnej. Jedynie sporadycznie obserwowano niewielkie, lokalne ogniska degeneracji komórek płciowych, które nie wpływały na ogólną strukturę gonady i mieściły się w zakresie zmian fizjologicznych. U samców wszystkie gonady odpowiadały stadium VII rozwojowym jąder, z dobrze wykształconymi, zwartymi kanalikami nasiennymi wypełnionymi gonocytami i bez oznak patologii.

U samic z grup eksponowanych na BPA zaobserwowano szeroki przekrój stadiów rozwojowych jajników (VI–IX), co wskazuje na większe zróżnicowanie tempa i przebiegu rozwoju w porównaniu z grupą kontrolną. Większość jajników miała prawidłową budowę, odpowiadającą danemu stadium rozwojowemu, i, w pojedynczych przypadkach, z niewielkimi ogniskami degeneracji komórek płciowych. W przypadku trzech gonad zaobserwowano obecność rudymentarnych metamerów: dystalnych, wyraźnie oddzielonych, mniejszych segmentów gonady, nietypowych dla tych etapów rozwojowych.

U samców eksponowanych na BPA, większość jąder odpowiadała stadium rozwojowemu VII, podobnie jak w grupie kontrolnej. We wszystkich stężeniach BPA u części osobników stwierdzono obecność zmian degeneracyjnych o różnym nasileniu. Obejmowały one zarówno niewielkie ogniska degeneracji, ograniczone do pojedynczych kanalików nasiennych, jak i bardziej rozległe zmiany obejmujące znaczną część gonady. W niektórych obserwowano zaawansowaną atrofię gonad, wskazującą na zanik lub przebudowę kanalików nasiennych. Dodatkowo u dwóch samców, podobnie jak u samic, stwierdzono obecność rudymentarnych metamerów.

Najwyraźniejsze zaburzenia w rozwoju gonad odnotowano w grupie eksponowanej na EE2. U samic rozwój jajników obejmował stadia VII–IX, przy czym dominowały gonady o bardziej zaawansowanym stadium rozwojowym (IX), niemal całkowicie wypełnione oocytami w fazie diplotenu. Jednocześnie w dwóch przypadkach

w gonadach zaobserwowano rozległe obszary pozbawione komórek płciowych, co wskazywało na zaawansowaną degenerację tkanki.

U dwóch osobników określonych jako *intersex* (wg nomenklatury zaproponowanej przez Lutz i in., 2008) stwierdzono obecność prawidłowo rozwiniętego jajnika we wczesnym stadium rozwoju (VI) po jednej stronie ciała oraz przeciwległego jądra o nieprawidłowej morfologii. Ponadto u jednego osobnika zaobserwowano gonadę o mieszanych cechach jądra i jajnika oraz przeciwległe jądro o nieprawidłowej budowie. U wszystkich 4 fenotypowych samców z grupy eksponowanej na EE2 nie stwierdzono odchyleń od prawidłowej struktury jąder, które odpowiadały stadium VII rozwoju.

Wskaźnik palcowy

Analiza wykazała, że u *P. ridibundus* DR nie jest dymorficzną cechą płciową: nie wykryto istotnych różnic w DR między samcami i samicami. W przypadku prawej kończyny przedniej nie stwierdzono również istotnego wpływu ekspozycji na BPA lub EE2. W przypadku pozostałych kończyn stwierdzono, że istnieje zależność między ekspozycją na badane związki a wartością DR, ale w interakcji z wielkością ciała.

Dla lewej kończyny przedniej w grupie EE2 większe osobniki charakteryzowały się niższymi wartościami wskaźnika 2D:4D. Podobną zależność znaleziono dla lewej kończyny tylnej u osobników eksponowanych na najwyższe stężenie BPA (10^{-7} M) oraz EE2, oraz dla prawej kończyny tylnej u osobników eksponowanych na najwyższe stężenie BPA oraz EE2. W grupie kontrolnej oraz w grupie eksponowanej na najniższe stężenie BPA (10^{-9} M) obserwowano odwrotną zależność: większe osobniki miały wyższe wartości DR.

Łącznie wyniki wskazały, że wpływ badanych związków na DR u *P. ridibundus* nie jest jednoznaczny, lecz zależy od wielkości ciała osobników i jest zróżnicowany między kończynami.

Dyskusja

Prace przeglądowe: Publikacja I i II

Analiza literatury dotyczącej badań nad wpływem EDCs (Frątczak i in., 2025c) na płazy ujawniła wyraźną dominację eksperymentów prowadzonych na wąskiej grupie gatunków modelowych, głównie z rodzaju *Xenopus*, na formach larwalnych płazów, w warunkach laboratoryjnych z ekspozycją w wodzie, bez dodatkowych czynników środowiskowych. Podejście to umożliwia precyzyjne analizy mechanizmów hormonalnych u gatunków relatywnie łatwych w hodowli (z całym cyklem życiowym odbywającym się w środowisku wodnym), jednak nie odzwierciedla ono ogromnego zróżnicowania filogenetycznego, fizjologicznego i ekologicznego płazów. Uzyskiwane wyniki trudno uogólnić na inne grupy płazów, które dominują obecnie w skali globalnej (Neobatrachia, Wake & Koo, 2018). Tymczasem dane pochodzące z badań na gatunkach niemodelowych wskazują, że reakcje na EDCs mogą różnić się zarówno kierunkiem, jak i siłą (Mackenzie i in., 2003; Piprek i in., 2012; Tamschick i in., 2016a-c), co może wynikać m.in. z odmiennych systemów determinacji płci (Miura i in., 2016), jak również typu i tempa różnicowania gonad (Storrs i Semlitsch, 2008).

Dodatkowo różnice w ekologii i fizjologii poszczególnych gatunków, m.in. dotyczące cyklu życiowego (Nolan i in., 2023), struktury skóry (Hillyard i in., 2008), sposobu żerowania (Fan i in., 2019) czy strategii zimowania (Swanson i in., 2018), mogą decydować o odmiennych drogach narażenia i stopniu wrażliwości na te związki chemiczne (Frątczak et al., 2025b). Głównym wnioskiem wynikającym z analizy literatury jest więc konieczność projektowania eksperymentów przy użyciu gatunków niemodelowych, w układach dostosowanych do ich biologii, a nie jedynie do wymogów standaryzacji laboratoryjnej (Luedtke i in., 2023).

Realistyczna ocena ryzyka związanego z działaniem EDCs wymaga rozszerzenia zakresu badań o gatunki reprezentujące różne linie filogenetyczne i strategie ewolucyjne (Tamschick i in., 2016 a-c; Wagner i Viertel, 2017). Niezbędne jest również prowadzenie eksperymentów w warunkach bardziej zbliżonych do naturalnych, uwzględnieniem obecności dodatkowych czynników, takich jak zmienność temperatur (Tang i in., 2020), promieniowanie UV (Croteau i in., 2009), obecność w zbiornikach wodnych osadów dennych (Fuentes i in., 2014), drapieżników (LaFiandra i in., 2008) czy patogenów (Paetow i in., 2023), co pozwoli uzyskać wyniki lepiej odzwierciedlające rzeczywiste scenariusze środowiskowe.

Istotnym kierunkiem dalszych badań pozostaje także rozwój nieinwazyjnych metod oceny płci oraz zaburzeń hormonalnych, które mogłyby być stosowane zarówno w badaniach eksperymentalnych, jak i terenowych (Orton i in., 2023). Jak wykazał drugi przegląd literatury (Frątczak i in., 2025c), związki endokrynnie czynne oddziałują nie tylko na budowę gonad i poziom hormonów, lecz także na zewnętrzne, dymorficzne cechy płciowe, oraz co kluczowe, biorąc pod uwagę luki w naszym zrozumieniu tego problemu, zachowania i komunikację między osobnikami. Zmiany te mogą obniżać sukces rozrodczy osobników, a jednocześnie stanowią potencjalnie cenne, nieinwazyjne wskaźniki działania EDCs, które pozostają jednak słabo poznane.

Podsumowując, dalszy postęp w badaniach nad wpływem EDCs na płazy wymaga odejścia od koncepcji jednego „idealnego" modelu laboratoryjnego na rzecz większej różnorodności gatunkowej, prowadzenia badań w warunkach bliższych środowisku naturalnemu (np. w mezokosmach) oraz poszukiwania alternatywnych biomarkerów zaburzeń endokrynnych. Jak zaproponowano w niniejszej rozprawie, jednym z takich indykatorów może być wskaźnik palcowy (DR), łączący łatwość pomiaru z możliwością odzwierciedlenia subtelnych zmian hormonalnych zachodzących w okresie wczesnego rozwoju.

Praca na okazach ze zbiorów: Publikacja III

Badania przeprowadzone na okazach ze zbiorów Katedry Zoologii Uniwersytetu Przyrodniczego w Poznaniu pozwoliły na szczegółową analizę DR u *Pelobates fuscus*, gatunku reprezentującego podrząd Mesobatrachia, dla którego dotychczas brakowało takich danych. Uzyskane wyniki podkreślają, że poprawna interpretacja wskaźników DR wymaga uwzględnienia allometrii, czyli zależności skalujących między długościami palców (Beaty i in., 2016; Lolli i in., 2017), czego nie brano pod uwagę w większości poprzednich prac. W przeprowadzonej na grzebiuszkach analizie nieuwzględniającej tego aspektu nie wykazano istotnych różnic między płciami, natomiast po wprowadzeniu korekty allometrycznej ujawniły się wyraźne różnice, z wyższymi wartościami wskaźnika palcowego 2D:4D u samców.

Uzyskany dla *P. fuscus* wynik, w którym wartości 2D:4D są wyższe u samców, jest zbieżny z obserwacjami u innych grup kręgowców, w tym gadów i ptaków (Leoni i in., 2008; Van Damme i in., 2015). W kontekście filogenetycznym, dane na temat DR u *P. fuscus* stanowią istotne uzupełnienie dotychczasowej wiedzy. Większość dotychczasowych badań obejmowała gatunki z rodzin Neobatrachia i Archaeobatrachia

(Balogová i in., 2015; Germano i in., 2011; Lofeu i in., 2017), podczas gdy informacje dotyczące Mesobatrachia pozostawały ograniczone (Alinezhadi i in., 2020).

Uzyskane wyniki poszerzają zatem wiedzę o gatunku z grupy o pośredniej pozycji filogenetycznej w stosunku do pozostałych rzędów płazów (Ivanova i in., 2017; Székely i Nemes, 2002) oraz wskazują, że analiza DR może być użytecznym narzędziem w badaniach nad dymorfizmem płciowym wśród płazów, pod warunkiem stosowania odpowiedniej metodyki.

Praca eksperymentalna: Publikacja IV

Kondycja ciała

W przeprowadzonych badaniach eksperymentalnych wykazano istotną zależność między ekspozycją na EDCs a kondycję ciała osobników, opisaną jako SMI. Wskaźnik ten odzwierciedla relację między masą a wielkością ciała płazów i koreluje z dalszym wzrostem osobników, a tym samym z ich potencjałem do osiągnięcia sukcesu rozrodczego (Hinderer i in., 2025). W niniejszym badaniu stwierdzono, że w porównaniu z grupą kontrolną wartości SMI były niższe w grupach eksponowanych na EE2 oraz na najwyższe stężenie BPA (10^{-7}), co sugeruje, że związki te w sposób jednoznaczny, negatywnie wpływają na kondycję żab.

W dotychczasowych badaniach dotyczących wpływu BPA i EE2 na płazy nie mierzono ich wpływu na kondycję ciała wyznaczaną przez wskaźnik SMI lub BMI, co utrudnia porównanie uzyskanych wyników. Zaobserwowane pogorszenie kondycji pod wpływem BPA jest jednak zgodne z doniesieniami o zaburzeniach wzrostu i rozwoju somatycznego u płazów, które mogą wynikać z oddziaływania tego związku chemicznego na procesy regulowane przez hormony tarczycy (Heimeier i Shi, 2010). W literaturze można również znaleźć dane dotyczące wpływu BPA na wielkość ciała płazów, chociaż przynoszą one niejednoznaczne wnioski. Przykładowo w części badań nie wykazano wpływu BPA ani na długość (SVL), ani masę ciała u *X. laevis* (Levy i in., 2004; Li i in., 2022; Pickford i in., 2003). Z drugiej strony Tamschick i in. (2016a) zaobserwowali spadek zarówno długości, jak i masy ciała pod wpływem BPA u *X. laevis* oraz u *H. arborea*, oraz brak takiego efektu u *B. viridis*.

Równie niejednoznaczne są dane dotyczące EE2. W kilku badaniach nie stwierdzono wpływu tego związku na długość (SVL) i masę ciała u *X. laevis* (Cevasco i in., 2008; Tamschick i in., 2016b), *X. tropicalis* (Pettersson i in., 2006) oraz *L. pipiens* (Hogan i in., 2008; Mackenzie i in., 2003). Jednak u innych gatunków pod wpływem

EE2 wykazywano zarówno wzrost SVL, u *L. sylvaticus* (Mackenzie i in., 2003), jak i jednoczesny wzrost SVL i masy ciała, u *B. viridis* (Tamschick i in., 2016b), a także spadek długości ciała przy niezmienionej masie, u *H. arborea* (Tamschick i in., 2016b). Tak duże rozbieżności sugerują, że reakcja na EE2 jest gatunkowo zależna, jak również może wynikać z warunków eksperymentalnych i okna czasowego ekspozycji.

Rozwój płciowy

Wyniki badań wykazały, że ekspozycja na etynyloestradiol (EE2) istotnie zaburza proces różnicowania płci fenotypowej u *P. ridibundus*. Zaobserwowane wyraźne przesunięcie stosunku płci w kierunku samic w grupie eksponowanej na EE2 sugeruje jego silne działanie feminizujące, szeroko udokumentowane w literaturze u innych gatunków. Efekt ten wykazano m.in. u *X. laevis* w stężeniach 10^{-9}– 10^{-10} M (Pettersson i Berg, 2007), *X. tropicalis* (10^{-7}– 10^{-9} M; Gyllenhammar i in., 2009; Pettersson i in., 2006), *R. temporaria* (10^{-10} M; Pettersson i Berg, 2007), *L. pipiens* (3×10^{-9}– 3×10^{-8} M; Mackenzie i in., 2003), *L. sylvaticus* ($3{,}22\times10^{-8}$ M; Tompsett i in., 2013) oraz *B. bufo* (3×10^{-6} M; Ujhegyi i Bókony, 2020). Efekt odwrócenia płci fenotypowej u samców dodatkowo potwierdzono analizami genetycznymi u *X. laevis* (10^{-8}– 10^{-9} M; (Tamschick 2016b), *X. tropicalis* (2×10^{-11}– 10^{-10} M; Hirakawa i in., 2013), *H. arborea* (10^{-8}– 10^{-9} M; (Tamschick i in., 2016b) oraz *B. viridis* (10^{-8}– 10^{-9} M; Tamschick i in., 2016b), co wskazuje na znaczący wpływ EE2 na mechanizmy determinacji i różnicowania płci.

Uzyskane w niniejszej pracy wyniki histologiczne rozszerzają te obserwacje, wskazując na znaczący wpływ EE2 na rozwój gonad u kolejnego gatunku płaza. W porównaniu do grupy kontrolnej, w grupie eksponowanej na EE2 obserwowano duże zróżnicowanie stadiów rozwojowych jajników. Odnotowano również kilka przypadków nieprawidłowej morfologii gonad. Stwierdzono wystąpienie jednego osobnika z gonadami o mieszanych cechach jądra i jajnika, jak również dwóch osobników *intersex*, u których jajnik o prawidłowej budowie na wczesnym stadium rozwojowym współwystępował z morfologicznie nieprawidłowym jądrem. Podobne zmiany opisywano wcześniej u innych gatunków płazów, w tym *X. laevis* (10^{-8}– 10^{-10} M; Cevasco i in., 2008; Tompsett i in., 2012, 2013), *X. tropicalis* (10^{-11} M; Hirakawa i in., 2013), *R. temporaria* (10^{-10} M; Pettersson & Berg, 2007), *R. septentrionalis* (~10^{-11} M; (Park i Kidd, 2005) oraz *L. pipiens* (5×10^{-9} M; Hogan i in., 2008). Notowano również inne zaburzenia rozwoju gonad u *H. arborea* (10^{-8} M; Tamschick i in., 2016a) oraz *B. viridis* (10^{-8}– 10^{-9} M; Tamschick i in., 2016b).

Wyniki uzyskane w części eksperymentalnej, w zestawieniu z danymi literaturowymi, wskazują, że EE2 wykazuje silne działanie feminizujące, przy czym charakter i nasilenie tego efektu zależą od gatunku oraz prawdopodobnie od warunków i sposobu ekspozycji. Konsekwencje tych zmian mogą mieć istotne znaczenie na poziomie populacyjnym. Wyraźne przesunięcie stosunku płci w kierunku samic może zaburzać dynamikę rozrodu, prowadząc do zwiększonej konkurencji o samce oraz zmian w intensywności doboru płciowego (Pröhl, 2002), a nawet zmian w systemie determinacji płci (Miura, 2007; Miura i in., 2016).

W przypadku rodzaju *Pelophylax* implikacje te mogą być szczególnie istotne ze względu na specyficzny system rozrodu. Gatunki z kompleksu *Pelophylax esculentus* funkcjonują w systemie hybrydogenezy, w którym podczas gametogenezy eliminowany jest genom jednego z gatunków rodzicielskich, a utrzymanie linii hybrydowej wymaga krzyżowania wstecznego z drugim gatunkiem (Berger, 1977; Dedukh i in., 2019). Zaburzenia stosunku płci, zwłaszcza niedobór samców jednego z gatunków, mogą zwiększać częstość kojarzeń z udziałem osobników hybrydowych, prowadząc do destabilizacji relacji między formami rodzicielskimi i hybrydami. W dłuższej perspektywie może to wpływać na przepływ genów, trwałość czystych genetycznie gatunków oraz stabilność całego systemu hybrydowego (Skierska i in., 2023).

W przeciwieństwie do EE2, wpływ bisfenolu A (BPA) na różnicowanie płci i rozwój gonad u płazów nadal nie jest jednoznaczny. Z badań przeprowadzonych wcześniej na *X. laevis* wynika, że ekspozycja na BPA w stężeniu 10^{-7} M może prowadzić do feminizacji gonad oraz przesunięcia stosunku płci w kierunku samic (Kloas i in., 1999; Levy i in., 2004). Jednocześnie zmiany patomorfologiczne w jądrach w cytowanych badaniach były stosunkowo rzadkie (Levy i in., 2004).

W przeprowadzonym w ramach niniejszej rozprawy doktorskiej badaniach nie zaobserwowano efektu feminizującego BPA. Stosunek płci w grupach eksponowanych na ten związek był zbliżony do 1:1 lub nieznacznie przesunięty w kierunku samców, co mieści się w granicach normy w populacjach płazów. Należy jednak podkreślić, że brak analizy genetycznej uniemożliwia jednoznaczne wykluczenie odwrócenia fenotypowej płci. Niezależnie od tego wykazano, że BPA wpływał na rozwój gonad, prowadząc do wystąpienia zmian patomorfologicznych. U części samców zaobserwowano rozległą degenerację tkanki jąder, obejmującą utratę komórek płciowych, co mogłoby się przekładać na obniżoną płodność lub potencjalnie całkowitą sterylność u osobników. Obserwacje te są podobne do wyników uzyskanych przez Tamschick i in. (2016a), którzy

wykazali zmniejszoną liczbę komórek płciowych oraz nieprawidłową budowę kanalików nasiennych, w tym ich poszerzenie, u *X. laevis* eksponowanych na BPA w stężeniach 10^{-6}, 10^{-7} i 10^{-8} M.

U samców z grup eksponowanych na BPA stwierdzono dodatkowo obecność dystalnych segmentów zawierających tkankę jąder, które można zinterpretować jako rudymentarne metamery (Piprek i in., 2015; Tamschick in., 2016a). Podobne zmiany opisywano u *H. arborea* i *B. viridis* eksponowanych na BPA (Tamschick i in., 2016a). W warunkach prawidłowego różnicowania u płazów, gonada początkowo ma budowę metameryczną, przy czym jądra rozwijają się wyłącznie z metamerów proksymalnych (przednich), natomiast metamery dystalne (tylne) ulegają degeneracji, rozpoczynającej się od IV stadium rozwoju gonad (Haczkiewicz i Ogielska, 2013). Obecność ich utrzymujących się form w gonadach dalszych stadiów rozwojowych wskazuje zatem na zaburzenie tego procesu. Interesującym wynikiem było stwierdzenie podobnych struktur w jajnikach trzech samic z grup eksponowanych na BPA. W warunkach prawidłowych metamery jajnikowe powstają w stadium III rozwoju gonad i następnie różnicują się w płaty jajnika (Ogielska i Kotusz, 2004; Piprek i in., 2015). W tym przypadku opisany stan można interpretować jako pozostałości metamerów, które nie uległy prawidłowemu przekształceniu w płaty jajnika. Obecność niedorozwiniętych struktur w późniejszych stadiach rozwoju sugeruje zahamowanie lub opóźnienie morfogenezy jajnika, co wskazuje na negatywny wpływ BPA na ten proces.

Wskaźnik palcowy

W niniejszych badaniach nie stwierdzono istotnych różnic w wartościach wskaźnika palcowego (2D:4D) między samcami a samicami *P. ridibundus*. Podobne wyniki uzyskano wcześniej u innych gatunków płazów, takich jak *Leptodactylus fuscus* (Lofeu i in., 2017), żaby z wyspy Maud *Leiopelma pakeka* (Germano i in., 2011) czy ropuchy agai *Rhinella marina* (Beaty i in., 2016) co sugeruje, że dymorfizm płciowy w zakresie DR nie jest zjawiskiem uniwersalnym w tej grupie kręgowców.

Jednocześnie brak różnic między płciami nie oznacza, że wskaźnik palcowy jest biologicznie nieistotny. Uzyskane wyniki wskazują, że DR może reagować na czynniki środowiskowe, w tym związki endokrynnie czynne. W niniejszym badaniu zarówno EE2, jak i BPA wpływały na wartości DR, przy czym efekt ten zależał od wielkości ciała (SVL) oraz kończyny, co bezpośrednio utrudnia interpretację zamian, które zaszły. Niemniej, istotny wpływ ekspozycji na EE2 w interakcji z SVL stwierdzono dla lewej kończyny

przedniej oraz dla obu kończyn tylnych, gdzie większe osobniki charakteryzowały się niższymi wartościami DR. W przypadku BPA w najwyższym stężeniu (10^{-7} M) podobny efekt obserwowano dla obu kończyn tylnych.

Interpretacja tych zmian nie jest jednoznaczna. U ssaków wyższe wartości 2D:4D są zwykle związane z silniejszym wpływem estrogenów lub słabszym działaniem androgenów w okresie rozwoju embrionalnego (Auger i in., 2013; Hönekopp i Watson, 2010). Podobne zależności sugerowano również u płazów: u *L. fuscus* ekspozycja na testosteron obniżała DR, co interpretowano jako efekt maskulinizujący (Lofeu i in., 2017). W tym kontekście, spodziewanym efektem byłoby wyższe DR u *P. ridibundus* pod wpływem związków estrogenowych. W niniejszym badaniu jednak EE2 i BPA prowadziły do obniżenia DR, szczególnie u większych osobników. Jest to sprzeczne z wynikami uzyskanymi u ssaków, gdzie BPA zwiększał wartości DR u samców szczurów (Auger i in., 2013). Wskazuje to, że odpowiedź długości palców na działanie związków endokrynnie czynnych może być cechą silnie gatunkową. U *P. ridibundus* związki estrogenowe mogą wpływać na długość palców poprzez ogólne zaburzenie równowagi hormonalnej lub działanie alternatywnych szlaków regulacyjnych.

Na odmienność mechanizmów kształtujących wskaźnik palcowy u płazów wskazują wyniki uzyskane w Publikacji III. W badaniach nad *P. fuscus* wykazano wyższe wartości DR u samców we wszystkich kończynach, co jest zjawiskiem odwrotnym do tego obserwowanego u ssaków (Frątczak i in., 2025a). Z kolei w innych badaniach własnych, przeprowadzonych na żabie moczarowej *Rana arvalis* oraz *R. temporaria*, stwierdzono istotnie niższe wartości skorygowanego allometrycznie DR u samców we wszystkich kończynach (Frątczak i in., 2025b), co byłoby zgodne ze spodziewanym wzorcem u płazów bezogonowych (Lofeu i in., 2017). Wyniki dotyczące *R. arvalis* i *R. temporaria* należy jednak interpretować z ostrożnością ze względu na trudności metodyczne w cytowanych badaniach, związane z odchowem płazów, które mogły wpłynąć na rozwój osobników i uzyskiwane wartości wskaźnika (Frątczak i in., 2025b). Kierunek dymorfizmu płciowego DR u płazów nie jest więc uniwersalny i może różnić się między gatunkami, co podkreśla złożoność mechanizmów regulujących tę cechę oraz konieczność jego ostrożnej interpretacji.

Istotnym elementem uzyskanych wyników jest zróżnicowana odpowiedź poszczególnych kończyn i stron ciała. U ssaków przyjmuje się, że prawa kończyna przednia jest bardziej wrażliwa na działanie hormonów w okresie rozwoju embrionalnego (Hönekopp i Watson, 2010), co potwierdzono m.in. w badaniach na szczurach

eksponowanych na BPA (Auger i in., 2013). W niniejszym badaniu efekt EE2 był natomiast najbardziej widoczny w lewej kończynie przedniej, co sugeruje, że u płazów występuje inna lateralizacja efektów hormonalnych.

Interpretację wyników dodatkowo utrudniają ograniczenia metodologiczne obecne w literaturze. Wzorzec DR u płazów może znacząco różnić się między gatunkami, a nawet populacjami (Kaczmarski i in., 2021). Ponadto błędy w oznaczaniu palców kończyn przednich (Kaczmarski i in., 2021) oraz brak uwzględnienia allometrii mogą prowadzić do zafałszowania wyników (Frątczak i in., 2025a).

Podsumowując, choć w niniejszym badaniu nie wykazano dymorfizmu płciowego wskaźnika palcowego u *P. ridibundus*, jego wrażliwość na działanie związków endokrynnie czynnych została potwierdzona. Wskazuje to na jego potencjał jako biomarkera zaburzeń endokrynnych u płazów. Dalsze badania powinny koncentrować się na mechanizmach rozwojowych odpowiedzialnych za tę zmienność oraz na ocenie przydatności DR, jako narzędzia bioindykacyjnego w różnych grupach płazów.

Podsumowanie i wnioski

Celem niniejszej rozprawy doktorskiej było pogłębienie wiedzy na temat znaczenia DR u płazów bezogonowych oraz ocena jego potencjalnej przydatności jako nieinwazyjnego wskaźnika płci i zaburzeń hormonalnych wywoływanych przez EDCs. Cel ten realizowano równolegle w kilku uzupełniających się kierunkach: przez podsumowanie i analizę literatury dotyczącej wpływu EDCs na płazy, udoskonalenie metodyki pomiaru i analizy DR oraz eksperymentalne sprawdzenie wpływu wybranych EDCs na płeć, budowę gonad, kondycję somatyczną i DR u przedstawiciela płazów bezogonowych.

Prace przeglądowe potwierdziły istnienie znaczących luk badawczych dotyczących wpływu EDCs na płazy, zwłaszcza nadreprezentację gatunków modelowych z rodzaju *Xenopus* oraz niedostatek eksperymentów prowadzonych w warunkach wychodzących poza klasyczne schematy laboratoryjne (Frątczak i in., 2025c). Wykazano również, że wpływ EDCs na cechy potencjalnie bioindykacyjne pozostaje słabo poznany (Frątczak i in., 2025d).

W badaniach na okazach znajdujących się w zbiorach Katedry Zoologii, zgodnie z postawioną wcześniej hipotezą potwierdzono, że uwzględnienie allometrii ma kluczowe znaczenie dla prawidłowej interpretacji wskaźnika palcowego u płazów. W badaniach nad *P. fuscus* dymorfizm płciowy DR ujawnił się dopiero po zastosowaniu korekty allometrycznej, co potwierdza, że analizy nieuwzględniające skalowania długości palców mogą prowadzić do błędnych wniosków. Jednocześnie uzyskano nowe dane dla słabo poznanego przedstawiciela *Mesobatrachia*, rozszerzając wiedzę o zróżnicowaniu DR u płazów bezogonowych (Frątczak i in., 2025a).

W części eksperymentalnej potwierdzono, że wybrane związki endokrynnie czynne: EE2 oraz BPA, mogą wpływać na rozwój płciowy osobników *P. ridibundus*, a zakres tego wpływu zależy od substancji i jej stężenia. Potwierdzono również hipotezę zakładającą wpływ BPA i EE2 na kondycję somatyczną osobników.

Hipoteza zakładająca, że wzór DR jest dymorficzną cechą płciową została potwierdzona u *P. fuscus* (Frątczak i in., 2025a) oraz odrzucona u *P. ridibundus* (Frątczak i in., 2026). Jednocześnie uzyskane wyniki z badań wskazały, że DR, zgodnie z postawioną hipotezą, reaguje na działanie EDCs, przy czym efekt ten zależy od wielkości ciała i różni się między kończynami. Sugeruje to, że DR nie jest uniwersalnym markerem płci u płazów, ale może mieć potencjalną wartość jako wskaźnik zaburzeń

endokrynnych. Jego interpretacja musi być jednak prowadzona z dużą ostrożnością, z uwzględnieniem specyfiki gatunku.

Podsumowując, prace składające się na niniejszą rozprawę porządkują stan wiedzy na temat wpływu EDCs na płazy, wskazują kluczowe ograniczenia dotychczasowych badań oraz dostarczają nowych danych na temat DR: metodyki jego analizy oraz powiązań z płcią i ekspozycją na EDCs u płazów bezogonowych.

Perspektywy dalszych badań

Badania zrealizowane na potrzeby niniejszej rozprawy doktorskiej są kontynuowane w ramach grantu Preludium 22, przyznanego przez Narodowe Centrum Nauki. Obecnie prowadzona jest dalsza część projektu, której celem jest ocena wpływu BPA i EE2 u gatunku reprezentującego grupę żab brunatnych: żaby moczarowej *R. arvalis*.

Gatunek ten różni się od *P. ridibundus* zarówno uwarunkowaniami ekologicznymi, jak i tempem dojrzewania gonad. *P. ridibundus*, przedstawiciel żab zielonych, jest gatunkiem ściśle związanym z wodnym środowiskiem. Większość życia, nie tylko w czasie rozwoju larwalnego i okresu godowego, spędza w zbiornikach wodnych i ich pobliżu. W przeciwieństwie do niej, *R. arvalis* jest narażona na zanieczyszczenia w zbiornikach wodnych właściwie tylko w okresie rozwoju larwalnego oraz w czasie godów. Dodatkowo, rozwój gonad u żab brunatnych przebiega wolniej w stosunku do rozwoju somatycznego niż ma to miejsce u żab zielonych (Ogielska i Kotusz, 2004). W dużej części zachodzi on już po metamorfozie, gdy osobniki opuszczają zbiorniki wodne. Może to wpływać na podatność gonad na związki endokrynnie czynne obecne w środowisku wodnym.

Dalsze badania pozwolą więc ocenić, w jakim stopniu pozycja filogenetyczna, tryb życia i tempo rozwoju płciowego modulują reakcję płazów na EDCs. Badania na *R. arvalis* dostarczą również kolejnych danych na temat DR i jego użyteczności jako markera płci oraz ekspozycji na EDCs. Dane uzyskane w badaniach wstępnych sugerują, że u gatunku tego, w przeciwieństwie do *P. ridibundus*, występuje dymorfizm płciowy DR (Frątczak i in., 2025a), co być może przełoży się również na wyraźniejszy wpływ EDCs na ten wskaźnik.

W dalszej perspektywie planowane są również badania terenowe, ukierunkowane na ocenę poziomu zanieczyszczenia środowiska związkami endokrynnie czynnymi oraz mikroplastikiem w siedliskach płazów wzdłuż gradientu antropopresji: od obszarów silnie zurbanizowanych i rolniczych po siedliska o charakterze naturalnym. Celem tych analiz będzie określenie, w jakim stopniu rzeczywiste zanieczyszczenie EDCs przekładają się na kondycję osobników, przebieg rozwoju gonad oraz sukces rozrodczy populacji płazów.

Ważne jest uzyskanie wyników, które będą mieć znaczenie nie tylko poznawcze, lecz także aplikacyjne, dla ochrony rodzimych gatunków oraz rozwoju skutecznych strategii monitoringu i ograniczania zanieczyszczeń środowiska.

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Assessing species bias in amphibian research on endocrine disruptors: beyond *Xenopus laevis*

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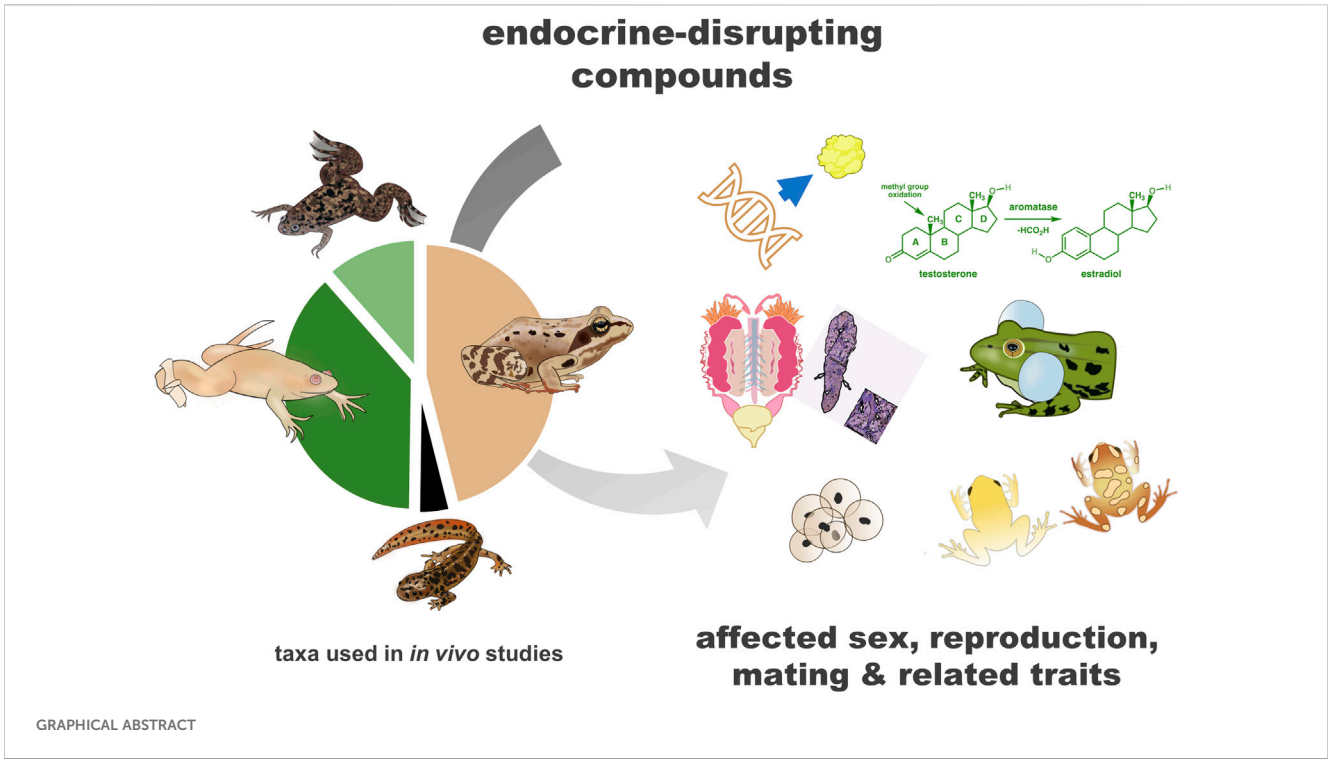
Due to their high sensitivity to hormonal agents, in recent years, amphibians have been proposed as bioindicators for the presence of endocrine-disrupting compounds (EDCs) in the environment. EDCs are a chemically diverse group of compounds, mainly of anthropogenic origin, that can interfere with hormone receptors. The escalating global environmental pollution with endocrine-disrupting compounds (EDCs) poses a significant threat to wildlife and human health. Amphibians are at high risk of exposure to EDCs in the environment, as they produce eggs without a protective shell, possess highly permeable skin, and most of them undergo an aquatic life phase, where they are chronically exposed to EDCs in the water. This exposure raises concerns about the contribution of EDCs to the dramatic decline of amphibian populations and underscores the necessity of environmental-relevant studies in this area. Despite the critical need, amphibians have attracted relatively little research focus in this regard. Although above 200 experimental studies on the topic of EDCs and sex, reproduction, and related traits in amphibians have been conducted, a significant portion of them rely on model species from the *Xenopus* genus, which do not fully represent the diverse group of amphibians. Additionally, these studies often use strict laboratory conditions that fail to mimic natural exposure scenarios. Our extensive review highlights the research gaps and emphasizes the importance of incorporating a broader range of amphibian species to understand the true impact of EDCs. We advocate for more studies in environmentally relevant settings and the use of native species to enhance the ecological validity of findings.

KEYWORDS

EDC, environmental pollution, model species, ecotoxicology, one health

1 Introduction

Amphibians have often been used in experiments concerning the impact of hormonal agents on reproductive parameters (Kloas et al., 2024). The most notable example includes the use of frogs from the *Xenopus* genus in the creation and validation of pregnancy tests (Wallingford, 2022). Due to their high sensitivity to hormonal agents, in recent years, amphibians have also been proposed as possible bioindicators of the presence of endocrine-disrupting compounds (EDCs) in the environment (Lambert et al., 2016). EDCs are concerning, mostly human-generated, exogenous substances that mimic the action of endogenous hormones (European Commission, 1996). EDCs are a chemically diverse group that includes mainly synthetic compounds present in, e.g., pesticides, plasticizers, surfactants, drugs, and personal care products. EDCs can interact with hormone receptors, or by other mechanisms, target estrogenic, androgenic, and thyroid pathways of action (for the review see



GRAPHICAL ABSTRACT

La Merrill et al., 2020). These compounds are found in significant quantities in soil, atmosphere, and surface waters worldwide (Ankley et al., 2018; Annamalai and Namasivayam, 2015; Caliman and Gavrilescu, 2009; Singh et al., 2023).

Amphibians are at high risk of exposure to EDCs in the environment, as they possess highly permeable skin and produce eggs without a protective shell (Wells, 2010). Moreover, most of them undergo an aquatic life phase during embryonic and larval development, where they are chronically exposed to all compounds, such as EDCs, in the surrounding water. In the context of the development of body systems and sex differentiation taking place at this time, they are extremely susceptible to the potential harmful effects of EDCs (Huang et al., 2005; Kloas et al., 1999; Kloas, 2002). Because of this, amphibians can act as excellent bioindicators of EDCs' presence in the environment (Lambert et al., 2016), which is important from the perspective of biomonitoring and human health, especially in a comprehensive approach, such as One Health. Studies on the impact of EDCs are crucial also because EDCs are suggested to contribute to the worldwide dramatic decline of amphibian populations, underlining the urgent need for more studies with environmental-relevant settings on this topic (Kloas et al., 2024; Luedtke et al., 2023; Salla et al., 2024a).

In comparison to other groups of vertebrates, particularly fish, amphibians are still an understudied group of animals in the context of endocrine disruptors (Johnson et al., 2017; Kloas and Lutz, 2006; Kloas et al., 2024). It is important to remember that limiting research to only selected taxa carries significant risks, with the most glaring example being the thalidomide scandal, which would not have had such disastrous effects had it been tested on a wider range of species. Thalidomide, a drug prescribed to pregnant women in the late 1950s and early 1960s, caused severe birth defects in thousands of children because its teratogenic effects were not detected in the limited animal models initially used for testing (Swaters et al., 2022). Apart from studying the impact of EDCs on different groups of vertebrates, the diversity of species should also be taken into account.

In the case of amphibians, some authors suggested, that popular ecotoxicological protocols, such as The Frog Embryo Teratogenesis Assay-Xenopus (FETAX) used by American Society of Testing and Materials (Dumont et al., 1983; Testing and Materials, 1998) for studying potential endocrine-disrupting effects of industrial substances are limited due to the use of the single species from the *Xenopus* genus. The good example is the ongoing discussion on the safety of pesticide atrazine (Hayes et al., 2002; Hoskins et al., 2019; LaFiandra et al., 2008; Spolyarich et al., 2010; Storrs and Semlitsch, 2008), which in the studies on *Xenopus* spp. and other amphibians produced different results. Species diversity in ecotoxicological studies is crucial when thinking about how these findings might apply to different ecosystems and the environment as a whole (Luedtke et al., 2023). The current study was designed as a literature review to explore research gaps in studies on the impact of endocrine-disrupting compounds (EDCs) on the amphibian reproductive system. A primary focus was placed on examining the diversity of species involved in existing research and identifying possible, correlated issues.

2 Methods of literature search

We extensively reviewed the studies published by the end of 2024 on the topic of EDCs and their impact on sex and sex-related traits of amphibians. As sex-related traits, we understood the sex accessory structures: genital ducts and derivatives of these structures; and the secondary sexual characteristics: all differences

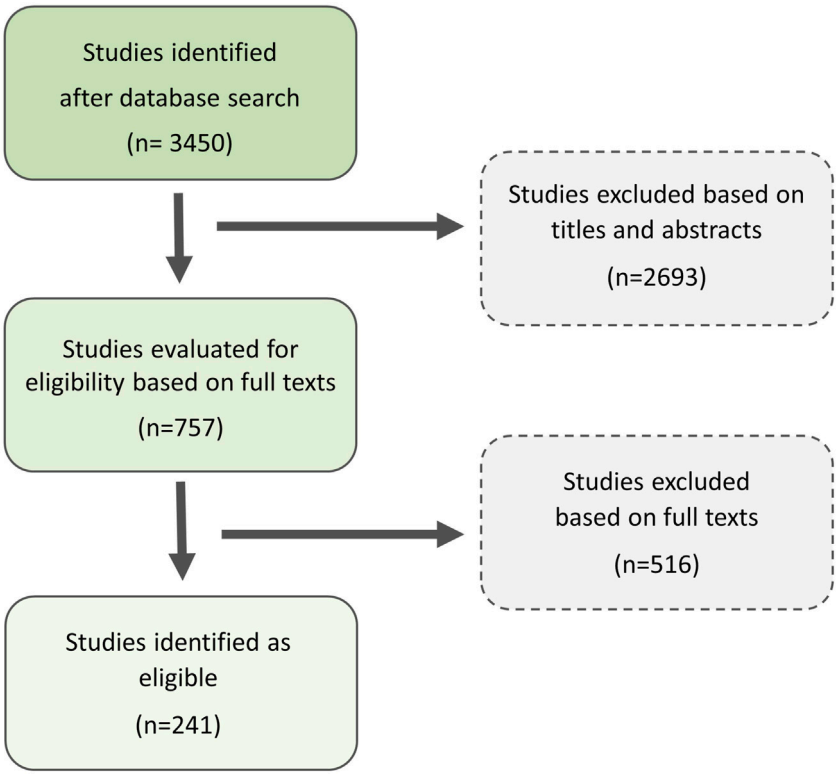


FIGURE 1
Scheme of literature search approach based on two electronic databases for the search: Web of Science Core Collection® and Google Scholar.

between the sexes resulting from sexual maturation, other than gonads and sex accessory structures (Sever and Staub, 2011). In the search, we also included the impact of EDCs on hormone signaling, expression of reproduction-related genes, sexually dimorphic behavior, mating, and fertility of amphibians. We focused on experimental studies. Analysis of research focused on EDCs and thyroid-dependent functions was beyond the scope of this review.

We used two electronic databases for the search: Web of Science Core Collection® and Google Scholar. In the database Web of Science Core Collection® the following Boolean search string was used: TS=(amphibia* OR frog* OR toad* OR newt* OR salamander* OR tadpole* OR caecilian* OR anura* OR caudata* OR urodela* OR apoda*) AND TS=(sex* OR gonad* OR test* OR ovar* OR hormone* OR vitellogenin OR reproduct* OR fertilit* OR oocyt* OR sperm* OR breeding* OR behaviour* OR nuptial pad* OR gene*) AND TI=("endocrine disruptor*`" OR endocrine* OR EDC* OR pollutant* OR pharm* OR estro* OR *estra* OR andro* OR *estradiol* OR EE2* OR *gestagen* OR progesterone* OR levonorgestrel* OR diethylstilbestrol* OR "aromatase inhibitor*" OR flutamide* OR finasteride* OR *phenol* OR *paraben* OR *cide* OR *azine* OR methoxychlor* OR chlorpyrifos* OR organochlorine* OR *dichlorodiphenyl* OR DDD* OR DDE* OR DDT* OR azocyclotin* OR glyphosate* OR Roundup* OR butachlor* OR *phosphate* OR thiophanate* OR triadimefon* OR *azole* OR vinclozolin* OR thiophanate* OR tamoxifen* OR pyrimethanil* OR tributyltin* OR triphenyltin* OR fludioxonil* OR metaxyl* OR MAXIM OR triclosan* OR fertilizer* OR nitrate* OR ammonium* OR "heavy metal*" OR arsenic* OR chromium* OR cadmium* OR lead* OR surfactant* OR detergent* OR plasticizer* OR "flame retardant*" OR TBBPA* OR TCBPA* OR *phenyl* OR PCB* OR PBDE* OR phthalate* OR styrene* OR *chlor* OR benzene* OR "UV filter*" OR camphor* OR 4-MBC* OR 3-BC* OR trenbolone* OR microcystin* OR MCLR* OR *acid* OR salt*).

A similar strategy of search strategy was used in Google Scholar. The keywords for the search were chosen based on a preliminary literature review and updated each time a new type of EDC or potential EDC was encountered during the search. To select potentially useful studies, the titles and abstracts of 3,450 articles found were first scanned for those remotely relevant to the topic. 757 potentially relevant studies were identified. Next, after reading the full papers, we were left with 241 works, which we included in the review (Figure 1). Studies were chosen if they fulfilled the inclusion criteria: (a) the publication was written in English; (b) the study was experimental and (c) the study provided data on the impact of the exposition of amphibians or amphibian tissue on the chemical compound on sex and/or at least one, defined by us, sex-related trait. The journal or date of publication of the paper was not relevant. Unpublished results from Master's and PhD Dissertation papers were not included.

The nomenclature of species mentioned in the article follows Frost (2024), with some modifications. A full list of English common and Latin names of all mentioned species, as well as their taxonomy, can be found in the Supporting Information (Supplementary Table S5). For describing changes within the

TABLE 1 Nomenclature, systematic position, sex determination system (GSD – genetic sex determination, TSD – temperature-dependent sex determination) and ecological traits of species of amphibians in experimental studies on the impact of endocrine-disrupting compounds on sex and sex-related traits with external exposition to compound. Please note that some studies used more than one species.

Order, Family	Latin species name	Sex determination system	Ecology of larvae	Diet of larvae	Ecology of adult individuals	IUCN Red List Status Account	Geographical distribution	Number of studies on larvae / on post-metamorphic forms
Anura, Bombinatoridae	*Bombina bombina*	unknown	lentic: benthic	primarily herbivorous	fossorial, terrestrial, aquatic	LC	Central and Eastern Europe	1/0
Anura, Bombinatoridae	*Bombina variegata*	XX/XY	lentic: benthic	primarily herbivorous	fossorial, terrestrial, aquatic	LC	terrestrial	1/0
Anura, Bufonidae	*Anaxyrus americanus*	unknown	lentic/lotic: benthic	herbivorous - detritivorous; scavenger	terrestrial	LC	Eastern North America	2/0
Anura, Bufonidae	*Bufo bufo*	ZW/ZZ	lentic: benthic	herbivorous - detritivorous	terrestrial, aquatic	LC	widespread in Europe, range extends east to Siberia and south to the North Africa	3/0
Anura, Bufonidae	*Bufo gargarizans*	ZW/ZZ	lentic/lotic: benthic	herbivorous - detritivorous	terrestrial, aquatic	LC	China, Taiwan	1/0
Anura, Bufonidae	*Bufotes viridis*	XX/XY	lentic/lotic: benthic	herbivorous - detritivorous	terrestrial, aquatic	LC	Central and Eastern Europe, Sweden, Estonia, range extends east to Siberia, south to the Middle East	5/0
Anura, Bufonidae	*Rhinella arenarum*	unknown	lentic/lotic: benthic[1]	herbivorous - detritivorous; carnivorous[1]	terrestrial, aquatic	LC	southern parts of South America	1/0
Anura, Bufonidae	*Sclerophrys regularis*	unknown	lentic: benthic	no data	terrestrial, aquatic	LC	sub-Saharan Africa, from West Africa to Central and East Africa, range extends into parts of North Africa and the Arabian Peninsula	1/0
Anura, Bufonidae	*Strauchbufo raddei*	XX/XY	lotic: benthic	herbivorous - detritivorous	terrestrial; populations in arid areas littoral	LC	China, Korea, Democratic People's Republic of Mongolia, Russian Federation	0/1
Anura, Bufonidae	*Hoplobatrachus rugulosus*	GSD unknown, observed TSD	lentic/lotic: suctorial[2]	herbivorous; carnivorous[2]	terrestrial, aquatic, arboreal	LC	Southeast Asia; widely farmed species	2/0
Anura, Bufonidae	*Rhinella icterica*	unknown	lentic/lotic: benthic	primarily herbivorous	terrestrial	LC	Argentina, Brazil, Paraguay	1/0
Anura, Dicroglossidae	*Euphlyctis cyanophlyctis*	ZW/ZZ, observed TSD	lotic: benthic	primarily detritivorous, scavenger, carnivorous	fossorial, terrestrial, aquatic	LC	South Asia	3/0
Anura, Dicroglossidae	*Fejervarya limnocharis*	XX/XY	lotic/lentic: pelagic, neustonic[3]	planktivorous-herbivorous[3]	terrestrial, aquatic, arboreal	LC	Southeast Asia; southern parts of Pakistan, Bhutan and China; Japan	0/1

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TABLE 1 (*Continued*) Nomenclature, systematic position, sex determination system (GSD – genetic sex determination, TSD – temperature-dependent sex determination) and ecological traits of species of amphibians in experimental studies on the impact of endocrine-disrupting compounds on sex and sex-related traits with external exposition to compound. Please note that some studies used more than one species.

Order, Family	Latin species name	Sex determination system	Ecology of larvae	Diet of larvae	Ecology of adult individuals	IUCN Red List Status Account	Geographical distribution	Number of studies on larvae / on post-metamorphic forms
Anura, Dicroglossidae	*Fejervarya vittigera*	unknown	lotic/lentic: pelagic, neustonic[4]	planktivorous-herbivorous[4]	terrestrial, aquatic, arboreal	LC	Philippines	0/1
Anura, Dicroglossidae	*Sphaerotheca pashchima*	unknown	lotic: benthic[5]	no data	terrestrial[5]	unknown	India	1/0
Anura, Hylidae	*Acris blanchardi*	unknown	lotic/lentic: benthic[6]	primarily herbivorous[6]	terrestrial, aquatic	LC	North America	3/1
Anura, Hylidae	*Dryophytes versicolor*	unknown	lotic/lentic	herbivorous - detritivorous	terrestrial, arboreal	LC	North America	3/0
Anura, Hylidae	*Hyla arborea*	XX/XY, observed TSD	lotic/lentic: benthic, pelagic	herbivorous - detritivorous	terrestrial, arboreal	LC	widespread in Europe, range extends north to Denmark and Sweden, east to Ukraine and southern part of Russia	5/0
Anura, Hylidae	*Hyla intermedia*	XX/XY	lotic/lentic: benthic, pelagic[7]	herbivorous - detritivorous[7]	terrestrial, arboreal	LC	Italy	2/0
Anura, Hyperoliidae	*Hyperolius argus*	unknown	lentic: benthic	primarily herbivorous	terrestrial, arboreal	LC	Southeast Africa	2/0
Anura, Hyperoliidae	*Hyperolius viridiflavus*	XX/XY	lentic/lotic: benthic[8]	primarily herbivorous[8]	terrestrial, arboreal	LC	West Africa	1/0
Anura, Limnodynastidae	*Limnodynastes peronii*	unknown	lentic: benthic[9]	primarily herbivorous[9]	terrestrial, aquatic, arboreal	LC	Eastern Australia	1/0
Anura, Limnodynastidae	*Limnodynastes tasmaniensis*	unknown	lentic: benthic[10]	primarily herbivorous[10]	terrestrial, aquatic, arboreal	LC	Australia	1/0
Anura, Pipidae	*Xenopus tropicalis*	ZWY	lentic: suspension feeder	primarily herbivorous	aquatic	LC	The range covers coastal areas and adjacent regions of West Africa	20/6
Anura, Pipidae	*Xenopus laevis*	ZW/ZZ	lentic: suspension feeder	primarily herbivorous	aquatic	LC	Sub-Saharan Africa (native range)	61/28
Anura, Ranidae	*Glandirana rugosa*	XX/XY or ZW/ZZ	lentic: suspension feeder[11]	primarily herbivorous[11]	terrestrial, aquatic	LC	Japan, Northern and Southwestern Korea, Northeastern China	4/0
Anura, Ranidae	*Indosylvirana caesari*	unknown	no data	no data	terrestrial, aquatic[12]	unknown	India	1/0
Anura, Ranidae	*Lithobates catesbeianus*	XX/XY, observed TSD	lentic: benthic	primarily herbivorous	terrestrial, aquatic	LC	North America; introduced: many parts of South America, Europe, Asia	4/0

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TABLE 1 (*Continued*) Nomenclature, systematic position, sex determination system (GSD – genetic sex determination, TSD – temperature-dependent sex determination) and ecological traits of species of amphibians in experimental studies on the impact of endocrine-disrupting compounds on sex and sex-related traits with external exposition to compound. Please note that some studies used more than one species.

Order, Family	Latin species name	Sex determination system	Ecology of larvae	Diet of larvae	Ecology of adult individuals	IUCN Red List Status Account	Geographical distribution	Number of studies on larvae / on post-metamorphic forms
Anura, Ranidae	*Lithobates clamitans*	XX/XY	lentic/lotic: benthic	herbivorous - detritivorous	terrestrial, aquatic	LC	North America	1/0
Anura, Ranidae	*Lithobates pipiens*	XX/XY	lentic: benthic	herbivorous - detritivorous	terrestrial, aquatic	LC	North America	11/1
Anura, Ranidae	*Lithobates sphenocephalus*	XX/XY	lentic: benthic	primarily herbivorous	terrestrial, aquatic	LC	North America	1/0
Anura, Ranidae	*Lithobates sylvaticus*	XX/XY, observed TSD	lentic: benthic	herbivorous - detritivorous; carnivorous	terrestrial, aquatic	LC	North America	7/1
Anura, Ranidae	*Pelophylax* kl. *esculentus*	unknown (hybridogenetic species; hemiclonal mode of reproduction)	lentic/lotic: benthic	primarily herbivorous	terrestrial, aquatic	LC	Widespread in Europe, range extends west to France, east to Russian Federation, north to Scandinavia, south to the Balkans. Introduced to Spain and United Kingdom	0/1
Anura, Ranidae	*Pelophylax nigromaculatus*	XX/XY	lentic/lotic: benthic	primarily herbivorous	terrestrial, aquatic	NT	East Asia, Southern part of Russian Federation	7/5
Anura, Ranidae	*Pelophylax perezi*	XX/XY	lentic/lotic: benthic	herbivorous - detritivorous	terrestrial, aquatic	LC	Iberian Peninsula, South of France	1/0
Anura, Ranidae	*Rana chensinensis*	unknown	lentic/lotic: benthic	herbivorous - detritivorous	terrestrial, aquatic, arboreal	LC	East Asia, south-east part of Russian Federation	1/2
Anura, Ranidae	*Rana dalmatina*	XX/XY, observed TSD	lentic: benthic[13]	herbivorous - detritivorous[13]	terrestrial, aquatic, arboreal	LC	Widespread in Europe, range extends north to the Sweden, Finland and Poland, west to the France, east to the Ukraine; introduced in Belgium.	3/0
Anura, Ranidae	*Rana temporaria*	XX/XY, observed TSD	lentic: benthic	herbivorous - detritivorous	terrestrial, aquatic, arboreal	LC	Widespread in Europe, range extends west to United Kingdom and Ireland, east to Russia, north to Scandinavia, south to Spain and Italy.	3/0
Anura, Rhacophoridae	*Polypedates cruciger*	unknown	lentic: benthic[14,15]	herbivorous, zooplanctivorous[14,15]	terrestrial, arboreal	LC	Sri Lanka	1/0
Urodela, Ambystomatidae	*Ambystoma tigrinum*	ZW/ZZ	lentic	carnivorous	fossorial, terrestrial, aquatic	LC	North America	1/0

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TABLE 1 (*Continued*) Nomenclature, systematic position, sex determination system (GSD – genetic sex determination, TSD – temperature-dependent sex determination) and ecological traits of species of amphibians in experimental studies on the impact of endocrine-disrupting compounds on sex and sex-related traits with external exposition to compound. Please note that some studies used more than one species.

Order, Family	Latin species name	Sex determination system	Ecology of larvae	Diet of larvae	Ecology of adult individuals	IUCN Red List Status Account	Geographical distribution	Number of studies on larvae / on post-metamorphic forms
Urodela, Cryptobranchidae	*Andrias davidianus*	ZW/ZZ, observed TSD	lotic	carnivorous	aquatic	CR	China, introduced: Taiwan	1/0
Urodela, Salamandridae	*Lissotriton boscai*	XX/XY	lentic/lotic	carnivorous	terrestrial, aquatic	LC	Portugal, Spain	0/1
Urodela, Salamandridae	*Lissotriton helveticus*	XX/XY	lentic/lotic	carnivorous	terrestrial, aquatic	LC	Western Europe, northern parts of Portugal and Spain	0/3
Urodela, Salamandridae	*Notophthalmus viridescens*	unknown	lentic/lotic	carnivorous	terrestrial, aquatic	LC	North America	0/1
Urodela, Salamandridae	*Pleurodeles waltl*	ZW/ZZ, observed TSD	lentic/lotic	carnivorous	terrestrial, aquatic	LC	Morocco, Portugal, Spain	2/0

Data on sex determination systems was sourced from the HerpSexDet database (Nemesházi and Bókony, 2023). Data on species ecology was primarily obtained from AmphibiaWeb (2024) and the AmphiBIO database (Oliveira et al., 2017). For specific cases, additional sources were used and are indicated as follows: [1]Duport Bru, 2020; [2]Traijitt et al. 2021; [3]Khan, 2018; [4]Salo and Solania, 2022; [5]Padhye et al. 2017; [6]Johnson and Christiansen, 1976; [7]Escoriza, 2014; [8]Viertel et al., 2007; [9]Barker et al., 1995; [10]Peterson and Boulton, 1999; [11]NatureServe Explorer, 2023; [12]Sayyed, 2015; [13]Ćirković et al., 2023; [14]Meegaskumbura et al. 2010; [15]Bowatte et al, 2013.

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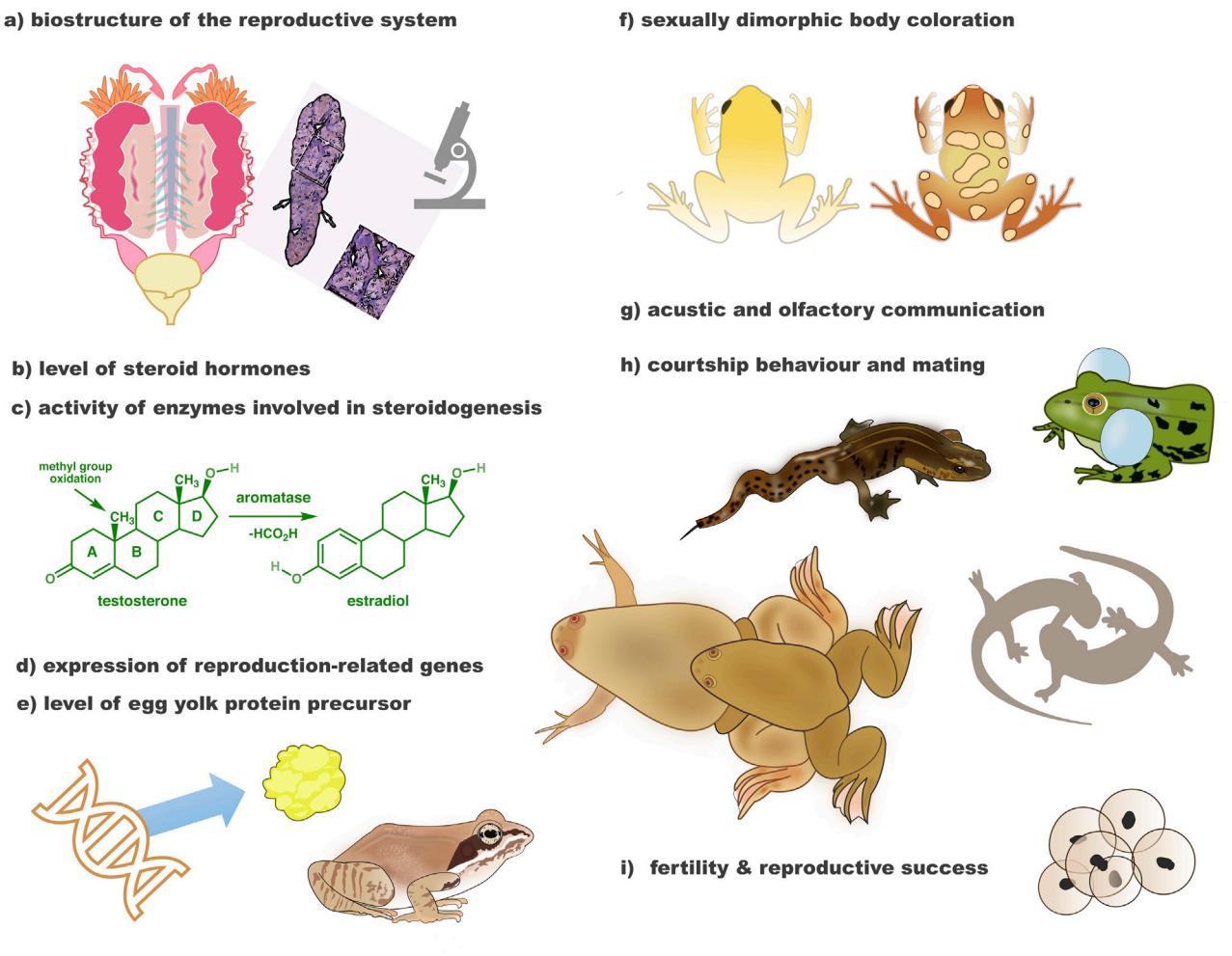

FIGURE 2
Endocrine-disrupting compounds affect many aspects of amphibian sex and reproduction. Among observed effects of EDCs in *in vivo* studies on amphibians were adverse impact on (morphology and/or histology) of the reproductive system **(a)**, level of sex steroid hormones in plasma and/or body organs **(b)**, activity of enzymes involved in steroidogenesis in liver and/or gonads **(c)**, expression of sexually-dimorphic and other reproduction-related genes in liver, gonads and/or brain **(d)**, level of egg yolk protein precursor–vitellogenin in liver and/or gonads **(e)**, as well as sexually dimorphic external traits **(f)**, sexual communication **(g, h)**, fertility, and reproductive success **(i)**.

reproductive system caused by EDCs, we use the nomenclature proposed by Lutz et al. (2008).

3 Results

We identified 241 experimental studies on the impact of EDCs on sex and sex-related traits of amphibians, from which 214 were *in vivo* studies. The majority of the experimental works were performed on developmental, aquatic forms of amphibians (see Table 1). In the Supplementary Information, detailed information about found experiments with waterborne exposition (90,19% of all *in vivo* studies) of amphibians to EDCs, including time of exposition, life stages, concentrations of EDCs, and the most significant results can be found (Supplementary Table S1). Among *in vivo* experiments, we identified only two articles in which the compound was ingested by animals (Tang et al., 2020; Ziková et al., 2013). In some, mostly older studies, amphibians were exposed to EDCs via injections or intraperitoneal implants (9,81% of all *in vivo* studies), and we consider them to be generally less environmentally relevant. Full lists of studies with these routes of exposition to EDCs (Supplementary Table S2) as well as *in vitro* and *ex vivo* studies (Supplementary Table S3), and species used in them, are included in the Supplementary Information (Supplementary Data Sheet S1). Moreover, in the Supplementary Information, we attach a table with abbreviations and IUPAC (International Union of Pure and Applied Chemistry) nomenclature of reviewed EDCs. (Supplementary Table S4). The most significant, recognized effects of EDCs on sex and sex-related traits of amphibians in the researched literature are presented in Figure 2.

We found that *in vivo* studies externally exposing amphibians to EDCs included 45 species (Table 1), constituting 0,52% of all species of amphibians known to date (31.12.2024). The two most frequently used species, from the same family Pipidae, account for almost half (49,78%) of all researched species (Figure 3).

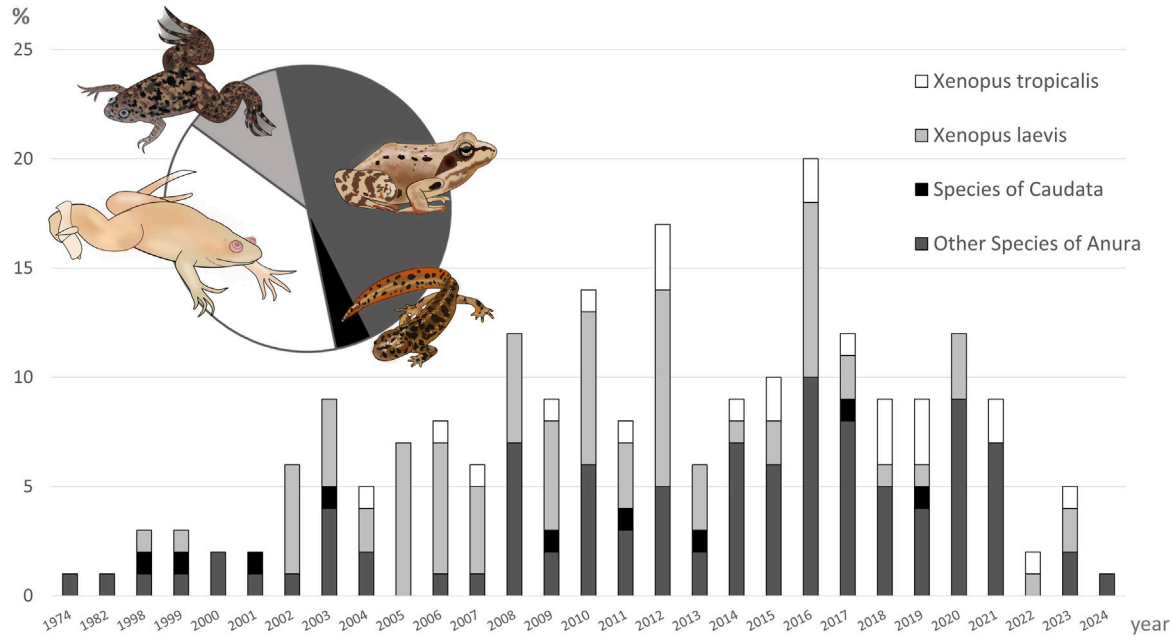

FIGURE 3
Annual trends in the number of amphibian taxa used in experimental studies on the impact of endocrine-disrupting compounds on sex and sex-related traits with the external exposition to compound. The y-axis represents the percentage of studies per year using particular taxa, while the x-axis the publication year (1974–2024). The pie chart illustrates the overall proportion of each taxonomic group used across all analyzed studies. Please note, that some studies used more than one species of amphibians.

4 Discussion: are model species the best choice?

4.1 The overreliance on *Xenopus* spp.

The African clawed frog *X. laevis* is still perceived as an ideal model for studying the effects of endocrine-disrupting compounds. It is due to its quick somatic and sexual development, the well-established knowledge about life stages, and available methods of genetic sex determination (Cong et al., 2006; Huang et al., 2005; Oka et al., 2006; Yoshimoto et al., 2008). It should be noted that *Xenopus laevis* is an evolutionarily conserved lineage, having diverged nearly 17.5 million years ago (Furman et al., 2015). While, for example, the American toad *Anaxyrus americanus* has existed for only 1.3 million years (Masta et al., 2002). The evolutionary conservation of *X. laevis* contributes to its utility as a model for human biology because it implies stability in its genetic and developmental pathways over millions of years. This stability makes it easier to study fundamental biological processes, such as cell division, gene regulation, and embryonic development, that are shared between humans and *X. laevis*. Its conserved nature ensures that findings in *X. laevis* are often applicable to humans, providing a reliable system for studying evolutionarily shared mechanisms (Blum and Ott, 2018; Gao and Shen, 2021; Nenni et al., 2019).

However, evolutionary conservation of *X. laevis* may limit its representativeness for amphibian biology. Its long divergence time means that *X. laevis* represents a unique and relatively ancient lineage compared to other amphibians, which may not well reflect the evolutionary and ecological traits of most of the contemporary species (Roelants et al., 2007). Another commonly used model closely related to *X. laevis* is the western clawed frog *Xenopus tropicalis*, which shares its advantages for toxicological research (Olmstead et al., 2010). Unfortunately, it does not add sufficient variance to account for responses to toxic substances (Cannatella, 2015; Robert and Cohen, 2011). Both species from the family Pipidae belong to the basal anurans (suborder Mesobatrachia), constituting less than 5% of currently known species (together with Archaeobatrachia), while most modern Anura belong to Neobatrachia. Therefore, the diversity of amphibians is still poorly represented in laboratory research (Wake and Koo, 2018).

The most commonly used species in ecotoxicology research are easy to obtain, common, and widely distributed (Schiesari et al., 2007), which is also evident in our study. Species bias in studies on endocrine disruptors may have significant consequences for the conservation of amphibians. The species most threatened by pollution with EDCs may be the ones difficult to obtain, living in areas with little interest and investment in ecotoxicological research (Schiesari et al., 2007). Another issue is the origin of animals used in the studies. Model species are usually obtained from laboratory populations with limited genetic variance and do not undergo natural selection like wild populations. They can also be a subject of unintentional selection for traits supporting survival in captivity conditions, of which researchers using such "domesticated" lines are not always aware. It has been shown that wild populations may develop tolerance to pollutants in the environment. Consequently, they can be less sensitive to the harmful effects of some toxicants than animals of the same species bred for generations in controlled, laboratory conditions (Romero-Blanco and Alonso, 2022).

4.2 Amphibians differ in sensitivity to EDCs

Results obtained from studies on non-model species often contradict those from studies on model ones. For example, significant differences in sensitivity of species to the effects of exogenous 17β-estradiol (E2) were found. E2 is widely present in surface waters, as it is excreted into the environment by humans and farm animals. In water-borne exposition studies, E2 was found to have a feminizing impact on morphology and histology of gonads in *X. laevis* (Hu et al., 2008; Lutz et al., 2008; Oka et al., 2006), the Indian skipper frog *Euphlyctis cyanophlyctis* (Phuge and Gramapurohit, 2015) and the Japanese wrinkled frog *G. rugosa* males (Ohtani et al., 2000; Oike et al., 2020). Moreover, exposition through the larval period to different concentrations of E2 caused female-biased to 100% female sex ratio in *X. laevis* (Bögi et al., 2002; Hu et al., 2008; Lutz et al., 2008; Oka et al., 2006), *E. cyanophlyctis* (Phuge and Gramapurohit, 2015), the common toad *Bufo bufo* (Petrini and Zaccanti, 1998), and *Glandirana rugosa* males (Ohtani et al., 2000; Oike et al., 2020), which suggested cases of sex reversal.

However, in the green frog *Lithobates clamitans,* injections with E2 for a long period did not significantly affect the sexual development of male and female tadpoles (Mintz et al., 1945). Also in the common reed frog *Hyperolius viridiflavus* exposition to E2 did not have any impact on gonadal morphology, even when a dose of E2 was very high (Richards, 1982), and in an outdoor study of the Blanchard's cricket frog *Acris blanchardi* E2 was found to have minimal effect on sexual development (Windle et al., 2021). Another study comparing the impact of E2 on the *X. laevis*, the European fire-bellied toad *Bombina bombina*, the yellow-bellied toad *Bombina variegata*, the European tree frog *Hyla arborea*, and the European green toad *Bufotes viridis*, found significant differences in the extent of induced changes within gonads of these species (Piprek et al., 2012).

More examples of differences in sensitivity to EDCs can be found in studies using a bacteriostatic compound triclosan (TSC), widely present in the environment as a residue from, e.g., personal care products. In the dark-spotted frog *Pelophylax nigromaculatus*, exposure to TSC during larval development caused a male-biased sex ratio, abnormalities within the gonads, and changed expression of reproduction-related genes (Chen et al., 2018). In comparison, TCS did not significantly impact sexual development and gonadal differentiation in *X. laevis* (Fort et al., 2017) or the expression of reproduction-related genes in adult *X. tropicalis* (Regnault et al., 2016).

Significant differences in sensitivity between species were also noted in other studies, exposing amphibians to a synthetic estrogen ethinyloestradiol (Tamschick et al., 2016b; Tamschick et al., 2016c), plasticizer bisphenol A (Tamschick et al., 2016a), nonylphenol, compound widely used in surfactants and detergents (Mackenzie et al., 2003) and a fungicide agent ethylenethiourea (Phuge et al., 2021).

Differences in sensitivity to EDCs between species could be largely due to various sex determination and differentiation mechanisms, as well as physiological and ecological differences, which we discuss below.

4.3 Differences in sex determination and differentiation in amphibians

The sexual determination system for most species of amphibians is still unknown. The limited data we have, however, show a huge variety of sex determination systems in this group of vertebrates (Nakamura, 2009). In this context, it becomes obvious that studying the effects of EDCs on only model species (such as *X. laevis* and *X. tropicalis*) is not enough to identify the true impact of these compounds on a huge variety of modern amphibian forms.

4.3.1 Genetic sex determination

Although it is generally accepted that the sex of amphibians is determined genetically, for most amphibian species, morphologically distinguishable sex chromosomes are not identified. Until now, the sex chromosome system has been recognized only for about 2% of the species (Bullejos et al., 2024). Data on sex-determining genes and gene regulatory networks involved in sexual differentiation are also limited (Nakamura, 2009; Ruiz-Garciá et al., 2021; Kuhl et al., 2024).

No universal sex-specific loci in amphibians were found, and at this moment, genetic determination of sex is possible only for a few species (Olmstead et al., 2010; Yoshimoto et al., 2008). It is particularly striking when considering the diversity of this taxonomic group. To date (31.12.2024), 8,816 amphibian species have been identified, and new species are discovered every year AmphibiaWeb (2024).

However, even this limited knowledge about genetic sex determination systems in amphibians shows a huge variability and plasticity within the group (Dufresnes and Crochet, 2022; Ma and Veltsos, 2021). The male (XX/XY) or female (ZZ/ZW) heterogametes in amphibian species can be found, as well as novel systems of chromosomal sex determination. Examples are the 00/W0 sex chromosome system in the Hochstetter's frog *Leiopelma hochstetteri* (Green, 1988), or the multiple sex chromosomes (=XXAA~/XXAY ~ type) system in the Puerto Cabello robber frog *Strabomantis biporcatus* (Schmid et al., 1992). Notably, in some phylogenetic lines of amphibians, the events of switching chromosomal sex determination systems occurred (Evans et al., 2012).

Variations of sex chromosomes have been identified even within a single species. *Xenopus tropicalis* is an interesting example, as in this species, three homomorphic sex chromosomes (Y > W > Z) were identified. As a result, males of *X. tropicalis* can possess ZZ, YZ, or YW chromosome combinations, while in females, ZW or WW chromosome sets can be found. Additionally, both sexes can be either heterogametic or homogametic. It should be taken into account in the studies on EDCs, as depending on the chromosome sets and types of gametes of parents, the sex ratio obtained in the offspring may differ from the conventionally assumed 1:1 (Furman et al., 2020; Roco et al., 2015). Another, somewhat extreme example, is the Japanese wrinkled frog *G. rugosa* the 6 geographic variants, with both XX/XY and ZZ/ZW sex chromosome systems, were found (Miura, 2007; Ogata et al., 2003; Oike et al., 2017; Mawaribuchi et al., 2023).

There are pieces of evidence that the sex determination system may impact the sensitivity of amphibians to hormonal compounds. *Glandirana rugosa* frogs from populations with novel chromosome sex determination systems were found to have lower sensitivity to hormonally induced gonadal sex reversal and the associated gene expression pattern (Miura et al., 2016). In theory, they could be less prone to the impact of EDCs. Thus, switching sex determination systems can have serious consequences for the survival of amphibian

populations. According to the theory of "asymmetrical sex reversal", homogametic sexes are more prone to phenotypical sex reversal and require weaker stimuli to undergo it (Nemesházi and Bókony, 2022).

4.3.2 Impact of steroidal sex hormones

Steroidal hormones play a crucial role in sex determination in amphibians (Hayes, 1998; Nakamura, 2013; Zaborski, 1986). They are also involved in the development and maintenance of sex accessory structures and secondary sexual characteristics in these animals (Harvey and Propper, 1997; Iwasawa and Kobayashi, 1974; Norris, 2011a). EDCs mimic steroidal hormone effects in sex determination, making amphibians extremely sensitive to these compounds. However, the role of steroidal hormones in sex determination and differentiation varies among species (Mali and Gramapurohit, 2016), which can be another reason for a species-specific sensitivity to the endocrine-disrupting compounds in the environment.

4.3.3 Impact of temperature on the sex of amphibians

The available evidence suggests that no amphibian species has exclusively temperature-dependent sex determination, although some of them retain sensitivity to it (Hayes, 1998; Nakamura, 2009; Ruiz-Garciá et al., 2021). Examples are some Urodela species: the Edough ribbed newt *Pleurodeles poireti* (Dournon et al., 1984), the iberian ribbed newt *Pleurodeles waltl* (Dournon et al., 1990; Zaborski, 1986), as well as Anura: the wood frog *Lithobates sylvaticus* (Lambert et al., 2018), the Dybowski's frog *Rana dybowskii* (Xu et al., 2022), the agile frog *Rana dalmatina* and the chinese edible frog *Hoplobatrachus rugulosus* (Mikó et al., 2021; Tang et al., 2020). For such species, outcomes of exposure to EDCs may differ depending on the interacting temperature. In the environment, amphibians are simultaneously subjected to many factors that can shape their phenotypical sex. In this context, the potential role of temperature in sex determination should not be overlooked.

4.3.4 Type of gonadal differentiation

In addition to a huge variety of sex determination systems, amphibian species differ in patterns of gonadal differentiation and rate of sexual development. It may significantly impact their sensitivity to endocrine disruptors, the time frame in which EDCs can affect their phenotypical sex, and the outcome of exposure to EDCs (e.g., feminization, masculinization, or none).

In amphibians can be found bipotential gonads. The differentiation of them may be categorized into three types. In most species, bipotential gonads develop directly into ovaries or testes (differentiated type). In some anuran species, however, the timing of differentiation of gonads in males and females is different, with testes developing from bipotential gonads much later than ovaries (semi-differentiated type). In this type of differentiation, some authors mistakenly recognize bipotential gonads in males as ovaries (explained by Ogielska, 2009). In rare cases, another type of gonadal differentiation can be found, in which in genetic males and females bipotential gonads first develop into ovaries, and then, in males, transform into testis (undifferentiated type) (Gramapurohit et al., 2000; Ogielska, 2009; Tanimura and Iwasawa, 1989).

Importantly, even populations of the same species may differ in the timing/type of gonadal differentiation (differentiated vs. semi-differentiated type) (Rodrigues et al., 2015). Studies on Urodela suggest that they undergo direct differentiation from bipotential gonad into ovary or testis (Dumond et al., 2008), although knowledge on gonadal differentiation in this group of amphibians is scarce.

4.3.5 Rate of gonadal differentiation

Amphibians also differ in the timing and rate of gonadal development and differentiation (for the review see Ogielska and Kotusz, 2004), with some having extremely slow gonadal differentiation in comparison to others (Piazza et al., 2023). The effects of EDCs on sex and sex-related traits may strongly depend on the type and timing of gonadal differentiation in species. *Xenopus laevis*, with direct and synchronized differentiation of bipotential gonad into ovaries or testis, may not be universally representative of the whole group of amphibians.

Data from experimental studies show that species with short ovarian development time, e.g., the Southern leopard frog *Lithobates sphenocephalus* (Storrs and Semlitsch, 2008), are more susceptible to the impact of EDCs than others. Species with quick sexual differentiation during the aquatic phase of life can be more prone to adverse effects of EDCs in surrounding water than species with slow sexual differentiation, extended to the post-metamorphic, terrestrial life stages. Worth noting, temperature can also have a significant impact on the rate of gonadal differentiation (Burraco et al., 2023).

4.3.6 Mixed-sex amphibians: norm in some species?

In many studies in which amphibians were exposed to EDCs, the presence of mixed-sex gonads (which contain both ovarian and testicular tissue) (Howe et al., 2004), intersex gonads (both testes and ovaries as separate structures in one individual) (Hayes et al., 2002), testicular oocytes (oocytes within testicular tissue) (Jeganathan and Rajakaruna, 2021) or completely sex reversed individuals (e.g., genetic males with regular, female gonads) was observed (Oka et al., 2006). However, there is a question: To what extent are these gonadal changes a natural phenomenon?

For example, mixed-sex individuals and testicular oocytes were observed in both experimental and control groups in several studies regarding impact of pesticide atrazine (Brande-Lavridsen et al., 2008; Coady et al., 2004; Jooste et al., 2005; Orton et al., 2006; Preez et al., 2008; Storrs-Méndez and Semlitsch, 2010) or EE2 (Brande-Lavridsen et al., 2008). Existences of testicular oocytes, mixed-sex or sex-reversed individuals in amphibian populations come also from field studies (Alho et al., 2010; Bókony et al., 2021; Lambert et al., 2019; Nemesházi et al., 2020; Preez et al., 2009; Reeder et al., 1998; Xu et al., 2022). Especially testicular oocytes, in many studies regarded as a pathology, may, in fact, be a natural phenomenon in some species or populations (Preez et al., 2009; Storrs-Méndez and Semlitsch, 2010). In some cases, observations of abnormal gonads in control groups could be, however, due to mistakes in experimental design (Hayes, 2005).

Some studies suggest that mixed-sex or intersex stages of gonads may be expected for some species (with semi-differentiated or undifferentiated type of gonadal development). Especially for toads (Bufonidae), not all cases of mixed-sex or intersex individuals should be perceived as anomalies. In Bufonidae rudimental hermaphroditism can be found. Adult males have Bidder's organs–undeveloped ovaries - located anterior to the

testes, in which oogenesis is still active, but oocytes do not reach maturity or are reduced. The exact function of the Bidder's organ is unknown (Petrini and Zaccanti, 1998). Additionally, differentiation of the bipotential gonads into testes in toads is delayed (retarded type of differentiation) (Storrs-Méndez and Semlitsch, 2010). As we underlined in the above section, *Differences in sex determination and differentiation in amphibians*, to accurately identify mixed-sex and intersex individuals, comprehensive knowledge of gonadal development, timing, and subsequent stages in a particular species is crucial.

4.4 Ecological differences

4.4.1 Aquatic vs. biphasic species

Apart from genetic differences between taxa, species also differ in ecology (Table 1), which can impact the main routes of exposure to EDCs in the environment. Most of our knowledge about the impact of EDCs on amphibians comes from studies with aqueous exposure (Slaby et al., 2019). The widely used standardized amphibian toxicity test, FETAX, using *X. laevis*, is based on exposure via water (Testing and Materials, 1998). However, the water environment may not be the primary route of exposure to pollutants for most species of amphibians (Brühl et al., 2013; Purucker et al., 2023). *Xenopus* spp., as fully aquatic species, do not represent the majority of amphibians with biphasic life cycles, which transition between aquatic and terrestrial habitats.

This transition exposes animals to additional stressors, such as pesticide contamination on land, different temperatures, and greater exposure to UV light (Nolan et al., 2023). Moreover, biphasic amphibians by bioaccumulation during the aquatic stage can transfer pollutants such as microplastics from the aquatic environment to the terrestrial one, thus contributing to the dissemination of them in the ecosystem (Park and Kidd, 2005; Szkudlarek et al., 2023). While the *Xenopus spp*. remain a convenient laboratory organism, its life history fails to capture the complexity of biphasic species' exposure to environmental stressors and processes occurring during the transition from the aquatic stage to the terrestrial one. To obtain a more accurate understanding of the impact of EDCs on amphibians, ecotoxicology studies must incorporate biphasic species (Nolan et al., 2023).

4.4.2 Terrestrial exposure

Terrestrial stage of life may be particularly problematic for amphibians living in agricultural areas, where they are exposed to significant quantities of endocrine-disrupting pesticides and fertilizers via contact with polluted soil and overspraying (Brühl et al., 2011; Churko et al., 2024; Cruz-Santiago et al., 2023; Fryday and Thompson, 2017). Pesticides, such as atrazine, may be absorbed by the highly permeable skin of amphibians, especially on the ventral side, and accumulate in the body (Purucker et al., 2023; Quaranta et al., 2009). This has been confirmed in the studies on, e.g., *Anaxyrus americanus* (Méndez et al., 2009), the juvenile barking tree frog *Dryophytes gratiosus,* and the southern leopard frog *Lithobates sphenocephala* (Belden et al., 2010).

However, species differ in their risk of exposure due to behavioral and ecological traits (Lenhardt et al., 2015). Nocturnal or fossorial species that remain underground during the day may avoid direct contact with contaminated surfaces, unlike diurnal, surface-active species (Adams et al., 2021; Belden et al., 2010).

Similarly, habitat use—particularly the proportion of time spent in terrestrial versus aquatic environments—modulates the likelihood of EDC exposure (Swanson et al., 2018). In this context, an interesting question is how the risk of exposure to EDCs differs between species with various overwintering strategies of adult forms. For example, species wintering in polluted water bodies in bottom sediment, where EDCs are accumulated (Fuentes et al., 2014; Swanson et al., 2018), are potentially more prone to exposure to EDCs than the species wintering on land.

4.4.3 Skin structure and physiology

Species ecology is closely tied to interspecific differences in skin structure and physiology, which influence both exposure to, and sensitivity to EDCs. Amphibian skin is not only the principal organ for respiration and osmoregulation but also the main route of chemical uptake from the environment (Quaranta et al., 2009). It varies in permeability, keratinization, glandular structure, and lipid content depending on species, developmental stage, and habitat (Hillyard et al., 2008). For example, toads living in arid regions have thicker, more keratinized skin to reduce water loss (Akat et al., 2022), which may also limit absorption of waterborne or soil-based pollutants.

In aquatic larvae, the skin is typically thin and highly vascularized, optimized for cutaneous respiration but also highly vulnerable to aqueous contaminants (Duellman and Trueb, 1994; Fox, 1977). As in some species larvae metamorphose into terrestrial juveniles or adults, keratinization increases to prevent desiccation—yet dermal absorption, especially of lipophilic compounds, remains a key exposure route (Akat et al., 2022). These physiological traits, coupled with their dual aquatic–terrestrial lifestyle, make amphibians sensitive bioindicators of environmental contamination (Kloas et al., 2006), but also demand a broader, more species-diverse approach in toxicological research.

Additionally, it is important to note that some EDCs are airborne (Annamalai and Namasivayam, 2015) and may reach amphibians through less-studied routes such as inhalation or direct dermal absorption from the atmosphere. This pathway remains largely unexplored in amphibians and its potential significance should not be overlooked. To our knowledge, the inhalation or dermal absorption of airborne EDCs and their effects have not yet been studied in amphibians, representing a critical gap in ecotoxicological research.

4.4.4 Feeding strategies

Additionally, it is worth considering the ecological and physiological differences in relation to feeding strategies. Notably, *Xenopus* spp. tadpoles are highly specialized filter feeders, primarily consuming suspended organic particles and zooplankton from the water column (Wassersug, 1996). In contrast, the majority of anuran tadpoles feed by grazing on biofilms and scraping detritus or algae from submerged surfaces using keratinized mouthparts (McDiarmid and Altig, 2000). The key differences in larval feeding strategies are summarized in Table 1.

These differences in feeding modes can substantially affect the route and intensity of EDCs exposure, as shown in fish (Fan et al., 2019; Müller et al., 2020). Filter-feeding larvae may be particularly vulnerable to dissolved or particle-bound EDCs suspended in the water, while surface-grazing tadpoles may ingest pollutants accumulated in periphyton or sediment layers. To our best knowledge, this hypothesis has not been tested yet.

Similar considerations apply to adult amphibians. Species feeding on aquatic invertebrates may remain more vulnerable to waterborne or sediment-associated pollutants (Veseli et al., 2022) whereas terrestrial-feeding species may primarily encounter EDCs present in, e.g., soil-dwelling invertebrates (Kwak and An, 2021). Moreover, trophic level may influence susceptibility: animals feeding on vertebrates or high trophic-level invertebrates may experience biomagnification of certain lipophilic EDCs (Mizukawa et al., 2009; Previšić et al., 2021; Ruhí et al., 2016).

Altogether, both the habitat and dietary preferences of amphibians—during larval and adult stages—contribute to interspecific variation in EDCs exposure and likely influence also physiological susceptibility to chemicals. These ecological and behavioral factors should be taken into account when assessing the environmental risks posed by EDCs across different species.

4.5 Species with unique life history traits

Among anuran amphibians, unique species with direct development, without an intermediate, morphologically distinct, free-living larval phase can be found (Callery et al., 2001). These direct developers, e.g., the Puerto Rican tree frog, *Eleutherodactylus coqui*, still undergo a thyroid hormone-dependent, rapid metamorphosis before hatching. It is interesting how EDCs present in the environment can influence the somatic and sexual development of such species. Are they less or more prone to the effects of endocrine disruptors?

The risk and consequences of exposure to EDCs are also unknown for Caecilians. Only about 5% of all described species of Caecilians have been studied in the topic of reproductive biology (Gomes et al., 2012). Very little is known about sex determination and gonadal development (Serrano-Perez and Ramírez-Pinilla, 2021), and almost nothing about the hormonal system in Caecilians (Brun et al., 2023; Norris, 2011b). Based on the study of two species, the Tenmalai caecilian *Gegeneophis ramaswamii* and *Gegeneophis* sp. (unspecified species), it is stated that Caecilians represent male heterogamety (XX/XY) (Govindappa and Venkatachalaiah, 2005). Because of the fossoriality of these animals, it is hard to observe them in nature and study and identify the environmental risks they are prone to.

Interestingly, some species of Caecilians and Urodela are ovoviviparous, or viviparous, with "pregnancy" controlled by hormones (Exbrayat, 1992). We already know that for many hydrophobic EDCs, the primary exposure route may be via maternal transfer (Kadokami et al., 2004; Liu et al., 2020). Field studies suggest that some EDCs can be transferred to eggs in oviparous amphibians (Du et al., 2019; Guan et al., 2024; Hopkins et al., 2006; Kadokami et al., 2004; Wu et al., 2009). However, to the best of our knowledge, there is currently no data on the impact of EDC on embryos in ovoviviparous and viviparous amphibians. Some clues may come from the study on reptiles (Squamata), where gravid females of northern watersnakes *Nerodia sipedon*, exposure to atrazine by ingestion altered the sex ratio of offspring, skewing it toward males (Neuman-Lee et al., 2014).

Worth noting, in all known oviparous species of Caecilians, as well as some species of Urodela, such as *Pseudoeurycea juarezi*, and Anura, such as the Rosenberg's treefrog *Boana rosenbergi*, parental care of offspring can be found (Gomes et al., 2012). It can be suspected that all these processes are, to some extent, controlled by reproductive hormones and possibly can be disrupted by external disruptors.

4.6 Differences in study design

The ecological characteristics of species significantly shape the optimal design of experimental setups to deliver truly environmentally relevant data. In *in vivo* waterborne exposure experiments, methodologies vary, including the flow-through water system, which maintains relatively stable concentrations of contaminants and effectively removes their metabolites or degradation products (Pickford et al., 2003). In contrast, the static renewal system allows for the accumulation of these substances within the water tank, potentially influencing experimental outcomes, especially if these by-products possess endocrine-disrupting properties (Hayes et al., 2003). Flow-through systems may more accurately mimic the environmental conditions of species that inhabit flowing waters. However, this setup might not be entirely suitable for species residing in stagnant water bodies, for example, ephemeral ponds, like puddles or phytotelmata. Moreover, natural aquatic environments often contain sediments and organic matter that can bind significant amounts of EDCs and their metabolites, thereby reducing their concentration in the water, which suggests that the real exposure levels to EDCs for amphibians in wild conditions could differ from those in controlled settings (Fuentes et al., 2014). Worth noting, the impact of the presence of sediments in water tanks may vary between amphibian species with benthic or pelagic specialized larvae (Table 1).

For investigating the complicated interactions between contaminants, their metabolic and degradation by-products, and various environmental factors, outdoor mesocosms offer a viable solution (Windle et al., 2021). Despite the challenges associated with their use in ecotoxicological studies, mesocosms provide conditions that closely resemble those in natural static water bodies. They incorporate multiple ecosystem components and account for additional stressors such as temperature (Tang et al., 2020), UV light (Croteau et al., 2009), pathogens (Paetow et al., 2023; Salla et al., 2024b) or predators (LaFiandra et al., 2008). Even more realistic conditions than mesocosms offer experimental lakes (Park and Kidd, 2005), which are, though, not a widely available solution.

5 Summary

In conclusion, amphibians are notably vulnerable to endocrine-disrupting compounds (EDCs), which pose a significant threat to their

survival and highlight their utility as sensitive bioindicators of environmental pollution. Almost half of the EDCs studies focus on just two amphibian model species, failing to adequately represent the group's diversity. This is particularly true for Caecilians and Urodela, where data on EDC impacts are scarce.

As shown in the previous study of Schiesari et al. (2007), there is a significant biogeographic bias in the general field of ecotoxicology of amphibians. The vast majority of research in this field uses few, common species with the least-concern threat status, primarily found in temperate zones of the northern hemisphere (Table 1). This bias may be due to the concentration of ecotoxicological researchers in these regions and the reliance on traditional, generic ecological risk-assessment methodologies. It should be noted, however, that our review took into account only publications written in English, which could potentially create a research bias (Chowdhury et al., 2022; Konno et al., 2020).

Nevertheless, our review underlines the need to recognize the natural diversity within amphibians in ecotoxicological studies. Alternative model species, representative of diverse taxonomic groups, should be considered (Salla et al., 2024b; Wagner and Viertel, 2017). Whenever possible, the effects of EDCs should be examined on regionally occurring species, under conditions that closely mimic their natural habitats. In studies on native species, non-invasive methods of sex identification and assessing endocrine disruptions are particularly useful (Orton et al., 2023) but still require development. Studies using non-model species in complex experimental setups may be challenging. However, intentional variations in ecotoxicological experiments may help gain a realistic view of the EDC's impact on amphibians in nature (Mikó et al., 2017; Romero-Blanco and Alonso, 2022) and contribute to their protection.

Author contributions

MF: Conceptualization, Investigation, Visualization, Writing – original draft, Writing – review and editing. MK: Conceptualization, Writing – review and editing. KS: Supervision, Writing – review and editing. PT: Conceptualization, Methodology, Supervision, Writing – review and editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Generative AI statement

The author(s) declare that Generative AI was used in the creation of this manuscript. Language editing assistance was provided using OpenAI's ChatGPT-4. No AI-generated content or ideas were included in the manuscript.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fenvs.2025.1556788/full#supplementary-material

SUPPLEMENTARY TABLE S1
Summary of the *in vivo* studies on the impact of endocrine-disrupting compounds on sex and sex-related traits in amphibians with waterborne exposition, including the type of compound, species studied, developmental stage, time of exposition and the most significant results.

SUPPLEMENTARY TABLE S2
Summary of the *in vivo* studies on the impact of endocrine-disrupting compounds on sex and sex-related traits of amphibians with alternative routes of exposition.

SUPPLEMENTARY TABLE S3
Summary of the *in vitro* and *ex vivo* studies on the impact of endocrine-disrupting compounds on sex and sex-related traits of amphibians.

SUPPLEMENTARY TABLE S4
Nomenclature of all chemical compounds and commercial products mentioned in the manuscript and Supplementary Information.

SUPPLEMENTARY TABLE S5
Nomenclature and taxonomy of all species of amphibians mentioned in the manuscript and in the Supplementary Information.

SUPPLEMENTARY DATA SHEET S1
List of references cited only in the Supplementary Materials.

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REVIEW ARTICLE OPEN ACCESS

Chemical Interference: A Review on Endocrine Disruptors and Reproductive Communication in Amphibians

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Keywords: acoustic communication | agrochemicals | hormones | olfactory communication | pheromones | pollution

ABSTRACT

Amphibians are highly vulnerable to anthropogenic pollution, primarily due to their permeable skin and eggs, as well as their habitat preferences. Endocrine-disrupting compounds (EDCs), prevalent in aquatic environments and soil, pose a significant threat to their survival. While the physiological effects of EDCs on amphibians have been extensively studied, their impact on behavior remains relatively unexplored. This paper reviews the existing literature on the impact of EDCs on the mating behavior of amphibians, including disruptions in acoustic, olfactory, and visual communication. Although it has been shown that amphibian reproduction can be affected by endocrine disruptors, there are still significant research gaps. We performed an extensive review of the literature, which yielded only 27 eligible studies—21 of which tested the effects on mating communication and behavior, and only 6 examined the impact on body coloration. There is a strong need for a deeper understanding of how EDCs, both alone and in combination with other stressors, affect the reproductive behavior of amphibians, as this may have serious implications for the dynamics and survival of entire populations and species.

1 | Introduction

Amphibians are a group of vertebrates particularly sensitive to anthropogenic changes, which threaten their survival globally (Luedtke et al. 2023). They are highly susceptible to contaminants due to their permeable eggs and skin, as well as their habitat preferences. Most species develop in aquatic environments (Wells 2008), whereas adult individuals often reside in areas prone to anthropogenic pollution such as agricultural lands (Băncilă et al. 2023; Brühl et al. 2011, 2013), wetlands (Sievers, Hale, Parris, et al. 2019) and forests (Chen et al. 2004; Lambert and Skelly 2016). Numerous contaminants found in these habitats, including pesticides, fertilizers, heavy metals, and plasticizers, are recognized as endocrine-disrupting compounds (EDCs) and are known to impact amphibian growth, development, and survival (Baker et al. 2013; Bókony et al. 2018; Goto et al. 2006; Lefcort et al. 1998; Papoulias et al. 2013; Wojtaszek et al. 2004). Additionally, the food base of amphibians in these areas, which often includes invertebrates and small vertebrates, can be a source of EDCs (González-Alcaraz et al. 2020; Markman et al. 2007).

The impact of EDCs on amphibian physiology has already been extensively studied (Gyllenhammar 2008; Hayes et al. 2002; Jagnytsch et al. 2006; Kloas 2002; Tamschick, Rozenblut-Kościsty, Ogielska, Kekenj, et al. 2016; Tamschick, Rozenblut-Kościsty, Ogielska, Lehmann, et al. 2016). A significant number of studies focus on the impact of EDCs on the reproductive

system of amphibians, in particular the anatomy and histology of gonads (for the review, see Frątczak et al. 2025). Among the most striking are cases of mixed-sex conditions or complete sex reversal (e.g., Cai et al. 2020; Chardard et al. 2003; Oka et al. 2006; Tamschick, Rozenblut-Kościsty, Ogielska, Kekenj, et al. 2016; Tamschick, Rozenblut-Kościsty, Ogielska, Lehmann, et al. 2016). However, the impact of EDCs on amphibian behavior—especially that related to communication, mating, and reproduction—remains far less explored (Sievers, Hale, Parris, et al. 2019; Orton et al. 2023).

In many amphibian species, reproductive behaviors rely heavily on communication between mates, involving a series of hormonally regulated actions such as mate location, courtship, and mating. These behaviors are often sexually dimorphic and mediated through acoustic, olfactory, and visual signals, depending on the species and ecological context (Sever and Staub 2011; Woodley 2011). For example, males of most anuran species produce advertisement calls to attract females and to signal sexual readiness (Wells and Schwartz 2006), while in many Caudata, pheromonal cues play a key role in mate attraction and stimulation (Kikuyama et al. 2002). In some taxa, visual traits such as body coloration (Bell 2005; Rojas et al. 2023) or limb movements, e.g., arm or tail waving (Halliday 1990), may serve as supplementary courtship signals. These traits are under endocrine control and may be potentially disrupted by exposure to EDCs (Orton et al. 2023), affecting mating success and, ultimately, reproductive output. These effects can have profound consequences on the survival of the populations and whole species of amphibians.

Therefore, understanding how EDCs affect amphibian behavior is essential for recognizing their true ecological impact. Taking this into account, we review the effects of EDCs on amphibians, focusing on their impact on reproductive behavior through disruptions of acoustic, olfactory, and potentially visual communication. We aim to highlight the existing research gaps and encourage greater interest in this topic.

2 | Endocrine Disruptors and Their Behavioral Implications

EDCs are substances that mimic or interfere with the endogenous hormones of organisms (European Commission 1996). The majority of EDCs found in the environment are of anthropogenic origin and represent a diverse group of compounds, including pesticides, plasticizers, surfactants, pharmaceuticals, and residues from personal care products. EDCs have the capacity to bind to hormone receptors, particularly affecting estrogenic, androgenic, and thyroid signaling pathways (La Merrill et al. 2020). They have been shown to influence sexual characteristics across all vertebrate groups (Kloas and Lutz 2006) and are associated with numerous reproductive disorders in animals (Ghosh et al. 2022).

In recent years, there has been growing awareness that EDCs can also modify behavior across a wide range of species (Clotfelter et al. 2004). Exposure to EDCs has been linked to altered reproductive behaviors in mammals, including humans (Gore et al. 2019; López-Rodríguez et al. 2021; Patisaul and Adewale 2009; Rosenfeld 2015). Additionally, EDCs have been associated with altered stress responses and mood regulation (Hudecova et al. 2018; Quinnies et al. 2015), as well as various neurobehavioral disorders (de Cock et al. 2012; Kajta and Wójtowicz 2013; Miodovnik et al. 2011).

The impact of EDCs on reproductive behavior is especially well documented in fish. Numerous studies have shown that EDCs can alter courtship displays, mate choice, territorial aggression, and spawning behavior (Saaristo et al. 2019; Söffker and Tyler 2012; Tomkins et al. 2018). In addition to mating behaviors, EDC exposure can interfere with parental care, including nest building and egg fanning (Coulter et al. 2019; Saaristo et al. 2010; Bell 2005). Social dynamics, such as dominance hierarchies and territoriality, have also been shown to shift following EDC exposure, likely due to disrupted androgen or cortisol signaling (Coe et al. 2008; Filby et al. 2012). These behavioral disruptions underscore the multi-level effects of EDCs, which may compromise not only individual reproductive success but also broader population viability.

Although fish and amphibians differ in many respects, they share important features such as external fertilization in most species, environmentally dependent reproductive cycles, and hormone-regulated communication systems (Arcand-Hoy and Benson 1998). Thus, findings from fish studies may offer mechanistic insights into how EDCs could interfere with comparable processes in amphibians (Kloas et al. 2009; Norris and Carr 2013). Nonetheless, amphibians possess taxon-specific reproductive traits and behaviors, including acoustic signaling, pheromone-based mate attraction, and sexually dimorphic skin coloration, that require direct examination (Sever and Staub 2011). Understanding how EDCs affect these specialized signaling pathways is essential for predicting the ecological consequences of pollution on amphibian populations. This is especially important because amphibians are highly sensitive to EDCs and serve as valuable, early indicators of environmental contamination (Kloas and Lutz 2006).

3 | Materials and Methods

We extensively reviewed the studies published up to 01.01.2025 on the topic of EDCs and their impact on sexual signal production and reproductive behavior in amphibians. In the preliminary search, we included many other biological traits related to the sex and reproduction of amphibians.

We used two electronic databases for the search: Web of Science Core Collection and Google Scholar. In the database Web of Science Core Collection, the following Boolean search string was used: TS = (amphibia* OR frog* OR toad* OR newt* OR salamander* OR tadpole* OR caecilian* OR anura* OR caudata* OR urodela* OR apoda*) AND TI = (endocrine disruptor* OR endocrine* OR EDC* OR pollu* OR pharm* OR estro* OR *estra* OR andro* OR *estradiol* OR EE2* OR *gestagen* OR progesterone* OR levonorgestrel* OR diethylstilbestrol* OR aromatase inhibitor* OR flutamide* OR finasteride* OR *phenol* OR *paraben* OR *cide* OR *azine* OR methoxychlor* OR chlorpyrifos* OR organochlorine* OR *dichlorodiphenyl* OR DDD* OR DDE* OR DDT* OR azocyclotin* OR glyphosate* OR Roundup* OR butachlor* OR *phosphate* OR thiophanate*

OR triadimefon* OR *azole* OR vinclozolin* OR thiophanate* OR tamoxifen* OR pyrimethanil* OR tributyltin* OR triphenyltin* OR fludioxonil* OR metaxyl* OR MAXIM OR triclosan* OR fertilizer* OR nitrate* OR ammonium* OR heavy metal* OR arsenic* OR chromium* OR cadmium* OR lead* OR surfactant* OR detergent* OR plasticizer* OR flame retardant* OR TBBPA* OR TCBPA* OR *phenyl* OR PCB* OR PBDE* OR phthalate* OR styrene* OR *chlor* OR benzene* OR UV filter* OR camphor* OR 4-MBC* OR 3-BC* OR trenbolone* OR microcystin* OR MCLR* OR *acid* OR salt*) AND TI = (sex* OR gonad* OR test* OR ovar* OR hormone* OR vitellogenin OR reproduct* OR fertilit* OR oocyt* OR sperm* OR breeding* OR gene* OR behavior * OR pheromone* OR scent* OR nuptial pad* OR vocal* OR acoustic* OR signal* OR call* OR communic* OR courtship* OR mate* OR color* OR phenotype*).

A similar search strategy was used in Google Scholar. The keywords for the search were chosen based on a preliminary literature review. To identify potentially relevant studies, the titles and abstracts of the retrieved articles were screened for any connection to the topic.

From this pool, studies were selected based on full-text screening if they met the following inclusion criteria: (a) the study was experimental and involved controlled exposure of amphibians to EDCs and (b) it reported data on traits directly related to mate communication (e.g., calling behavior, pheromone production) or on traits with potential signaling function (e.g., vocal sac development, skin coloration). We focused solely on the English-language publications, which potentially may represent a limitation of our review (Chowdhury et al. 2022; Konno et al. 2020). The journal or date of publishing the paper was not limited. Unpublished results from Master's and PhD Dissertation papers were not included. Unless stated otherwise, all studies discussed in the review were conducted under laboratory conditions using waterborne exposure and adult individuals. When mentioning changes within the reproductive system caused by EDCs, we used the nomenclature proposed by Lutz et al. (2008).

4 | Results

The initial search in both Web of Science Core Collection and Google Scholar yielded over 3000 articles; the majority of which focused on EDC effects on gonadal development, hormone levels, or gene expression in amphibians. Among them, we identified only 27 eligible studies (Figure 1), from which 21 tested the effects on mating communication and behavior, and only 6 examined the impact on body coloration. This highlights how incredibly understudied this area remains, underscoring the urgent need for further research into how EDCs may influence amphibian behavior, particularly in the context of reproductive communication.

Studies, in which EDCs were found to disrupt acoustic and olfactory communication of amphibians, are summarized in Table 1, while studies on EDCs and body coloration are presented in Table 2. In the Supporting Information, a more detailed summary of all found studies, including studies in which compounds did not impact amphibians, with concentrations of EDCs calculated in different units, and additional results related

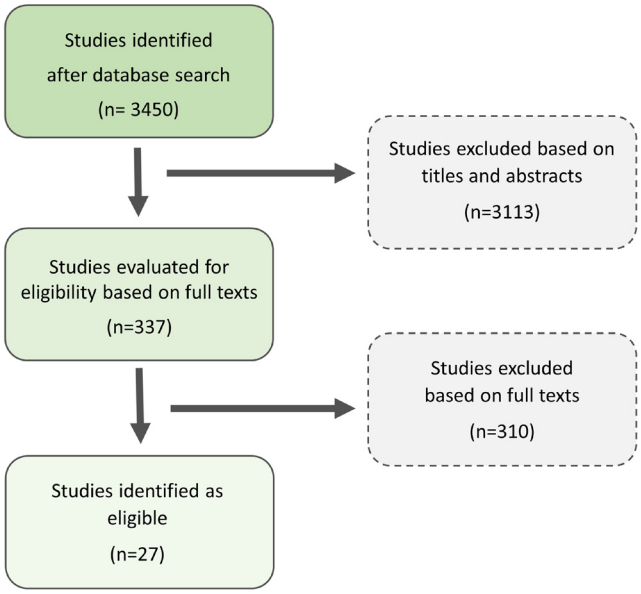

FIGURE 1 | Scheme of literature search approach based on two electronic databases for the search: Web of Science Core Collection and Google Scholar.

to other sex-related traits (Table S1) can be found. The names, categories, source, and environmental relevance of EDCs included in the review are presented in Table 3.

5 | Impact on Behavior by Disruption of Acoustic Communication

Hormones play a key role in the development and maintenance of secondary sexual characteristics and reproductive behaviors in amphibians, including sexual communication, partner receptivity, and mating (for reviews, see Sever and Staub 2011; Woodley 2011). These hormonally mediated traits may be susceptible to disruption by the presence of EDCs in the environment. In the majority of anuran species, acoustic communication is the primary mechanism used to locate and attract a mate and initiate reproduction (Colafrancesco and Gridi-Papp 2016; Wake and Koo 2018; Wells and Schwartz 2006). Research indicates that both the production and perception of acoustic signals in amphibians are modulated by hormones such as androgens, estrogens, gonadotropin-releasing hormone, and neuropeptides, including arginine vasotocin (for reviews, see Arch and Narins 2009; Woodley 2011). As a result, EDCs that interfere with these hormonal pathways may disrupt acoustic communication, ultimately impairing mate selection and breeding success.

The well-established model for studying the effects of EDCs on vocal signals in amphibians is the African clawed frog *Xenopus laevis* (Hall and Kelley 2021). Males of this species produce a variety of vocalizations, including ticking, rasping, croaking, and growling. Advertisement calls, which signal readiness to mate, consist primarily of ticking and croaking. Growling—a low-frequency, continuous vibration—is typically used to deter rival males, while rasping is thought to be associated with aggressive interactions or a reduced level of sexual arousal (Leininger and Kelley 2015).

TABLE 1 | Summary of studies and reported concentrations at which the compound affected mating behavior or related traits in amphibians.

Reference	Compound[a]	Species	Developmental stage[b]	Sex (if specified)	Time of exposure	C [mol/L][c]	Assessed traits and parameters	The most important results
Behrends et al. (2010)	Flutamide	*Xenopus laevis*	Adult	Male	3 days	1.00E-06	Calling activity	Decreased calling activity
Behrends et al. (2010)	Flutamide	*Xenopus laevis*	Adult	Male	3 days	1.00E-08	Calling activity	Decreased calling activity
Efosa et al. (2017)	Diclofenac	*Xenopus laevis*	Adult	Male	8 days	9.99E-08	Calling activity; characteristics of advertisement calls	Decreased number of advertisement calls
Hayes et al. (2010)	Atrazine	*Xenopus laevis*	Hatched tadpoles	Male	2–3 years post-metamorphosis	1.16E-08	Histology of larynx; morphology and histology of nuptial pads; number of successful copulations with females	Changed color of nuptial pads; changed histology of larynx; lower number of successful copulations
Hoffmann and Kloas (2010)	Vinclozolin	*Xenopus laevis*	Adult	Male	96 h	1.00E-06	Calling activity; characteristics of advertisement calls	Decreased calling activity; changed advertisement call characteristics (call indicating less aroused males)
Hoffmann and Kloas (2010)	Vinclozolin	*Xenopus laevis*	Adult	Male	96 h	1.00E-08	Calling activity; characteristics of advertisement calls	Decreased percentage of advertisement calls; changed call characteristics (call indicating less aroused males)
Hoffmann and Kloas (2010)	Vinclozolin	*Xenopus laevis*	Adult	Male	96 h	1.00E-10	Calling activity; characteristics of advertisement calls	Decreased percentage of advertisement calls; changed call characteristics (call indicating less aroused males)
Hoffmann and Kloas (2012a)	17α-ethinylestradiol	*Xenopus laevis*	Adult	Male	96 h	1.00E-12	Calling activity	Decreased percentage of advertisement calls; changed call characteristics (call indicating less aroused males)
Hoffmann and Kloas (2012a)	17α-ethinylestradiol	*Xenopus laevis*	Adult	Male	96 h	1.00E-11	Calling activity	Decreased percentage of advertisement calls; changed call characteristics (call indicating less aroused males)

(Continues)

TABLE 1 | (Continued)

Reference	Compound[a]	Species	Developmental stage[b]	Sex (if specified)	Time of exposure	C [mol/L][c]	Assessed traits and parameters	The most important results
Hoffmann and Kloas (2012a)	17α-ethinylestradiol	*Xenopus laevis*	Adult	Male	96 h	1.00E-10	Calling activity	Decreased percentage of advertisement calls; changed call characteristics (call indicating less aroused males)
Hoffmann and Kloas (2012a)	17α-ethinylestradiol	*Xenopus laevis*	Adult	Male	96 h	1.00E-08	Calling activity; female receptivity to the call (number of females expressing phonotaxis toward male call)	Decreased percentage of advertisement calls; changed call characteristics (call indicating less aroused males); fewer females expressing phonotaxis toward male call (lower attractiveness of males)
Hoffmann and Kloas (2012a)	17α-ethinylestradiol	*Xenopus laevis*	Adult	Male	96 h	1.00E-06	Number and characteristics of advertisement calls	Decreased percentage of advertisement calls; changed call characteristics (call indicating less aroused males)
Hoffmann and Kloas (2012b)	17α-ethinylestradiol	*Xenopus laevis*	Adult	Male	96 h	1.00E-10	Calling activity; characteristics of advertisement calls	Decreased percentage of advertisement calls; changed call characteristics (call indicating less aroused males)
Hoffmann and Kloas (2012c)	Levonorgestrel	*Xenopus laevis*	Adult	Male	96 h	1.00E-07	Calling activity; characteristics of advertisement calls	Increased percentage of advertisement calls; changed call characteristics (call indicating more sexually aroused males)
Hoffmann and Kloas (2012c)	Levonorgestrel	*Xenopus laevis*	Adult	Male	96 h	1.00E-08	Calling activity; characteristics of advertisement calls	Increased percentage of advertisement calls; changed call characteristics (call indicating more sexually aroused males)
Hoffmann and Kloas (2012c)	Levonorgestrel	*Xenopus laevis*	Adult	Male	96 h	1.00E-10	Calling activity; characteristics of advertisement calls	Increased percentage of advertisement calls; changed call characteristics (call indicating more aroused males)

(Continues)

TABLE 1 | (Continued)

Reference	Compound[a]	Species	Developmental stage[b]	Sex (if specified)	Time of exposure	C [mol/L][c]	Assessed traits and parameters	The most important results
Hoffmann and Kloas (2012d)	MDHT	Xenopus laevis	Adult		96 h	1.00E-07	Male calling activity; characteristics of advertisement calls; female receptivity to the calls of unexposed males (number of females expressing phonotactic behaviour toward male call, latency to respond to the call, time spent near the source of male call)	Increased percentage of advertisement calls; changed call characteristics (call indicating more aroused males)
Hoffmann and Kloas (2012d)	MDHT	*Xenopus laevis*	Adult		96 h	1.00E-05	Male calling activity; characteristics of advertisement calls; female receptivity to the calls of unexposed males (number of females expressing phonotactic behaviour toward male call, latency to respond to the call, time spent near the source of male call)	Increased percentage of advertisement calls; changed call characteristics (call indicating more aroused males); higher female receptivity to male calls (more females expressing phonotactic behaviour toward male call; quicker response to the male call)
Hoffmann and Kloas (2012d)	MDHT	*Xenopus laevis*	Adult		96 h	1.00E-04	Male calling activity; characteristics of advertisement calls; female receptivity to the calls of unexposed males (number of females expressing phonotactic behaviour toward male call, latency to respond to the call, time spent near the source of male call)	Increased percentage of advertisement calls; changed call characteristics (call indicating more aroused males)
Hoffmann and Kloas (2016)	17α-ethinylestradiol	*Xenopus laevis*	Adult	Male	4 days	1.00E-10	Calling activity; characteristics of advertisement calls	Decreased percentage of advertisement calls; changed call characteristics (call indicating less aroused males)

(Continues)

TABLE 1 | (Continued)

Reference	Compound[a]	Species	Developmental stage[b]	Sex (if specified)	Time of exposure	C [mol/L][c]	Assessed traits and parameters	The most important results
Hoffmann and Kloas (2016)	Vinclozolin	*Xenopus laevis*	Adult	Male	4 days	1.00E-10	Calling activity; characteristics of advertisement calls	Decreased percentage of advertisement calls; changed call characteristics (call indicating less aroused males)
Hoffmann and Kloas (2016)	p,p′DDE	*Xenopus laevis*	Adult	Male	4 days	1.00E-09	Calling activity; characteristics of advertisement calls	Changed advertisement call characteristics (call indicating less aroused males)
Hoffmann and Kloas (2016)	p,p′DDE	*Xenopus laevis*	Adult	Male	4 days	1.00E-11	Calling activity; characteristics of advertisement calls	Decreased percentage of advertisement calls; changed call characteristics (call indicating less aroused males)
Huang et al. (2015)	Cadmium	*Pelophylax nigromaculatus*	Adult	Male	60 days	1.00E-06	Characteristics of advertisement calls; number of males responding with advertisement call to female call; time to first movement toward female call	Changed advertisement call characteristics (longer calls, fewer croaks); fewer males responding with advertisement call to receptive female call; longer time to first movement toward female call
Huang et al. (2015)	Cadmium	*Pelophylax nigromaculatus*	Adult	Male	60 days	1.00E-07	Characteristics of advertisement calls; number of males responding with advertisement call to female call; time to first movement toward female call	Changed advertisement call characteristics (longer calls, fewer croaks); fewer males responding with advertisement call to receptive female call; longer time to first movement toward female call
Huang et al. (2015)	Cadmium	*Pelophylax nigromaculatus*	Adult	Male	60 days	1.00E-08	Characteristics of advertisement calls; number of males responding with advertisement call to female call; time to first movement toward female call	Changed call characteristics (initially decreased and then increased call latency; higher call rate); longer time to first movement toward female call
Li et al. (2021)	Octylphenol	*Rana chensinensis*	Adult	Male	30 days	1.00E-06	Mating behavior (number of pairings)	Decreased number of pairing

(Continues)

TABLE 1 | (Continued)

Reference	Compound[a]	Species	Developmental stage[b]	Sex (if specified)	Time of exposure	C [mol/L][c]	Assessed traits and parameters	The most important results
Park et al. (2001)	Endosulfan	*Notophthalmus viridescens*	Adult	Female	4 days	1.20E-08	Attractiveness of females (visual selection by males; olfactory selection by males); mating success	Less males attracted toward water scented by exposed females; lower mating success; changes in pheromone gland morphology
Park et al. (2001)	Endosulfan	*Notophthalmus viridescens*	Adult	Female	4 days	2.50E-08	Attractiveness of females (visual selection by males; olfactory selection by males); mating success	Lower mating success; changes in pheromone gland morphology
Schwendiman and Propper (2012)	17β estradiol	*Xenopus tropicalis*	Adult		30 days	1.00E-08	Number of calls of males; number of mating behavior events: approaching mate; touching mate; arm waves; amplexus events; number of males expressing sexual behavior	Increased number of calls and arm waves in males; more males expressing sexual behavior
Schwendiman and Propper (2012)	Octylphenol	*Xenopus tropicalis*	Adult		30 days	1.00E-07	Number of calls of males; number of mating behavior events: approaching mate; touching mate; arm waves; amplexus events; number of males expressing sexual behavior	Increased number of calls and arm waves in males; more males expressing sexual behavior
Schwendiman and Propper (2012)	Octylphenol	*Xenopus tropicalis*	Adult		30 days	1.00E-08	Number of calls of males; number of mating behavior events: approaching mate; touching mate; arm waves; amplexus events; number of males expressing sexual behavior	Increased number of calls in males; more males expressing sexual behavior

(Continues)

TABLE 1 | (Continued)

Reference	Compound[a]	Species	Developmental stage[b]	Sex (if specified)	Time of exposure	C [mol/L][c]	Assessed traits and parameters	The most important results
Secondi et al. (2009)	Sodium nitrate	*Lissotriton helveticus*	Adult	Male	10 days	0.882	Secondary sexual characteristics (tail height; hind-foot web area); attractiveness of males (visual selection by females; olfactory selection by females)	Fewer females attracted to water scented by exposed males
Secondi et al. (2013)	Sodium nitrate	*Lissotriton helveticus*	Adult	Male	10 days	0.882	Courtship behavior (occurrence of courtship behavior; number and duration of fanning; number of spermatophore deposition and transfer)	More males expressing courtship behavior
Zhang et al. (2022)	NAs	*Xenopus tropicalis*	Adult	Male	5 days	20 mg/L[d]	Calling activity; characteristics of advertisement calls	Decreased calling activity

[a]MDHT, methyldihydrotestosterone; NAs, naphthenic acids; p,p′DDE, dichlorodiphenyldichloroethylene.
[b]Gosner stage – developmental stage according to Gosner (1960); NF stage – developmental stage according to Nieuwkoop and Faber (2020).
[c]Calculated assuming dilute aqueous solution of density equal to the density of water in normal conditions (997 kg/m^3).
[d]Can't calculate concentration/provide molar mass, due to substance tested being a mixture of different compounds.

TABLE 2 | Summary of studies and concentrations at which the compound affected body coloration of amphibians.

Reference	Compound[a]	Species	Developmental stage[b]	Time of exposure	C [mol/L][c]	Assessed traits and parameters	The most important results
Greenberg (1942)	Testosterone	*Acris gryllus*	Juvenile	3 weeks	implants under the skin, unspecified dosage	Sexually dimorphic body coloration, presence of vocal sacs	Coloration characteristic for adult females in males and females
Greenberg (1942)	Testosterone	*Acris gryllus*	Juvenile	5 weeks	implants under the skin, unspecified dosage	Sexually dimorphic body coloration, presence of vocal sacs	Coloration characteristic of adult females in males and females
Greenberg (1942)	Testosterone	*Acris gryllus*	Juvenile	3 months	implants under the skin, unspecified dosage	Sexually dimorphic body coloration, presence of vocal sacs	Coloration characteristic of adult females in males and females
Hayes and Menendez (1999)	17β estradiol	*Hyperolius argus*	Gosner stage 21	Up to Gosner stage 46	3.67E-05	Body coloration; presence of vocal sacs	Coloration characteristic for adult females in 100% of individuals; no vocal sacs
Hayes and Menendez (1999)	17β estradiol	*Hyperolius argus*	Gosner stage 21	Up to Gosner stage 46	3.67E-04	Body coloration; presence of vocal sacs	Coloration characteristic for adult females in 100% of individuals; no vocal sacs
Noriega and Hayes (2000)	17β estradiol	*Hyperolius argus*	Within 24 h of forelimb emergence	20 days	2.80E-07	Sexually dimorphic body coloration	Premature body coloration in females
Noriega and Hayes (2000)	17β estradiol	*Hyperolius argus*	Within 24 h of forelimb emergence	20 days	3.67E-07	Sexually dimorphic body coloration	Premature body coloration in females
Noriega and Hayes (2000)	17β estradiol	*Hyperolius argus*	Within 24 h of forelimb emergence	20 days	3.67E-06	Sexually dimorphic body coloration	Premature body coloration in females

(Continues)

TABLE 2 | (Continued)

Reference	Compound[a]	Species	Developmental stage[b]	Time of exposure	C [mol/L][c]	Assessed traits and parameters	The most important results
Noriega and Hayes (2000)	o,p′DDT	*Hyperolius argus*	Within 24 h of forelimb emergence	20 days	2.80E-07	Sexually dimorphic body coloration	Premature body coloration in females
Noriega and Hayes (2000)	o,p′DDT	*Hyperolius argus*	Within 24 h of forelimb emergence	20 days	2.80E-06	Sexually dimorphic body coloration	Premature body coloration in females
Noriega and Hayes (2000)	o,p′DDE	*Hyperolius argus*	Within 24 h of forelimb emergence	20 days	2.80E-06	Sexually dimorphic body coloration	Premature body coloration in females
Noriega and Hayes (2000)	o,p′DDD	*Hyperolius argus*	Within 24 h of forelimb emergence	20 days	2.80E-06	Sexually dimorphic body coloration	Premature body coloration in females
Richards (1982)	Testosterone	*Hyperolius viridiflavus*	From the start of feeding	Up to the emergence of forelimbs; 1-min bath per day	1.73E-04	Sexually dimorphic body coloration	Coloration characteristic for adult females in males and females
Richards (1982)	17β estradiol	*Hyperolius viridiflavus*	From the start of feeding	Up to emergence of forelimbs; 1-min bath per day	1.83E-07	Sexually dimorphic body coloration	Coloration characteristic of adult females in males and females
Richards (1982)	17β estradiol	*Hyperolius viridiflavus*	From the start of feeding	Up to the emergence of forelimbs; 1-min bath per day	3.66E-07	Sexually dimorphic body coloration	Coloration characteristic of adult females in males and females
Richards (1982)	17β estradiol	*Hyperolius viridiflavus*	From the start of feeding	Up to emergence of forelimbs; 1-min bath per day	1.83E-06	Sexually dimorphic body coloration	Coloration characteristic of adult females in males and females
Richards (1982)	17β estradiol	*Hyperolius viridiflavus*	From the start of feeding	Up to the emergence of forelimbs; 1-min bath per day	3.66E-06	Sexually dimorphic body coloration	Coloration characteristic of adult females in males and females
Scaia et al. (2019)	Nonylphenol	*Lithobates catesbeianus*	Gosner stage 32-35	14 days	4.54E-06	Body coloration	Darker body pigmentation

(Continues)

TABLE 2 | (Continued)

Reference	Compound[a]	Species	Developmental stage[b]	Time of exposure	C [mol/L][c]	Assessed traits and parameters	The most important results
Scaia et al. (2019)	Nonylphenol	*Lithobates catesbeianus*	Gosner stage 32-35	14 days	4.54E-05	Body coloration	Darker body pigmentation
Scaia et al. (2019)	Nonylphenol	*Lithobates catesbeianus*	Gosner stage 32-35	14 days	4.54E-04	Body coloration	Darker body pigmentation
Ujhegyi and Bókony (2020)	Glyphogan Classic	*Bufo bufo*	Gosner stage 25	Up to Gosner stage 42	1.31E-05	Sexually dimorphic body coloration	3 mixed-sex individuals with changes in body coloration

[a]DDD, dichlorodiphenyldichloroethane; DDE, dichlorodiphenyldichloroethylene; DDT, dichlorodiphenyltrichloroethane.
[b]Gosner stage – developmental stage according to Gosner (1960); NF stage – developmental stage according to Nieuwkoop and Faber (2020).
[c]Calculated assuming dilute aqueous solution of density equal to the density of water in normal conditions (997 kg/m^3).

Studies on *X. laevis* have demonstrated that androgens are critical for the development of the male vocal apparatus, including the larynx, laryngeal muscles, and motoneurons. The size, structure, and innervation of the larynx, as well as the neural circuits responsible for call production in the brain, are sexually dimorphic in this species (Hall and Kelley 2021; Zornik and Yamaguchi 2008). It has been shown that EDCs can interfere with the normal development of this complex system when exposure occurs during early life stages, thereby disrupting acoustic communication (Carr et al. 2003). Notably, EDCs may also affect vocal signals in adult individuals. This is likely due to the fact that adult amphibians retain a certain degree of plasticity within their vocal system. For example, ovariectomized females of *X. laevis* exposed to testosterone (T) via implants for 13 weeks began producing advertisement calls typical for males. This modification of the call was linked to changes within the vocal apparatus (Potter et al. 2005).

5.1 | Disruption of Vocal Signals by Endocrine Disruptors: Estrogenic and Anti-Androgenic Compounds

Evidence confirming that estrogenic and anti-androgenic compounds commonly found in the environment can alter amphibian vocal behavior is provided by 10 studies (see Tables 1 and 3). In one of them, Behrends et al. (2010) demonstrated that brief waterborne exposure to the anti-androgen flutamide at a low concentration (1×10^{-8} M) significantly suppressed the mating calls of adult male *Xenopus laevis*. Similarly, exposure to the non-steroidal anti-inflammatory drug diclofenac (DCF)—which is known to interfere with estrogenic pathways—at an environmentally relevant concentration (1×10^{-7} M) resulted in a notable reduction of the vocal activity of *X. laevis* males (Efosa et al. 2017). Given that vocalizations in many anuran species play a key role in attracting mates, a reduction in call output could significantly impair male reproductive success in natural settings. However, this hypothesis has not yet been directly tested in the studies cited (Behrends et al. 2010; Efosa et al. 2017), and the ecological consequences of reduced vocal activity remain largely speculative.

It is also important to note that experimental studies investigating the effects of EDCs on amphibian vocalizations often involve methodological constraints that may influence outcomes. In all reviewed studies, males were pharmacologically stimulated with human chorionic gonadotropin (hCG) prior to chemical exposure (see Table S1). This technique is used to trigger vocal behavior in laboratory conditions, particularly outside the breeding season (Schmidt 1966). However, the use of hCG may not accurately reflect natural physiological states and could affect the animal's calling patterns or hormone sensitivity (Efosa et al. 2017).

This concern was illustrated in the study by Efosa et al. (2017), where male frogs exposed to diclofenac (DCF) without hCG pretreatment showed a somewhat different vocal response compared to hCG-stimulated males. They expressed not only lower vocal output but also vocalized a higher proportion of rasping, which can be interpreted as an additional signal of low sexual arousal (Efosa et al. 2017). These findings suggest that hormonal

TABLE 3 | Summary of the endocrine-disrupting compounds included in the review.

Common name	Group of compounds	Description and relevance	Reference
17α-ethinylestradiol (EE2)	Estrogens	Synthetic estrogen used in contraceptive and replacement therapy drugs, commonly found in the environment	Wise et al. (2010)
17β-estradiol (E2)	Estrogens	Natural hormone excreted by animals, present also in therapeutic drugs, commonly found in the environment	Wise et al. (2010)
Ammonium nitrate	Fertilizers	Ingredient of fertilizers, intensively used in agriculture, commonly found in the environment	Daam et al. (2020)
Atrazine (ATZ)	Herbicides	Herbicide intensively used in agriculture, commonly found in the environment	Rohr and McCoy (2010)
Cadmium (Cd)	Heavy metals	Used in e.g., Pesticides, nickel-cadmium batteries, color pigments, the wood industry, commonly found in the environment	Järup and Åkesson (2009)
Dichlorodiphenyldichloroethane (DDD)	Insecticides	Breakdown product of DDT, highly persistent and commonly found in the environment	Agency for Toxic Substances and Disease Registry (2024)
Dichlorodiphenyldichloroethylene (DDE)	Insecticides	Breakdown product of DDT, highly persistent and commonly found in the environment	Agency for Toxic Substances and Disease Registry (2024)
Dichlorodiphenyltrichloroethane (DDT)	Insecticides	Insecticide used in large quantities in agriculture and vector control in the past, highly persistent and commonly found in the environment	Agency for Toxic Substances and Disease Registry (2024)
Diclofenac (DCF)	Pharmaceuticals	Widely used anti-inflammatory drug, commonly found in the environment	Efosa et al. (2017)
Endosulfan	Insecticides	Insecticide intensively used in agriculture, commonly found in the environment	Silva and Gammon (2009)
Flutamide (FLU)	Antiandrogens	Therapeutic drug with little environmental relevance, sporadically can occur in wastewater	Behrends et al. (2010)
Fulvestrant	Antiestrogens	Therapeutic drug with little environmental relevance, sporadically can occur in wastewater	Parrella et al. (2014)
Levonorgestrel (LNG)	Gestagens	Synthetic analog of progesterone, used in contraceptive drugs, hormonal replacement and cancer therapies, commonly found in the environment	Oropesa and Guimarães (2021)
Methyldihydrotestosterone (MDHT)	Androgens	Synthetic androgen currently withdrawn from the medical application, used as a model androgen in studies on EDCs, with little environmental relevance alone	Hoffmann and Kloas (2012a, 2012b, 2012c, 2012d)

(Continues)

TABLE 3 | (Continued)

Common name	Group of compounds	Description and relevance	Reference
Naphthenic acid fraction compounds (nafcs)	Petroleum ingredients	Carboxylic acids found in crude oils, fuel additives, emulsifiers, surfactants, enter freshwater ecosystems mainly after oil spills	Robinson et al. (2023)
Naphthenic acids (nas)	Petroleum ingredients	Carboxylic acids found in crude oils, fuel additives, emulsifiers, surfactants, enter freshwater ecosystems mainly after oil spills	Zhang et al. (2022)
Nonylphenol (NP)	Phenols	Compound widely used in surfactants and detergents, commonly found in the environment	Mosconi et al. (2002)
Octylphenol (OP)	Phenols	Compound widely used in surfactants and detergents, commonly found in the environment	Mosconi et al. (2002)
Progesterone (P)	Gestagens	Natural gestagen used in human medicine and veterinary drugs, commonly found in the environment	Lange et al. (2002)
Sodium nitrate	Fertilizers	Ingredient of fertilizers, intensively used in agriculture, commonly found in the environment	Poulsen et al. (2018)
Tamoxifen (TAM)	Antiestrogens	Therapeutic drug with moderate environmental relevance, sporadically can occur in wastewater	Orias et al. (2015)
Testosterone (T)	Androgens	Natural hormone excreted by animals, present also in therapeutic drugs, commonly found in the environment	Thomas et al. (2002)
Vinclozolin (VIN)	Fungicides	Fungicide intensively used in agriculture, commonly found in the environment	Feijó et al. (2021)

induction protocols may interact with tested compounds, potentially confounding experimental results. Therefore, when possible, stimulating animals to vocal activity by additional substances should be avoided to capture a more realistic view of EDCs' action.

In other experimental studies, the calling activity of adult *Xenopus laevis* males was reduced following exposure to agrochemicals known for their anti-androgenic action, including the insecticide dichlorodiphenyldichloroethylene (DDE) (Hoffmann and Kloas 2016) and the fungicide vinclozolin (VIN) (Hoffmann and Kloas 2010, 2016). Males exposed to relatively low and environmentally relevant concentrations of DDE (1×10^{-11} M) and VIN (1×10^{-10} M) produced significantly fewer advertisement calls; in the case of VIN, they exhibited a notable increase in the proportion of ticking sounds (Hoffmann and Kloas 2016). Furthermore, the advertisement calls of VIN-exposed males were characterized by longer pauses between clicks and a lower click rate. This pattern of calling, especially when combined with a reduced number of signals, may decrease the male's chances of attracting a female.

Importantly, DDE had a slightly different impact on males depending on the concentration (Hoffmann and Kloas 2016). Males exposed to 1×10^{-11} M DDE produced more growling sounds (deterring other males), while in the higher concentration (1×10^{-9} M) more ticking sounds (expressing higher sexual arousal). It remains unclear how such modifications may impact the chances of males to successfully reproduce.

The synthetic estrogenic compound 17α-ethinylestradiol (EE2) has also been shown to significantly affect the calling behavior of *X. laevis* males, at environmentally relevant concentrations of 1×10^{-10}M (Hoffmann and Kloas 2012b, 2016) and 1×10^{-12}M (Hoffmann and Kloas 2012a). As expected for an estrogenic compound, exposed males produced fewer advertisement calls (Hoffmann and Kloas 2016). In addition, the composition of the calls was altered. Males produced a higher amount of rasping and growling than the respective control animals, reflecting a lower sexual arousal state (Hoffmann and Kloas 2012a, 2012b, 2016). Both the spectral (Hoffmann and Kloas 2012a, 2012b, 2016) and temporal parameters (Hoffmann and Kloas 2012a, 2012b) of advertisement calls were affected, indicating a shift in signal

structure. As a result, fewer females moved toward the modified calls of males (Hoffmann and Kloas 2012a).

Importantly, the effect of EE2 on male calls disappeared 6 weeks after exposure, indicating that the impact of the compound was temporary (Hoffmann and Kloas 2012a). Interestingly, the effect of EE2 was also reduced when males were simultaneously exposed to anti-estrogenic compounds, such as tamoxifen or fulvestrant, which can suppress the action of synthetic estrogens (Hoffmann and Kloas 2012c). These findings highlight the importance of studying the combined effects of EDCs, especially those that amphibians are likely to encounter together in natural environments.

The natural estrogen 17β-estradiol (E2) and octylphenol (OP), a compound with estrogenic properties, have also been found to significantly affect the acoustic communication of amphibians. Interestingly, in a study using another model species—the Western clawed frog (*Xenopus tropicalis*)—these estrogenic compounds had a stimulating, rather than suppressing, effect on males. Males exposed to E2 and OP at a concentration of 1×10^{-8} M produced a higher number of advertisement calls. Additionally, males exposed to OP at 1×10^{-7} M showed increased arm waving, a behavior potentially linked to pheromone release. These changes could be interpreted as a sign of increased sexual arousal. In contrast, female behavior remained unchanged following exposure to either E2 or OP (Schwendiman and Propper 2012).

These unexpected results highlight the importance of comparing the effects of different EDCs—even within the same chemical group. For example, could the natural estrogen E2 have different effects than the synthetic compound EE2 used in previous studies? Similarly, do males of *X. tropicalis* respond differently to estrogenic compounds compared to *X. laevis*? These questions underline the need for species-specific research and a more nuanced understanding of how EDCs influence amphibian behavior.

Disruption of mating calls in adult *X. tropicalis* males was also observed following exposure to naphthenic acids (NAs)—petroleum-derived compounds suspected to have weak estrogenic and anti-androgenic properties—at a concentration of 20 mg/L (Zhang et al. 2022). Males exposed to this environmentally relevant concentration showed reduced vocal output and shorter calling duration, which may reflect lower sexual arousal. Interestingly, the effect of NAs was reversible: after 2 weeks of recovery in clean water, the calling activity of males returned to normal (Zhang et al. 2022).

In contrast, exposure of the wood frog (*Lithobates sylvaticus*) to naphthenic acid fraction compounds (NAFCs)—chemically related substances that may also have anti-androgenic properties—at concentrations of 5 or 10 mg/L did not alter mating behavior (Robinson et al. 2023). This difference may be due to chemical variation between NAs and NAFCs or species-specific sensitivity to these compounds. These findings reinforce the importance of conducting comparative studies across multiple amphibian taxa to better understand the diverse effects of EDCs and to identify species that may be particularly vulnerable to environmental contamination (Frątczak et al. 2025).

5.2 | Disruption of Vocal Signals by Endocrine Disruptors: Other Compounds

To date, only one study has investigated the effects of a typical androgen on the vocal activity of amphibians (Hoffmann and Kloas 2012d). It can be hypothesized that androgens and anti-estrogenic compounds may have a stimulating effect on male mating behavior. An experiment on adult male *Xenopus laevis* showed that exposure to the androgenic compound methyldihydrotestosterone (MDHT) at concentrations as low as 1×10^{-7} M altered their advertisement calls, with males producing fewer rasping elements—vocalizations usually linked to a less sexually aroused state—suggesting enhanced sexual arousal. Interestingly, MDHT also affected female behavior: exposure to a concentration of 1×10^{-5} M increased female receptivity to normal male advertisement calls played through speakers (Hoffmann and Kloas 2012d).

Another understudied group of EDCs in the context of amphibian behavior are gestagens (Hoffmann and Kloas 2012c; Säfholm et al. 2014). Notably, interesting results were observed after exposing adult *X. laevis* males to levonorgestrel (LNG)—a synthetic progestin—at a concentration as low as 1×10^{-7} M. Exposed males produced altered advertisement calls, with a higher number of calls and a lower proportion of rasping, indicating a more sexually aroused state. It can be hypothesized that such call patterns may increase the likelihood of attracting a mate (Hoffmann and Kloas 2012c).

Interestingly, the natural analog progesterone did not affect male calling parameters in the same way. This difference may be due to the mildly androgenic activity of LNG (Hoffmann and Kloas 2012c). These findings underscore that the effects of synthetic analogs can differ significantly from those of natural hormones. This highlights the importance of carefully selecting compounds for experimental studies, ensuring that they reflect the types of substances amphibians are likely to encounter in their environment. Furthermore, the choice of positive controls requires special attention, as natural hormones may act differently than their synthetic counterparts.

Heavy metals, such as cadmium (Cd), can also significantly affect the vocal activity of amphibians (Huang et al. 2015). These effects are believed to result either from weak interactions with estrogenic pathways or through neurotoxic mechanisms (Huang et al. 2015; Järup and Åkesson 2009). This was demonstrated in the dark-spotted frog (*Pelophylax nigromaculatus*), a species in which males produce advertisement calls during the breeding season to attract females (Cheong et al. 2013). According to Huang et al. (2015), receptive females respond vocally, prompting males to emit specific reply calls and approach the source of the sound. In their long-term study, exposure to cadmium (Cd) altered male responses to female calls. Males exposed to higher Cd concentrations (1×10^{-6} and 1×10^{-7} M) produced longer calls with fewer croaks, while at lower concentrations (1×10^{-8} M), call latency initially decreased and then increased, and an elevated call rate was observed. Additionally, exposed males were less likely to approach female calls, and a significant proportion did not vocalize at all.

It was confirmed in a later study that low doses of cadmium cause histological changes in the larynx of both male and female *P. nigromaculatus* (Duan and Huang 2016). These structural alterations may explain the changes in vocal signaling and, importantly, suggest that the effects could be permanent.

6 | Impact on Behavior by Disruption of Olfactory Communication

In amphibian species that do not rely on vocalizations for mate attraction, pheromones serve as key signals for communication between individuals (Kikuyama et al. 2002). Although chemical communication in amphibians has been studied for several decades, particularly in Caudata, many aspects remain poorly understood (Woodley 2015). Foundational work in salamanders identified the presence of courtship pheromones produced by mental glands (Houck and Reagan 1990; Kikuyama et al. 2002), and more recent studies have characterized specific pheromones and their behavioral effects (Rollmann et al. 1999; Woodley 2015). However, the chemical identity, variation, and perception of these signals remain underexplored across most amphibian taxa, especially in Anura (Woodley 2015).

Available data indicate that pheromone production and reception in amphibians are regulated by prolactin and steroid hormones (Kikuyama et al. 2002; Sever and Staub 2011). As a result, EDCs present in the environment may alter these hormonal pathways, thereby affecting both the production and perception of pheromones.

For example, the insecticide endosulfan, suspected of anti-androgenic and weakly estrogenic activity (Silva and Gammon 2009), has been shown to disrupt pheromone signaling in the red-spotted newt (*Notophthalmus viridescens*). Female newts exposed to endosulfan at a concentration as low as 1.2×10^{-8} M exhibited changes in their pheromonal glands, and their scent became less attractive to males (Park et al. 2001).

Another compound, the fertilizer sodium nitrate, affected the courtship behavior of the palmate newt (*Lissotriton helveticus*) at a concentration of 8.82×10^{-4} M. Exposed males showed a higher probability of displaying courtship behavior, which may indicate that sodium nitrate disrupts pheromone production, prompting males to compensate with increased visual displays (Secondi et al. 2013). Supporting this interpretation, *L. helveticus* females were less attracted to the scent of males exposed to the same concentration of sodium nitrate in another study (Secondi et al. 2009).

In contrast, ammonium nitrate, another common fertilizer ingredient, had no significant effect on the courtship behavior of the Iberian newt (*Lissotriton boscai*) under similar experimental conditions (Ortiz-Santaliestra et al. 2009). The mechanisms of endocrine disruption for both sodium and ammonium nitrates remain poorly understood, and their effects do not align clearly with classical androgenic or estrogenic pathways (Poulsen et al. 2018; Daam et al. 2020).

7 | Potential Effects of Endocrine Disruptors via Disruption of Physical Traits

EDCs can reduce amphibian reproductive success not only by disrupting mating behaviors directly but also by altering hormone-dependent traits involved in communication between mates. Several such traits mentioned throughout this review—such as the presence of vocal sacs (Hayes and Menendez 1999), laryngeal structure (Huang et al. 2015; Duan and Huang 2016), and nuptial pad morphology (Hayes et al. 2010)—are strongly hormone-dependent and play a critical role in producing or receiving mating signals. Altered secondary sexual traits, such as tail shape and foot webbing in *Lissotriton helveticus*, may also significantly influence female mate choice (Secondi et al. 2009, 2013).

Another hormone-regulated trait with a signaling function that may be disrupted by EDCs is skin coloration. It is a hormone-regulated trait that plays an important role in sexual communication in many amphibian species (Sever and Staub 2011). In anurans, for example, sexually dimorphic coloration often develops during the breeding season and may serve as a visual cue for mate recognition, assessment of sexual maturity, or indication of reproductive readiness (Rojas et al. 2023). The presence of sex steroid hormone receptors in the skin of both larvae and adults (Chieffi et al. 1975; Sever and Staub 2011) supports its role as a hormone-responsive signaling trait. Additionally, the genetic basis of coloration has been linked to sex chromosomes in some species, such as *Rana rugosa*, where a sex-linked color gene has been identified (Miura et al. 2011).

Given this background, skin coloration may be susceptible to disruption by EDCs, potentially altering communication between mates (Ujhegyi and Bókony 2020). However, to date, this hypothesis has not been experimentally tested. As such, we present data on substances that affect coloration as a case study of a physiological trait with potential indirect consequences for sexual communication, and as an area that needs further investigation due to its possible significance for amphibian reproductive success.

7.1 | Disruption of Sexually Dimorphic Body Coloration

One of the best-documented examples of EDC-induced disruption of sexually dimorphic coloration comes from studies on reed frogs. In the Argus reed frog (*Hyperolius argus*), body coloration is a sexually dimorphic trait that plays an important role in mate recognition (Rojas et al. 2023). The organochlorine insecticide dichlorodiphenyltrichloroethane (DDT) and its congeners, known for their anti-androgenic activity (Agency for Toxic Substances and Disease Registry 2024), have been found to influence body coloration in this species. Females exposed to o,p′-DDT at concentrations of 2.8×10^{-7} M and 2.8×10^{-6} M, as well as to o,p′-DDD and o,p′-DDE at 2.8×10^{-6} M, prematurely developed adult-type skin coloration. In contrast, exposure to positional isomers of these compounds, p,p′-DDT, p,p′-DDE, and p,p′-DDD did not result in observable changes in coloration (Noriega and Hayes 2000).

In another study, *H. argus* males exposed to estradiol (E2) at relatively high concentrations (3.65×10^{-4} M and 3.65×10^{-5} M) during the larval stage developed dorsal coloration typical of females. These males also failed to develop vocal sacs. In contrast, exposure to testosterone (T) led to the development of the male phenotype, including male-typical coloration and development of vocal sacs, in both genetic males and females (Hayes and Menendez 1999).

Interestingly, in the common reed frog (*Hyperolius viridiflavus*), exposure to both E2 (1.83×10^{-7} M) or T (1.73×10^{-4} M) during larval and juvenile stages caused the development of a phenotype typical for adult females: bright green or yellow body coloration, in genetic males and females. Importantly, this effect was reversible; after a few weeks without further exposure, individuals returned to their normal coloration (Richards 1982).

Similar effects of hormone manipulation or EDC exposure on body coloration have also been observed in other amphibian species, further supporting the idea that skin pigmentation is hormonally regulated and susceptible to disruption. In *Acris gryllus* (cricket frog), testosterone implants (dose not precisely reported) caused both juvenile males and females to develop male-typical breeding coloration, including yellow and dark throat pigmentation (Greenberg 1942).

In the common toad (*Bufo bufo*), dorsal coloration is moderately sexually dimorphic, with males typically showing brighter, more yellow-green skin, and females appearing darker and more reddish. Larval exposure to the synthetic estrogen 17α-ethinylestradiol (EE2) or the estrogenic herbicide glyphosate led to the appearance of mixed-sex individuals with duller, reddish-brown coloration, distinct from that of both normal males and females. Treatment with 1 μg/L EE2 resulted in 100% female phenotypes with typical female coloration, while mixed-sex individuals occurred at 1 ng/L EE2 and 3 μg/L glyphosate. However, it should be noted that the number of mixed-sex individuals observed was very small (Ujhegyi and Bókony 2020).

Additionally, in the American bullfrog (*Lithobates catesbeianus*), short-term exposure (14 days) of females to nonylphenol (NP) resulted in darkening of body pigmentation (Scaia et al. 2019). Worth noting, effects on skin coloration of amphibians may be useful as a potential biomarker of EDC exposure in wild populations (Orton et al. 2023).

8 | Summary and Future Directions

EDCs can significantly impact the mating behavior of amphibians. As we show in our review, endocrine disruptors interfere with acoustic, olfactory, and potentially visual communication of amphibians, which are essential for mate selection and further stages of courtship and breeding. Such disruptions may negatively impact not only the reproductive success of individuals but also the dynamics of entire populations and ultimately the survival of amphibian species. Notably, even relatively short exposure periods induce significant changes, highlighting this as an important direction for future research.

There are still significant research gaps in our understanding of the impact of EDCs on amphibian mating behavior. First of all, many studies test only the immediate impact of EDCs on the behavior of amphibians (e.g., Behrends et al. 2010; Hoffmann and Kloas 2012a–d; Hoskins et al. 2017; Park et al. 2001, for more examples see Table S1). There is a strong need for more research on the long-term and multi-generational consequences of chronic exposure to EDCs on the mating behavior and reproduction of amphibians.

Moreover, most studies on EDCs and amphibian behavior focused only on the effects of single chemicals and no additional stressors (See Table S1). The exception is the study of Hoffmann and Kloas (2012b), in which the effect of EE2 was tested in combination with anti-estrogenic compounds. It should be taken into account that in the natural environment, amphibians have contact with many different substances of natural (Iglesias-Carrasco et al. 2017) and anthropogenic origin (Swanson et al. 2018), as well as other stressors, such as competition with other mates (Uzendoski et al. 1993), presence of pathogens (Paetow et al. 2023), parasites (DuRant et al. 2015), and predators (LaFiandra et al. 2008; Uzendoski et al. 1993). These stressors can have synergistic or antagonistic actions that cannot be observed by studying a single contaminant in the simplified experimental protocols (Awkerman et al. 2024).

As we highlighted earlier, studies on EDCs need to encompass various substances, even those that are theoretically similar, such as natural and synthetic counterparts. Even if their chemical structures are similar, it seems that they can exhibit significant differences in their impact on amphibians (Hoffmann and Kloas, 2012). Additionally, synthetic substances may differ from natural ones in terms of environmental stability (Jürgens et al. 2002), which can affect their availability and bioactivity in organisms. We need studies on a wide range of compounds to better understand the potential risks associated with exposure to EDCs and enable more accurate predictions of their impact on organisms in natural environments.

Bias in the use of the model species, mainly *X. laevis*, is a universal problem in all studies encompassing the impact of pollutants on amphibians (Frątczak et al. 2025; Schiesari et al. 2007). Species of amphibians may have different sensitivities to EDCs (Rozenblut-Kościsty et al. 2019; Tamschick, Rozenblut-Kościsty, Ogielska, Kekenj, et al. 2016; Tamschick, Rozenblut-Kościsty, Ogielska, Lehmann, et al. 2016), as well as varying degrees of risk of exposure to EDCs in the environment due to their ecology. For example, while *X. laevis* (Zhang et al. 2022) is a fully aquatic species, *L. sylvaticus* (Robinson et al. 2023) is a terrestrial species with a short, explosive mating season (AmphibiaWeb, n.d.). Furthermore, *X. laevis*, used in the majority of EDC studies, employs a unique underwater vocalization mechanism distinct from the air-driven calls typical of terrestrial Neobatrachian species (Walkowiak 2006). These ecological and mechanistic differences highlight the need for studies to include a broader range of amphibian taxa to better understand the diverse impacts of EDCs on communication across species.

Taken together, existing evidence demonstrates that EDCs pose a serious but still underexplored threat to amphibian reproductive communication. To fully understand their ecological impact, future research must move beyond simplified models and experimental conditions. Only then can we accurately assess

the risks EDCs pose to amphibian biodiversity and reproductive success in the wild.

Author Contributions

Martyna Frątczak: conceptualization (equal), investigation (lead), visualization (lead), writing – original draft (lead), writing – review and editing (equal). **Mikołaj Kaczmarski:** conceptualization (equal), writing – review and editing (equal). **Katarzyna Szkudelska:** supervision (equal), writing – review and editing (equal). **Piotr Tryjanowski:** conceptualization (equal), methodology (lead), supervision (equal), writing – review and editing (equal).

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

All data used in the review are available in the Supporting Information.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section. **Table S1:** Summary of the studies on the impact of endocrine-disrupting compounds on mating behavior, sexual communication and body coloration of amphibians, including the type of compound, species studied, developmental stage, time of exposure and the most significant results.

Publikacja III

Frątczak, M., Kaczmarski, M., Jankowiak, Ł., Klessa, J., Bielicki, K., Lyskov, B., & Tryjanowski, P.

Digit ratio in the common spadefoot toad *Pelobates fuscus* (Anura: Mesobatrachia: Pelobatidae): patterns and correlations.

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Digit ratio in the common spadefoot toad *Pelobates fuscus* (Anura: Mesobatrachia: Pelobatidae): patterns and correlations

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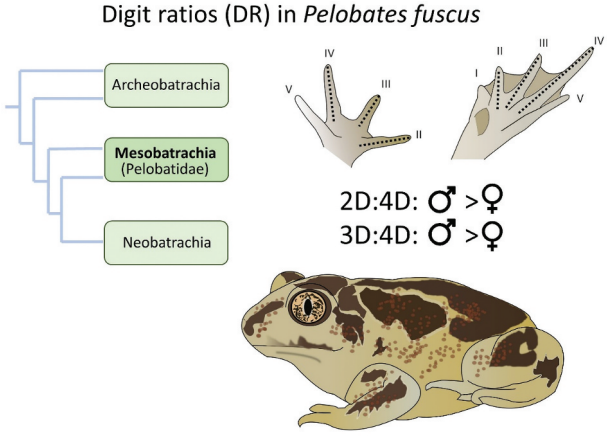
Abstract
Digit ratio (DR or 2D:4D), an indicator based on the relative length of the second and fourth digits, has been linked to embryonic sex hormone levels and is regarded as a biomarker for ontogenetic changes and evolutionary traits across species. While DR patterns often display a sex bias, they vary among taxa: in most mammals and tailed amphibians, females exhibit higher 2D:4D ratio than males, whereas in birds and reptiles, the trend is reversed. However, data on DR in Anuran amphibians remains limited, particularly within the Mesobatrachia, a relatively primitive group. This study investigates DR in the common spadefoot toad (*Pelobates fuscus*), a little-studied and secretive species, using 68 archival specimens, and two analytic approaches. In the simple correlation approach, there were no significant sex differences in DR in this species. However, when DRs were adjusted allometrically by digit length, regression analysis showed significant effects of both sex (male) and snout–vent length (SVL). Males had significantly larger DRs, mainly 2D:4D and 3D:4D, in all limbs, while SVL had a significant negative effect on most DRs. Further research is needed to clarify the factors influencing DR in amphibians.



Keywords: *Amphibian*, *biomarker*, *fossorial species*, *allometry*, *2D:4D*

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1. Introduction

Digit ratio (DR or 2D:4D) is an indicator based on the relative lengths of the second and fourth digits. It has been shown to correlate with levels of sex hormones (mostly androgens) during development in the embryonic period (Manning et al. 1998; Manning 2002) and is associated with many physical and behavioral traits in *Homo sapiens* (Klimek et al. 2014; Pasanen et al. 2022) and other vertebrate species (Ribeiro et al. 2016; Lofeu et al. 2017). DR is considered a potentially important biomarker of ontogenetic changes and biologically significant traits in the evolution of species (Lofeu et al. 2017).

It has been proposed that sexual dimorphism in 2D:4D could be universal across pentadactyl tetrapods, as the evolutionary development of the limbs (polydactyly) and genital bud may have occurred through the recruitment of common HOX genes (Manning 2002). In theory, since HOX genes are highly conserved across taxa and influenced by the endocrine system, the digit ratio (2D:4D) could potentially be similar among different taxonomic groups (Manning et al. 2003; Voracek & Manning 2003). Studies on DR in vertebrates have shown that for many, DR is sex-biased; however, this pattern is not consistent (Romano et al. 2005; Auger et al. 2013; Lofeu et al. 2017). For example, in most mammals (Fuse & Sawada 2019) and tailed amphibians (Kaczmarski et al. 2015), the 2D:4D ratio is higher in females than in males, while the opposite pattern can be found in birds and reptiles (Leoni et al. 2008; Van Damme et al. 2015; Kaczmarski et al. 2020). In some taxa, however, DR differs between the sexes even in closely related species, e.g. lizards from the family Tropidurus (Miranda et al. 2021). It is worth mentioning that many further studies (e.g. Rubolini et al. 2006; Kaczmarski et al. 2021) included analyses of other combinations of DRs (e.g. 2D:3D, 3D:5D).

Amphibians are still not well studied among modern tetrapods regarding DR patterns and correlations. Previous studies have described DR in five species within the order Urodela (Balogová et al. 2015; Kaczmarski et al. 2015) and at least 10 species within the order Anura (for a review, see Chang 2008; Alinezhadiert al. 2020; Kaczmarski et al. 2021; Javanbakht et al. 2022). A recent study challenged previous assumptions about DR numbering schemes and analytical methods, showing that DR in amphibians requires further research and revision of prior assumptions due to the adoption of incorrect digit numbering (Kaczmarski et al. 2021). Notably, we now know that during the development of the forelimbs, the primary digit is reduced, and the four present digits are, from a developmental perspective, digits II to V (Fabrezi & Alberch 1996; Fabrezi & Barg 2001). As some digits in the forelimbs have nuptial pads on digits II to IV (the first finger is reduced, as noted), these digits are important due to their role in grasping (or clasping) females during amplexus (Carvajal-Castro et al. 2020). At the same time, the third digit can be used for tapping behavior, which is also important during foraging by attracting prey (Vergara-Herrera et al. 2024).

Different amphibian groups exhibit variations in amplexus type (Carvajal-Castro et al. 2020), which could potentially be linked to sexual dimorphism in digit lengths, and thus DR. Most species within the Mesobatrachia and Archaeobatrachia exhibit the ancestral inguinal (lumbar) amplexus, with the male embracing the female in front of the hind legs, while most Neobatrachia exhibit axillary amplexus (Duellman & Trueb 1986; Wells 2010). Current DR studies are biased, focusing mainly on representatives of Neobatrachia (about 96% of anuran species) and Archaeobatrachia (around 0.4% of anuran species). However, research has shown that these groups lack a clear DR pattern, even though our understanding of how this trait develops has significantly increased (Lofeu et al. 2017; Kaczmarski et al. 2021). Mesobatrachia, which comprises approximately 3.6% of anuran species from five fossorial families, is a relatively primitive group of amphibians (AmphibiaWeb 2024), and data on DR in this taxon remain scarce. Only one study has investigated DR in a Mesobatrachia species, the eastern spadefoot toad *Pelobates syriacus*, in a relatively small sample, and the authors did not provide details on forelimb digit numbering (Alinezhadi et al. 2020). Moreover, most current studies on amphibians did not take into account the possible effect of allometry on DR results. This could bias our understanding of DR in vertebrates, as it has been revealed that in some species DR sexual dimorphism correlations may partly result from size differences between sexes (Beaty et al. 2016; Lolli et al. 2017).

The goal of our study was therefore to examine DR patterns and correlations with sex and size in a Mesobatrachia representative, the common spadefoot toad (*Pelobates fuscus*), using a correct digit numbering scheme, and two analytical approaches, including the possible effect of allometry, to provide new data on DR in this group of amphibians.

2. Materials and methods

The study was based on an archival collection of 68 specimens of *P. fuscus*, preserved in 70% ethanol

solution, housed in the Department of Zoology, Poznan University of Life Sciences. Sex determination of the individuals was performed through anatomical assessment of gonads during dissection. Each specimen was measured using a manual caliper (accuracy: 0.01 mm) for snout–vent length (SVL) (which in anurans corresponds to total body length) and head width (HW), by two independent observers. The level of technical measurement error was calculated using the intraclass correlation coefficient (ICC).

After manual measurements, photographs of each specimen's four limbs were taken with a Pentax Optio WG-5 digital camera, featuring a microscope function with an LED-backlit ring placed directly on the lens. The camera was positioned on plate glass with a scale marker (PEAK S-1983-S), allowing a consistent distance between the camera and the fixed object. While taking photographs, constant lighting conditions were maintained using the built-in LED. The limb images were used for further analysis. Individual digits of the forelimbs were numbered from II to V, and those of the hindlimbs from I to V, according to Kaczmarski et al. (2021). Computerized measurements of DR, using landmarks (see Figure S1 in the Supplementary Material), were performed according to Kaczmarski et al. (2015). Tmorph Gen 6 (Sheets 2000) was used to measure the distance between points (landmarks) corresponding to the length of the surveyed digits. This procedure was repeated for each limb of every specimen.

In the statistical analysis, we applied two distinct approaches. The first approach followed a previously established method that did not account for allometry, focusing solely on raw DRs without adjusting for digit length (Kaczmarski et al. 2015). In this approach, before modeling, multicollinearity among predictors was checked, revealing a high correlation between SVL and HW. To address this, principal component analysis (PCA) was performed, and the resulting scores were used as a single-size variable in the models. Linear models for each digit ratio (e.g. FL_2D3D, FR_3D4D) included sex and size (the PCA's first component) as predictors, testing both their additive and interactive effects. Models were fitted in R (2024, v. 4.3.3) with significance assessed via F-tests. Residuals were checked for normality and homoscedasticity using histograms, Q-Q plots, and Levene's test; if non-normal, the response variable was transformed using square root or log functions. For each left–right pair of digit ratios, paired t-tests assessed significant differences, with effect sizes calculated using Cohen's d (Hedges' correction). The sexual dimorphism index (SDI) for each digit length and ratio was calculated as per Gomes and Kohlsdorf (2011).

The second approach incorporated allometric adjustment to control for digit length differences, using a principal component derived from digit-related variables. This adjustment allowed us to isolate the effect of sex on DRs by accounting for variations in digit length, providing a clearer understanding of potential sexual dimorphism (Lolli et al. 2017). To achieve this, first Pearson's correlation tests were conducted between each pair of digit lengths (e.g. 2D and 4D) within each sex group. If a significant correlation ($p < .05$) was found, two models were fitted: a power function ($\log(2D) \sim \log(4D)$) and a linear model with an intercept ($2D \sim 4D$). The best-fitting model was selected based on the Akaike information criterion (AIC). Residuals from the selected model were added to the mean of the first digit to compute adjusted digit lengths. The adjusted DR (e.g. 2D_adj/4D) was then calculated for each sex group. If no significant correlation was observed, no further analysis was conducted. All statistical analyses were performed in (R Development Core Team 2024). Models from both analyses can be found in the online Supporting Information.

3. Results

When DRs were not allometrically controlled by digit length, there were no statistically significant differences in DR between males and females of the *P. fuscus*. Mean values (with SDI) of sexual differences in DR are presented in Tables S1 and S2. Data on values of SVL, HW, and digit length can be found in Tables S3–S5. For the digits of the forelimbs, particularly for 3D:5D and 4D:5D ratios, there was a positive relationship with size in males, roughly the same for both left and right sides (Figure S2). The single effects of size or sex alone did not show any significant relationship in any case.

When DRs were adjusted allometrically by digit length, regression analysis showed significant effects of both sex (male) and SVL (Table I). In this analytical approach, sex had a clear impact on some DRs, particularly 2D:4D and 3D:4D ratios in all limbs, which were higher in males. A significant effect of the male sex was observed also for the 2D:3D ratio on the left forelimb. Additionally, SVL had a significant negative effect on most DRs (Figure 1), with very low p values ($p \leq .001$), suggesting that as SVL increases, DRs tend to decrease.

Table I. Regression estimates for different digit ratios adjusted allometrically (e.g., 2D:4D - ratio of second to fourth digit) in forelimbs (FR – front right, FL – front left) and hindlimbs (HR – hind right, HL – hind left) of the common spadefoot toad (*Pelobates fuscus*), sex and snout-to-vent length (SVL), with standard error (SE).

Digit ratio	Intercept	Sex (male) (SE)	SVL (SE)
FL_2D3D_adj	1.860	0.057 (0.018)**	−0.016 (0.003)***
FL_2D4D_adj	1.129	0.040 (0.013)**	−0.009 (0.002)***
FL_2D5D_adj	0.775	0.047 (0.047)	0.009 (0.006)
FL_3D4D_adj	0.961	0.028 (0.011)*	−0.007 (0.002)***
FL_3D5D_adj	1.061	0.053 (0.037)	−0.001 (0.005)
FL_4D5D_adj	2.160	0.093 (0.055)	−0.012 (0.008)
FR_2D3D_adj	1.998	0.020 (0.020)	−0.019 (0.003)***
FR_2D4D_adj	1.207	0.026 (0.012)*	−0.011 (0.002)***
FR_2D5D_adj	1.444	−0.001 (0.039)	−0.006 (0.005)
FR_3D4D_adj	1.019	0.038 (0.011)***	−0.009 (0.001)***
FR_3D5D_adj	1.493	0.035 (0.030)	−0.011 (0.004)**
FR_4D5D_adj	2.508	0.028 (0.046)	−0.020 (0.006)**
HL_2D3D_adj	0.921	0.023 (0.012)	−0.008 (0.002)***
HL_2D4D_adj	0.530	0.014 (0.005)**	−0.005 (0.001)***
HL_2D5D_adj	1.113	0.005 (0.018)	−0.010 (0.002)***
HL_3D4D_adj	0.942	0.018 (0.009)*	−0.009 (0.001)***
HL_3D5D_adj	2.004	0.002 (0.030)	−0.019 (0.004)***
HL_4D5D_adj	3.649	0.018 (0.054)	−0.035 (0.007)***
HR_2D3D_adj	0.952	0.017 (0.012)	−0.009 (0.002)***
HR_2D4D_adj	0.495	0.012 (0.006)*	−0.004 (0.001)***
HR_2D5D_adj	1.040	0.016 (0.016)	−0.010 (0.002)***
HR_3D4D_adj	0.868	0.025 (0.010)*	−0.007 (0.001)***
HR_3D5D_adj	1.833	0.039 (0.027)	−0.017 (0.004)***
HR_4D5D_adj	3.344	0.052 (0.047)	−0.031 (0.006)***

*** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$.

4. Discussion

In this study, we conducted a detailed analysis of digit ratio (DR) patterns in *P. fuscus*, a species representing the Mesobatrachia. The primary objective was to determine whether there are differences in DRs between males and females. In the initial analysis, our results showed no clear pattern in DR between sexes in *P. fuscus*. This aligned with findings from other studies, that used a similar analytical approach without taking into account allometry, in other species, such as a representative of Neobatrachia (South American white-lipped grassfrog *Leptodactylus fuscus*) and a species of Archaeobatrachia (Maud Island frog *Leiopelma pakeka*), as well as some salamanders, e.g. the fire salamander *Salamandra salamandra* (Germano et al. 2011; Balogová et al. 2015; Lofeu et al. 2017). It is worth mentioning that a study on a closely related species, *P. syriacus*, found that the 2D:4D ratio in left hindlimbs was sex-related and higher in males (Alinezhadi et al. 2020), although it was performed on a very small number of specimens.

When our analysis accounted for allometric adjustments based on digit length, a different pattern emerged, showing that males had significantly higher values for particular DRs. It has been advocated, that DR analysis without allometric correction may show patterns that are partly a result of digit lengths and not just the influence of sex (Lolli et al. 2017). It should be considered that individuals with generally longer digits may exhibit different DRs due to digit length alone, rather than as a direct result of sex (Beaty et al. 2016; Lofeu et al. 2020). Therefore, results may potentially exaggerate or mask true sex differences in DRs (Lolli et al. 2017). Therefore, considering the possible effect of digit length allows us to achieve more reliable results.

Importantly, in our analysis of the allometrically controlled DRs, a "classical" DR − 2D:4D – was significantly higher in males, in all limbs. A similar pattern, with 2D:4D higher in males than in females, can be observed in birds and reptiles (Leoni et al. 2008; Van Damme et al. 2015). Additionally, SVL had a significant negative effect on most DRs adjusted by digit length, suggesting that as SVL increases, DRs tend to decrease in both sexes. This may suggest that the development of these proportions is to some extent related to overall body growth in this species.

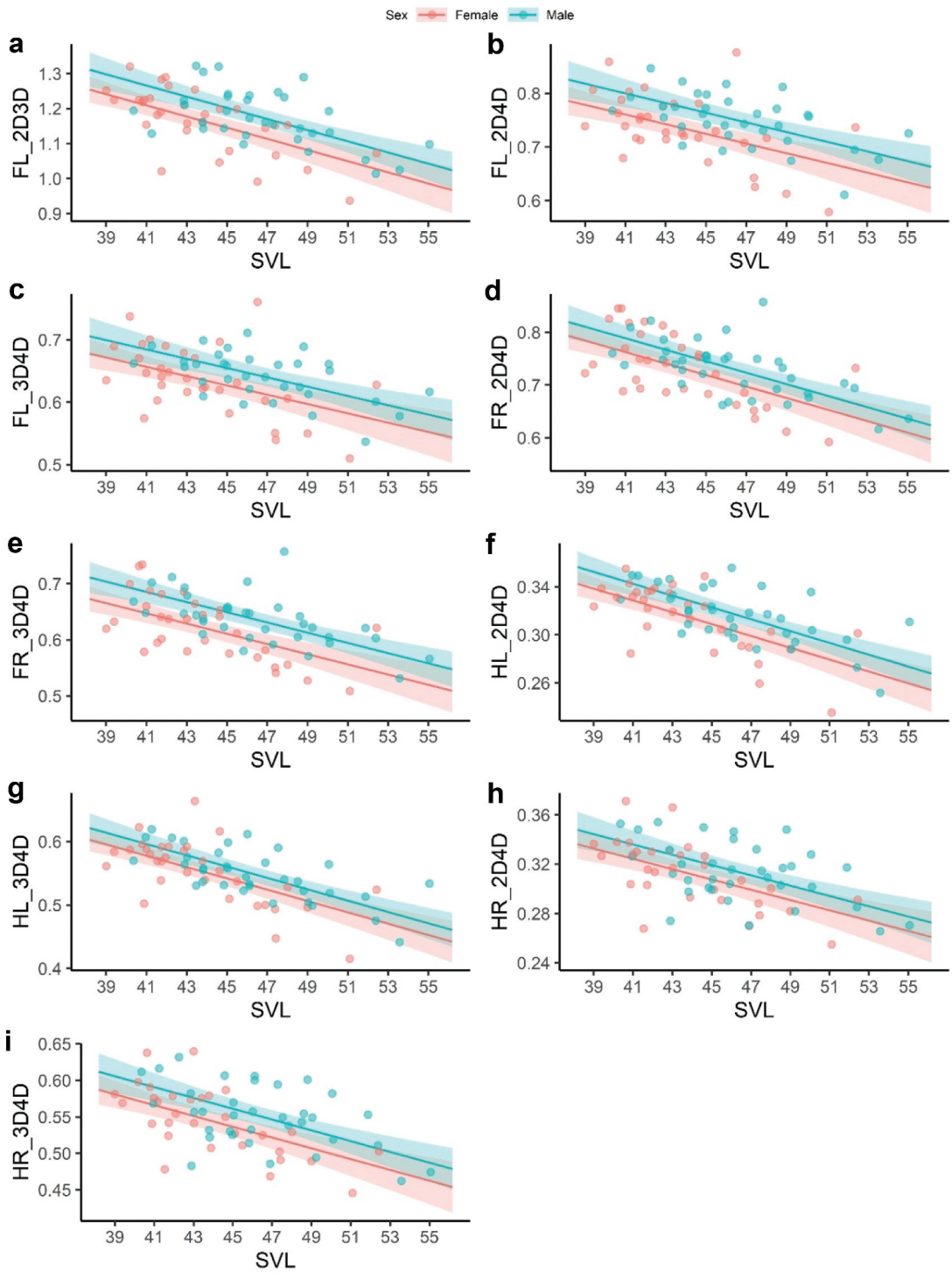

Figure 1. Scatter plots showing the relationship between snout–vent length (SVL) and digit ratios in males (blue) and females (red) of the common spadefoot toad *Pelobates fuscus*. Each panel represents a different digit ratio for forelimbs (FR – front right, FL – front left) and hindlimbs (HR – hind right, HL – hind left).
The regression lines indicate a general negative correlation between SVL and digit ratios, with males consistently having higher ratios than females across all comparisons.

It should be mentioned that the accuracy of the results of DR analysis depends greatly on measurement methods and the condition of preserved specimens (Rubolini et al. 2006; Direnzo & Stynoski 2012; Woodhead et al. 2018). To avoid mistakes in our assessment, we used previously validated

measurement methods and engaged two independent observers (Kaczmarski et al. 2015). While using live specimens would be ideal, we decided to utilize the valuable archival collection of this relatively rare and little-studied species, which has a very secretive, nocturnal lifestyle (Székely & Nemes 2002; Ivanova et al. 2017).

To sum up, our research shows that *P. fuscus* exhibits significant sex differences in DR. However, further studies on a larger number of representatives of Mesobatrachia, as well as other amphibian groups, and taking into account allometry, are necessary to better understand and interpret DR patterns in this tetrapod lineage.

Disclosure statement

No potential conflict of interest was reported by the author(s).

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Data availability statement

Statistical models used in the study are available in the online Supplemental Information. Raw data is available on request.

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Publikacja IV

Frątczak, M., Szkudelska, K., Grobelna, M., Hermaniuk, A., Jankowiak, Ł., Kaczmarski, M., Myczko, Ł., Rozenblut-Kościsty, B., & Tryjanowski, P.

Impact of bisphenol A and ethinyloestradiol on sex, body condition and digit ratio of the marsh frog *Pelophylax ridibundus* in the mesocosm exposure system.

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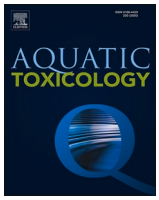

Impact of bisphenol A and ethinyloestradiol on sex, body condition and digit ratio of the marsh frog *Pelophylax ridibundus* in the mesocosm exposure system

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ABSTRACT

This study examined the impact of two environmentally widespread endocrine-disrupting compounds (EDCs), bisphenol A (BPA) and 17α-ethinylestradiol (EE2), on the marsh frog *Pelophylax ridibundus*. The analysis focused on somatic condition, gonadal differentiation, and the second-to-fourth digit ratio (DR, 2D:4D), tested as a potential non-invasive biomarker of endocrine disruption. Tadpoles were reared in outdoor mesocosms and exposed weekly to three environmentally-relevant concentrations of BPA (BPA7 - 10^{-7}, BPA8 - 10^{-8}, BPA9 - 10^{-9} M) and one concentration of EE2 (10^{-9} M), acting as a positive control. The exposition was conducted in a static-renewal system. While neither treatment nor sex influenced snout–vent length (SVL) or body weight, Scale Mass Index (SMI) was significantly reduced in the highest concentration BPA group (–0.139 ± 0.036) and in EE2 (–0.173 ± 0.036), indicating impaired somatic condition. EE2 caused a strikingly female-biased sex ratio (≈9:1 F:M), increasing the odds of being female by 5.2-fold, whereas BPA did not affect sex ratios. Histological analyses revealed two intersex and one mixed-sex individuals in the EE2 group. In the number of BPA-exposed males, significant gonadal degeneration and rudimentary metamers were observed, with similar structures found in females, suggesting disturbed gonadal development. Although DR did not differ between sexes, both EE2 and BPA altered it in a size-dependent manner, with significant treatment × SVL interactions in hindlimbs (EE2, BPA7) and the left forelimb (EE2), where larger individuals exhibited lower ratios under exposure. These environmentally relevant findings demonstrate that BPA and EE2 profoundly disrupt somatic health, gonadal differentiation and sex-related traits in a non-model amphibian species, with clear implications for wildlife conservation.

1. Introduction

Endocrine-disrupting compounds (EDCs) are exogenous chemicals that interfere with the endocrine system by mimicking, blocking, or otherwise altering hormonal signaling pathways (La Merrill et al., 2020). The majority of environmental EDCs originate from anthropogenic sources, such as industrial chemicals, pharmaceuticals, and personal care products. These compounds have been shown to affect various hormonal axes, particularly those involving estrogens, androgens, and thyroid hormones, with documented effects across a broad range of vertebrate taxa (Kloas et al., 2024; La Merrill et al., 2020).

EDCs are widespread in surface waters across Europe and globally (Puri et al., 2023), where they may affect reproduction, development, and survival of aquatic organisms. Amphibians, with their permeable skin and external development, are particularly vulnerable and are recognized as sensitive bioindicators of aquatic pollution (Frątczak

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et al., 2025b; Kloas et al., 2024). Understanding how EDCs influence amphibian development is therefore important not only from a physiological perspective but also for biodiversity conservation and ecological risk assessment (Heimeier and Shi, 2010; Kloas et al., 2024).

Among the most prevalent EDCs detected in surface waters are bisphenol A (BPA) and 17α-ethinylestradiol (EE2). BPA is a high-production-volume chemical commonly used in the manufacture of plastics and epoxy resins. As a xenoestrogen, BPA interacts with estrogen, thyroid, and androgen receptors, disrupting multiple endocrine pathways (Heimeier and Shi, 2010; Li et al., 2022). In amphibians, exposure to BPA has been associated with feminization of gonads, thyroidal dysfunction, and altered sex ratios, although responses are often species-specific and findings remain inconsistent (Kloas et al., 1999; Tamschick et al., 2016a). EE2, a synthetic estrogen widely used in oral contraceptives, is another potent endocrine disruptor frequently found in aquatic environments. It was found to have strong feminizing effects in several amphibian species, frequently resulting in mixed-sex phenotypes or complete male-to-female sex reversal (Mackenzie et al., 2003; Pettersson and Berg, 2007; Tamschick et al., 2016b; Tompsett et al., 2012; Ujhegyi and Bókony, 2020).

In many amphibian species, monitoring the impact of EDCs in a non-invasive manner poses a significant challenge (Ujhegyi and Bókony, 2020). Most species lack external sexually dimorphic traits, and for many, genetic sexing tools are unavailable (Bullejos et al., 2024). For that reason, there is a growing interest in the identification of non-invasive biomarkers of sex and the impact of EDCs on amphibians (Ujhegyi and Bókony, 2020). In this study, we aimed to explore the potential of the second-to-fourth digit ratio (DR, 2D:4D) as a non-invasive indicator of sex and endocrine disruption in amphibians. DR is a morphological trait believed to reflect early hormonal influences and shown to correlate with sex and other physiological characteristics across a range of taxa (Lofeu et al., 2017; Manning, 2002). However, research on DR in amphibians remains poorly explored, and many existing studies rely on problematic assumptions, such as order of digit's numbering, that have recently been critically reassessed (Frątczak et al., 2025a; Kaczmarski et al., 2021).

The present study aimed to examine the effects of BPA, with EE2 chosen as positive control, on gonadal differentiation and external morphological traits, including the 2D:4D digit ratio, in a non-model species, the marsh frog *Pelophylax ridibundus* (Pallas, 1771). *Pelophylax ridibundus* is a widespread European amphibian, characterized by a predominantly aquatic lifestyle (Berger, 1977), which leads to continuous year-round exposure to waterborne pollutants. It breeds in deep water bodies and large rivers (Berger, 1977), often exposed to wastewater effluents, making it a particularly relevant species for assessing the ecological risks of EDCs. By focusing on gonadal differentiation and digit ratio in this species, the study sought to clarify how EDCs impact amphibian sexual development and to evaluate the potential of DR as a non-invasive biomarker of endocrine disruption.

2. Materials and methods

2.1. Animals

Fertilized eggs of *P. ridibundus* were obtained from a licensed commercial breeder (Frogs Po-Land, https://frogs-po-land.com) from 3 parental pairs. Due to the hybridogenetic system in the European water frog complex *Pelophylax esculentus* (Berger, 1977), the genotype of parental pairs was verified using molecular methods to confirm that the experimental specimens originated from non-introgressed *P. ridibundus*. Nuclear genome species assignment was conducted using amplification of the *serum albumin intron 1* (SAI-1) marker following the method of Hauswaldt et al., 2012, as modified by Ermakov et al., 2019. Additionally, mitochondrial DNA haplotypes were determined using primers specific for *P. lessonae* (L-mtDNA) and *P. ridibundus* (R-mtDNA), following methods described by Jośko and Pabijan, 2020.

2.2. EDCs concentrations

Doses of BPA and EE2 chosen for the experiment were aimed to reflect environmentally relevant levels of these compounds in European surface waters (Frątczak et al., 2025c; Ślósarczyk et al., 2021; Wilk et al., 2019). In Poland, BPA has been reported across a range of aquatic environments, with concentrations strongly dependent on the source and degree of anthropogenic pressure. The highest BPA levels were detected in landfill leachates, reaching 9.65×10^{-6} M (Wilk et al., 2019). Lower concentrations were reported in treated municipal wastewater, ranging from 4.75×10^{-8} M (Ślósarczyk et al., 2021) to 7.67×10^{-9} M (Kotowska et al., 2014). BPA has also been detected in natural surface waters, including rivers of the Greater Poland Voivodeship region, where concentrations of approximately 4.16×10^{-10} M were reported (Czarczyńska-Goślińska et al., 2017). EE2 has likewise been detected in Polish surface waters at environmentally relevant concentrations, including 1.22×10^{-10} M (Kudłak, 2010) and 1.69×10^{-10} M (Czerwonka and Kaca, 2012). Accordingly, in the study were applied 3 different BPA concentrations: 10^{-7} M, 10^{-8} M, and 10^{-9} M, to encompass environmentally relevant upper and intermediate ranges reported for freshwater systems. EE2 was tested at a single concentration (1×10^{-9} M) and included as a positive control, given its high estrogenic potency and well-documented effects at this level (Mackenzie et al., 2003; Pettersson and Berg, 2007; Tamschick et al., 2016b).

2.3. Rearing conditions

Tadpoles were reared since April to August 2024 in an outdoor, semi-open setup in mesocosms (Frątczak et al., 2025c). The system consisted of tanks with large water volumes, exposed to ambient environmental conditions, including natural temperature fluctuations, precipitation, and full-spectrum solar radiation, with water chemistry maintained within ranges representative of regional freshwater habitats.

Eggs of the obtained specimens were kept in carbon-filtered (FCCBL, AquaFilter) tap water and raised until obtaining hatchlings at the Gosner stage 21, when gonadal formation typically begins (Gosner, 1960; Ogielska, 2018). A total of 300 tadpoles were used in the experiment. Individuals at Gosner stage 21 were randomly assigned to one of five experimental conditions: a control group (C), and four treatment groups exposed to different concentrations of bisphenol A (BPA7, BPA8, BPA9, at the concentrations of BPA 10^{-7} M, 10^{-8} M, or 10^{-9} M accordingly) or ethinylestradiol (EE2, at the concentration of 10^{-9} M). Each treatment and control group included three replicate tanks with 20 tadpoles each. The mesocosms tanks with a water volume of 150 L were made of high-density polyethylene (HDPE), a material certified for contact with food and drinking water, to prevent chemical leaching and interaction with experimental conditions. Control tanks contained only carbon-filtered tap water, while experimental tanks contained one of the EDCs.

For the preparation of the treatment solutions, BPA or EE2 were dissolved in 96 % ethyl alcohol, and then given to tanks containing 150 liters of filtered tap water. Initially both compounds were dissolved and diluted in ethanol. The mixtures prepared this way in the volume of 2 ml were further transferred into 2 L of water. The prepared mixtures were poured into tanks with 150 L of water and remixed to obtain final concentrations mentioned above.

We adopted a repeated pulse exposure design with a weekly static-renewal system. The entire volume of control water and EDC solutions in the tanks was renewed weekly. Tanks before preparing new solutions were cleaned from developing biofilm. The system was intentionally designed without sediment, macrophytes, or established multi-trophic biological communities, to maintain controlled and comparable aqueous exposure conditions for EDCs. The inclusion of additional elements would be expected to alter EDC availability in the water column through significant sorption, microbial degradation, and biotic uptake, thereby introducing additional sources of variability in exposure that

were not the focus of the present study. Accordingly, the experimental design represented an intermediate, outdoor exposure system that increased environmental realism relative to laboratory assays while retaining control over key physicochemical drivers of EDC exposure (Macaulay et al., 2025).

Tadpoles were fed boiled *Urtica dioica* leaves twice per week, i.e. until the entire portion of food was eaten. Exposure continued until the onset of tail resorption, around the 43 Gosner stage (Gosner, 1960), after which individuals were transferred to semi-aquatic enclosures to complete metamorphosis for around two weeks, until reaching Gosner stage 46. At this stage, they were fed crickets or cockroaches twice per week *ad libitum*.

2.4. Monitoring of water conditions

The concentrations of target solutions of EDCs in randomly chosen, replicate tanks from each group were measured straight after preparing new solutions in the mesocosm. Samples were taken every week during the first 4 weeks of exposure, and then every other week until the end of the experiment. Water was collected in pre-cleaned glass bottles, cooled (<4 °C), and delivered to the laboratory within 24 h for analysis within the following day. Samples were coded to ensure blind analysis. To confirm target concentrations of BPA and EE2, LC-MS/MS with on-line SPE extraction analysis (Grobin et al., 2023) was performed in the commercial laboratory. The limit of detection (LOD) and limit of quantification (LOQ) for the analysed compounds were 0.005 μg/L and 0.015 μg/L for EE2, and 0.05 μg/L and 0.10 μg/L for BPA, respectively.

Measurements of temperature, pH, conductivity, salinity, and total dissolved solids (TDS) were taken weekly, after preparing fresh solutions of EDCs, from three randomly chosen tanks, using a multiparameter water quality meter (AZ8603, AZ Instruments). Additionally, a continuous registration of temperature was taken in three randomly chosen tanks, with the use of analog data loggers (Thermochron iButton, model DS1921G). For the collected data, normality of distribution was assessed using the Shapiro-Wilk test. Temperature, conductivity, salinity, and TDS did not follow a normal distribution in any group ($p < 0.05$), whereas pH was normally distributed ($p > 0.05$). Pairwise comparisons between tanks were conducted using the Wilcoxon (Mann–Whitney) test for normally distributed parameters, and one-way ANOVA for pH. Additionally, Kruskal-Wallis tests were used to assess differences in mean daily water temperature, recorded by data loggers, between tanks for each day of the 90-day experiment.

2.5. Dissection and tissue analysis

Following metamorphosis, frogs were anesthetized and then euthanized for analysis using 0,5 % tricaine mesylate (MS-222; Sigma-Aldrich), following immersion protocols described in Dedukh et al. (2019). Anesthesia was confirmed by the absence of vital signs. All procedures were conducted under the approval granted by the Local Ethical Committee for Animal Experiments in Poznań (Application No 2/2024/P1, approval date: February 27, 2024).

Each frog was dissected for gonadal analysis. The body cavity was opened along the ventral midline, and the gonads were examined *in situ* under a stereomicroscope (Olympus SZX7). Phenotypic sex was assessed based on the gross anatomy of the gonads, and any abnormalities were recorded. Then, the gonads were fixed in Bouin's solution (Sigma-Aldrich) for 4 h to preserve the tissue. After rinsing in several rounds in 70 % ethanol to remove residual fixative, samples were stored in ethanol until further processing. Then gonads from a subset of 105 individuals were embedded in Paraplast, sectioned at 7 μm (Leica RM 2255), and stained with Mallory's trichrome. Sections were examined with a Zeiss Axioskop 20 microscope and photographed with a cooled Carl Zeiss Axio-Cam HRc CCD camera.

Histological evaluation was performed on 20 individuals per group (10 females and 10 males), except for the EE2 group, in which only 4 males were available for assessment due to a strong sex ratio shift. For this reason, in the EE2 group, 20 individuals recognized as phenotypic females were analyzed. One or both gonads per individual were analyzed depending on the preservation quality of the samples. In total, 16 to 20 gonads per sex and group were evaluated (details in Supplementary Table S1). In intersex or phenotypically ambiguous individuals, both gonads were assessed separately. Gonads were analyzed to confirm sex, assess developmental stage, and identify potential mixed-sex or intersex features or histopathological changes. Gonadal structures were classified following developmental stages described by Haczkiewicz and Ogielska (2013). For the description of gonadal abnormalities, we used the nomenclature proposed by Lutz et al., 2008, defining mixed-sex as the case of possessing both ovarian and testicular tissue within one gonad, and intersex as the case of possessing ovary and testis as separate structures in one individual.

2.6. Photographs and measurements

Euthanized specimens were photographed using a Pentax Optio WG-6 camera mounted above a glass plate with a scale marker. Limbs were photographed under standardized lighting. The obtained images of limbs were used for further analysis. Individual digits on limbs were numbered in accordance with the pattern presented by Kaczmarski et al., 2021. Digit lengths were measured using Tmorph Gen 6 software (Sheets HD, 2000) with landmarks placed following the methodology described by Kaczmarski et al., 2021, using digital calipers accurate to 0.01 mm. Each limb was measured twice in random order. Additional metrics—snout-vent length (SVL) and head width (HW)—were recorded using a digital caliper (accuracy: 0.01 mm) twice. Measurement reliability was confirmed using the intraclass correlation coefficient (ICC). All morphometric measurements demonstrated very high repeatability (ICC = 0.906–0.998).

2.7. Data processing

As a measure of somatic condition, the Scaled Mass Index (SMI) was calculated, separately for males and females to account for sexual dimorphism in body condition. For each sex, a linear model was fitted with log-transformed weight as the response variable and log-transformed SVL as the predictor. The scaling exponent (bsma) was derived by dividing the slope of this model (betaOLS) by the Pearson correlation coefficient between log(weight) and log(SVL). The SMI was then computed for each individual using the formula: SMI = weight × (L0 / SVL)^bsma, where L0 represents the mean SVL for the respective sex (Peig and Green, 2009).

The DR was calculated as the length of the second digit divided by the length of the fourth digit (2D:4D) for each limb. Calculated DRs were then adjusted for allometric effects to remove the influence of body size separately for males and females, in order to better isolate sex-related differences in digit ratios (Frątczak et al., 2025a). For each limb's DR, the correlation between the lengths of the second and fourth digits was assessed using Pearson's correlation test. If the correlation was significant ($p < 0.05$), two models were fitted: a power function model (log(digit II) ~ log(digit IV)) using a generalized linear model, and a linear model (digit II ~ digit IV). The model with the lower Akaike Information Criterion (AIC) was selected, and residuals from this model were used to adjust the second digit length by adding the mean digit length to the residuals. The adjusted DR was then calculated as the adjusted second digit length divided by the fourth digit length. If no significant correlation was found, the original ratio was retained as the adjusted DR.

2.8. Data analysis: somatic indices and digit ratio

Multicollinearity among predictors was evaluated by calculating the Variance Inflation Factors (VIF). Predictors included SVL, HW, weight, and SMI, with VIF values inspected to ensure independence among

variables. Because of high correlation and high VIF between HW, weight, and SVL, for the further analysis, we used only SVL as the body size indicator. There was no issue of multicollinearity in the case of SVL and SMI (VIF = 1.061). The correlation plot is shown in the supplementary materials (Fig S1).

Body size (SVL) and condition (log(SMI)) were analyzed using linear models with experimental treatment (EXP), sex (SEX), and their interaction as predictors. Model simplification was performed using the drop1 function with F-tests to remove non-significant interactions and main effects, retaining significant terms based on p-values < 0.05. Model diagnostics were performed, and significant effects were visualized with predicted values and pairwise comparisons using the emmeans function with Tukey adjustment **(emmeans package in R)**.

Sex ratios were analyzed using a generalized linear model (GLM) employing a binomial family and logit link function. The model assessed the proportion of females across experimental groups, with the control group (C) as the reference level. The post-hoc pairwise comparisons were conducted using emmeans with Tukey adjustment.

Linear models were employed to analyze the adjusted DRs (FL_2D4D_adj, FR_2D4D_adj, HL_2D4D_adj, HR_2D4D_adj) for each limb. Initial models included experimental treatment (EXP), Scaled Mass Index (SMI), sex (SEX), Snout-Vent Length (SVL), and their interactions (EXP×SMI, EXP×SVL, EXP×SEX) as predictors. Model simplification was performed using the drop1 function with F-tests to remove non-significant interactions and main effects, retaining significant terms based on p-values < 0.05. Final models for each limb were selected based on these results.

All statistical analyses were performed in R (version 4.1.3). Packages utilized included lme4, emmeans, ggplot2, performance. Residual normality, homoscedasticity and influential cases (Cook's $D > 0.5$) were checked graphically and we did not find violation of assumptions.

3. Results

3.1. Conditions in water tanks

Measured in random tanks weekly, target BPA concentrations ranged, for BPA7 group from $1{,}00 \times 10^{-7}$ M to $1{,}48 \times 10^{-7}$ M, for BPA8 group from $1{,}00 \times 10^{-8}$ M to $1{,}28 \times 10^{-8}$ M and for BPA9 group from 1×10^{-9} to $2{,}27 \times 10^{-9}$. BPA was not detected in the negative control or EE2 group. Concentration of EE2 in the positive control group ranged from $1{,}36 \times 10^{-9}$ M to $2{,}1 \times 10^{-9}$ M. EE2 was not detected in either the negative control or BPA groups. Results from the analysis of EDCs are summed up in Table S2.

There were no significant differences in weekly measurements of temperature, CON, SALT, TDS or pH (all $p > 0.05$) between tanks. There were also no significant differences in mean temperature between tanks on any individual day ($p = 0.37$). Mean values of weekly measured water parameters by tank are shown in Fig. S2, and their weekly variation across tanks is presented in Fig. S3. Daily mean temperature between tanks is presented in Fig. S4.

3.2. Body size and somatic condition

Neither the endocrine treatments nor sex affected snout–vent length (SVL) or body weight (data not shown). A reduced model that contained only the main terms for treatment (EXP) and SEX explained 1.8 % of the no significant coefficients ($p \geq 0.21$).

Log-transformed Scale-Mass Index (SMI) varied among treatments (overall $F_{4,242} = 11.27$, $p < 0.001$) but not between sexes ($p = 0.21$) and showed no EXP × SEX interaction. Relative to controls, SMI was lower in BPA7 (–0.139 ± 0.036 SE, $p < 0.001$) and EE2 (–0.173 ± 0.036, $p < 0.001$) groups, whereas BPA8 and BPA9 did not differ from controls. Significant post hoc results of all comparisons are showed on Fig. 1A. All performed models are showed in the Supplementary Materials (Data File S1).

3.3. Sex ratio

The generalized linear model for sex ratio showed a significant effect of treatment on the proportion of females ($\chi^2 = 42.096$, df = 4, $p < 0.002$). The EE2 group produced a markedly female-biased sex ratio (≈9:1 F:M, corresponding to a female proportion of 0.90 ± 0.04). Odds of being a phenotypic female were 5.2-fold higher in EE2 relative to controls. Significant post hoc results of all comparisons are shown in Fig. 1B All performed models are showed in the Supplementary Materials (Data File S1).

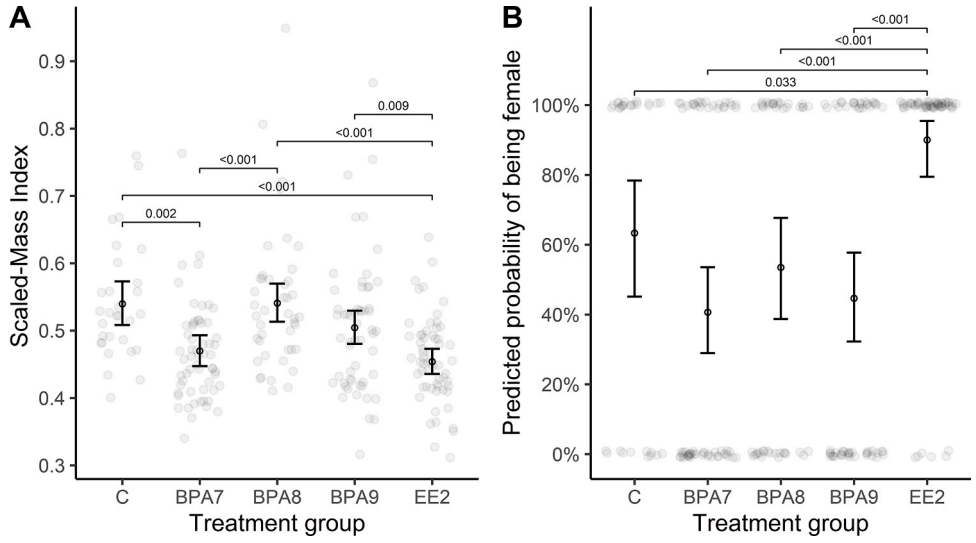

Fig. 1. Endocrine treatments effect on somatic condition, indicated by the Scale-Mass Index, and sex ratio in tadpoles of the marsh frog *Pelophylax ridibundus*. Treatment groups: *C* = control; BPA7, BPA8, BPA9 = bisphenol A at the concentration 10^{-7} M, 10^{-8} M, or 10^{-9} M accordingly, EE2 = ethinylestradiol at the concentration of 10^{-9} M. A: Scaled-Mass Index (SMI; mean ± 95 % CI) for each treatment group. Grey circles are individual values; horizontal brackets indicate significant Tukey pairwise differences (α = 0.05). B: Model-predicted probability of being female (mean ± 95 % CI) derived from the binomial GLM; grey points show raw outcomes (1 = female, 0 = male). Brackets denote significant Dunnett comparisons.

3.4. Histological examination of gonads

3.4.1. Females

All histological findings are summarized in Table 1. In gonads from individuals identified as phenotypic females based on gross gonadal anatomy, the following observations were made: in the control group, ovarian development followed the expected pattern, with most ovaries corresponding to stages VII–VIII (Ogielska and Kotusz 2004). Ovaries at the stage VII contained clusters of oogonia and meiocytes in the cortex, with the central region with some diplotene oocytes. In ovaries at the stage VIII, diplotene oocytes predominated, and early germ cells were sparse (Fig. 2A). The ovarian cavity was visibly reduced due to cortical expansion. Only one ovary in the control group exhibited a small, localized area of degenerating germ cells, without significant tissue damage.

In the BPA7 group, the majority of ovaries were classified as stage VII. One ovary displayed a separated, distal segment of the gonad, recognized as a rudimentary metamer (Piprek et al., 2015) and focal degenerative changes of germ cells, although without extensive tissue damage (Fig. 2B). In the BPA8 group, ovaries reached stages between VII (Fig. 2C) and IX. Ovaries at the stage IX were almost entirely filled with diplotene oocytes, with only a few residual oogonia and early meiocytes at the cortical margin (germ patches), and the ovarian cavity reduced due to oocyte accumulation. A rudimentary metamer was observed in one ovary, while another showed small areas of localized degeneration of germ cells. In the BPA9 group gonads were assigned to stages between VI (Fig. 2D) and IX, and, similarly to BPA8 group, distal, rudimentary metamer was found in one ovary.

In the EE2 group (Fig. 3), ovarian development ranged from stage VII to IX, with most ovaries classified as stage IX (Fig. 3C). Two ovaries exhibited significant abnormalities with large part of the gonad devoid of germ cells, suggesting advanced degeneration. Additionally, two individuals exhibited clear intersex conditions, with one ovary, at the developmental stage VI, recognized by the presence of a thick cortex with numerous meiocytes and primary oogonia, only few diplotene oocytes in the center, and a well-defined ovarian cavity, and a contralateral testis with abnormal morphology (Fig. 3A). Moreover, another individual showed mixed-sex tissue in one gonad and an abnormal testis in the other (Fig. 3B).

3.4.2. Males

All males in the control group exhibited morphologically normal testes corresponding to stage VII of development (Fig. 4A), characterized by the presence of compact and well-organized seminiferous tubules filled with gonocytes (male germ cells). No secondary spermatogonia or meiocytes were observed at this stage, which aligns with this developmental phase (Haczkiewicz and Ogielska, 2013; Ogielska, 2018). In most individuals, the rete testis was already visible, further confirming the stage of maturation. Similarly, all males in the EE2 group ($n = 4$) exhibited normal testicular structure consistent with stage VII of development.

In all BPA exposure groups, the majority of testes were also classified as developmental stage VII (see Table 1). In all concentrations, testes with mild degeneration limited to individual seminiferous tubules were observed, as well as gonads with more extensive degeneration affecting seminiferous tubules in at least half of the gonadal tissue (Fig. 4B). Additionally, 2 individuals from BPA8 and BPA9, exhibited pronounced gonadal atrophy, indicating loss or remodeling of seminiferous tubules (Fig. 4C). Moreover, rudimentary metamers were identified in two individuals, in the BPA7 and BPA8 groups (Fig. 4B, 4D).

Table 1
Histological findings in the gonads of phenotypic females and males across experimental groups.

Phenotypic females					
Group	C	BPA7	BPA8	BPA9	EE2
n	10	10	10	10	20
	Developmental stage[1]				
Stage VI	0	0	0	1	0
Stage VII	2	7	2	2	5
Stage VIII	8	2	4	3	5
Stage IX	0	1	4	4	7
	Abnormalities[2]				
Minor degeneration[3]	1	1	1	0	0
Major degeneration[4]	0	0	0	0	2
Rudimentary metamer[5]	0	1	1	1	0
Intersex condition[6]	0	0	0	0	2
Mixed-sex condition[7]	0	0	0	0	1
Phenotypic males					
Group	C	BPA7	BPA8	BPA9	EE2
N	10	10	10	10	4
	Developmental stage[1]				
Stage VI	0	2	1	1	0
Stage VII	10	8	9	2	4
	Abnormalities[2]				
Minor degeneration[3]	0	3	2	3	0
Major degeneration[4]	0	3	3	4	0
Atrophy[8]	0	0	1	1	0
Rudimentary metamer[5]	0	1	0	1	0

C = control; BPA7, BPA8, BPA9 = bisphenol A at the concentration 10^{-7} M, 10^{-8} M, or 10^{-9} M accordingly, EE2 = ethinylestradiol at the concentration of 10^{-9} M.

[1] Ovarian and testicular developmental stages were classified following Ogielska (2018). In some individuals, due to the widespread abnormalities, developmental stage of the gonad was not assessed.

[2] Minor degeneration refers to the presence of degenerating cells without extensive tissue damage.

[3] Major degeneration refers to degeneration affecting more than half of the gonad.

[4] Rudimentary metamer describes a separated, distal segment of the gonad.

[5] Intersex refers to individuals possessing an ovary and a testis as two separate gonads.

[6] Mixed-sex condition describes a gonad containing both ovarian and testicular tissue.

[7] Atrophy refers to the lack of seminiferous tubules in a part of the male gonad.

3.5. Digit ratio

In the statistical analysis, the final model for the front left digit ratio included treatment (EXP), Scaled Mass Index (SMI), sex (SEX), and the interaction between treatment and Snout-Vent Length (EXP×SVL). The interaction term was significant ($F = 3.7702$, $p = 0.0054$), indicating that the effect of treatment on the adjusted DR depends on body size. Specifically, for the EE2 treatment, there was a significant negative interaction with SVL (Estimate = −0.0209, $p = 0.0353$), suggesting that larger individuals in the EE2 group have lower adjusted DRs (Fig. 5A). All models with tests and estimates are presented in Supplementary Materials (Data File S1). For the front right adjusted DR, the final model included treatment (EXP), SMI, sex (SEX), and SVL. However, none of the terms were significant (all $p > 0.05$), indicating no significant effect of treatment, body condition (SMI), sex, or body size on the front right adjusted DR.

For both hind adjusted DRs, the final models included treatment (EXP), body condition (SMI), sex (SEX), and the interaction between treatment and body size (EXP × SVL). Significant interactions between treatment and SVL were detected in both limbs, indicating that the effect of treatment depended on body size. In the hind left limb, the interaction was significant ($F = 2.65$, $p = 0.034$), while in the hind right limb, it was highly significant ($F = 5.42$, $p < 0.001$). In both cases, BPA7 and EE2 treatments showed significant negative interactions with SVL, meaning that larger individuals exposed to these treatments had lower adjusted DRs. Specifically, for the hind left limb, BPA7 (Estimate = –0.0127, $p = 0.020$) and EE2 (Estimate = –0.0128, $p = 0.040$) showed this pattern (Fig. 5B), while for the hind right limb, the effects were even stronger for BPA7 (Estimate = –0.0199, $p < 0.001$) and EE2 (Estimate = –0.0136, $p = 0.023$) (Fig. 5C).

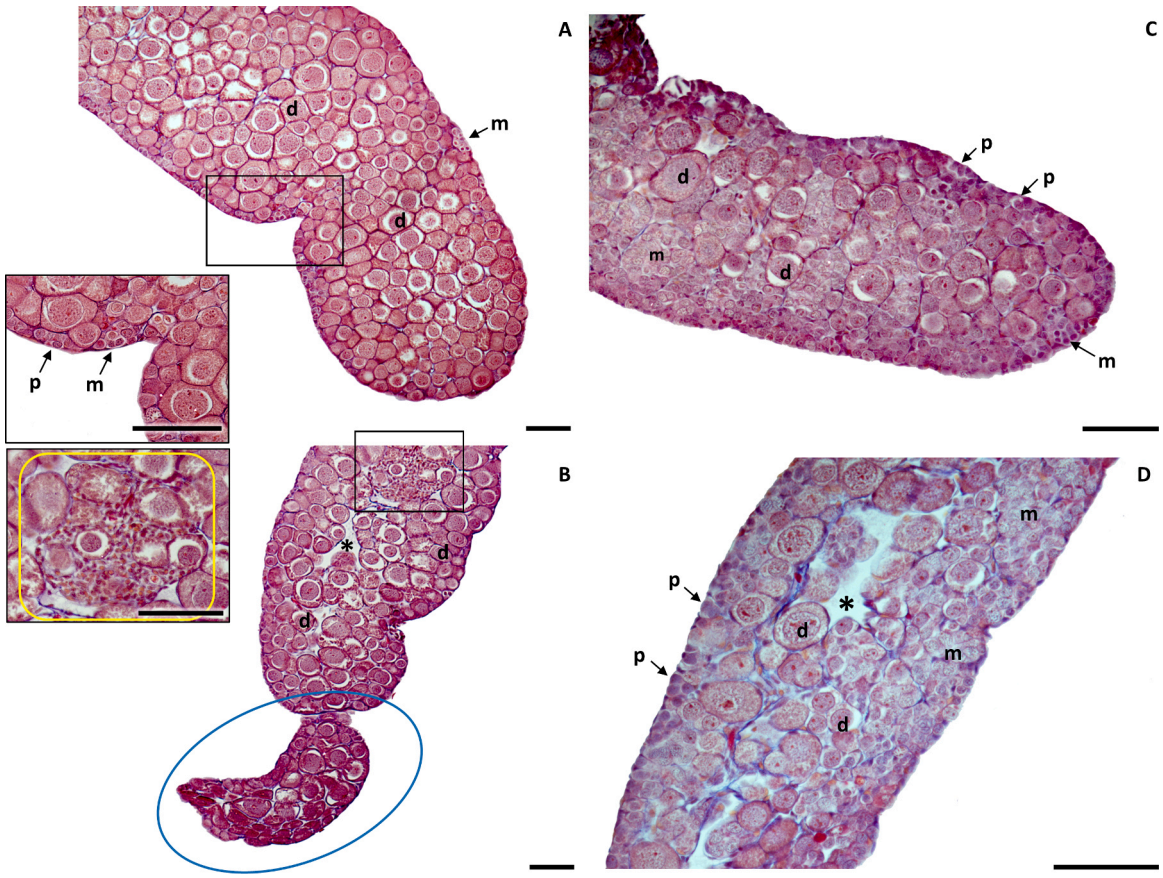

Fig. 2. Histological sections of ovaries from different exposure groups. A: Control group. The ovary appears normal and corresponds to developmental stage VIII. Numerous diplotene oocytes (d) fill the gonad, while at the periphery, densely packed meiotic cells (m) and primary oogonia (p) form a discontinuous layer, accumulating at the ends of the ovarian sacs. B: BPA7 group. Ovary at developmental stage VIII. A region of localized degeneration with the remaining somatic cells is present (framed in yellow), as well as a rudimentary metamer (circled in blue). C: BPA8 group. Ovary at developmental stage VII. Diplotene oocytes (d) vary considerably in size. The outer cortical layer is thick and composed of younger germ cell classes. Numerous clusters of meiotic cells (m) are present. D: BPA9 group. Ovary at developmental stage VI. Diplotene oocytes are scarce. The cortical layer is thick and populated by numerous meiocytes (m) and primary oogonia (p). The ovarian cavity (indicated by asterrisk) is clearly defined and still visible. Please note, that cytoplasmic shrinkage in the central region of some diplotene oocytes suggests suboptimal fixation, rather than abnormality.

4. Discussion

4.1. Conditions in water tanks

In this study, we adopted a mesocosm system to provide more environmentally relevant exposure conditions than standard laboratory assays. Mesocosms combine large water volumes with semi-natural settings, allowing experimental animals to develop under conditions that better approximate natural habitats, while still permitting control of key variables. Differences in target EDCs concentrations between tanks (Table S2) likely reflected natural heterogeneity and minor sampling differences, but overall measured values remained within the expected order of magnitude. Working with mesocosms is inherently challenging (Boone and James, 2005; Ford and Green, 2021) as high water volumes and outdoor conditions limit the possibility of continuous flow-through exposure and fully stable concentrations of studied compounds. At the same time, maintaining a balance between experimental control and natural variability is essential for ecological relevance. For this reason, we adopted a repeated pulse exposure with weekly static renewal as a practical compromise.

Environmental parameters (pH, conductivity, salinity, TDS and temperature) did not differ significantly between tanks and remained stable over the course of experiment. Conductivity (mean: 756 μS/cm) and salinity (mean: 0.39 ppt), while relatively high, were within ranges commonly reported for surface waters in Poland and representative for freshwater habitats in the study region (City of Poznań, 2025; Wojtasik and Kupiec, 2023), where stable *Pelophylax ridibundus* populations occur (Kolenda et al., 2024). These values are also consistent with ranges accepted in standard husbandry protocols for anuran models (Green, 2009).

4.2. Body size and somatic condition

We observed significant differences in SMI between treatment groups, indicating an impact on somatic condition. The scaled-mass index (SMI) has been validated as a reliable indicator of body condition in amphibians, positively correlating with future growth and thus serving as a meaningful indicator of fitness (Hinderer et al., 2025). Our analysis showed, that relative to controls, SMI was lower in BPA7 and EE2. Thus, it can be interpreted, that the highest concentration of BPA and EE2 had negative impact on condition of frogs.

As many studies on EDCs in amphibians is focus primarily on gonadal development and sexual characteristics (Brande-Lavridsen et al., 2008; Cai et al., 2020; Gyllenhammar et al., 2009; Hirakawa et al., 2013; Kloas et al., 1999; Pettersson and Berg, 2007; Tompsett et al., 2012; 2013) information on the impact of BPA and EE2 on body size and somatic condition is very limited. Previous studies did not find the impact of BPA on SVL (Pickford et al., 2003) and weight (Levy et al., 2004; Li et al., 2022; Pickford et al., 2003) in *Xenopus laevis*, while Tamschick et al. (2016a) observed a negative impact of BPA on SVL and weight in

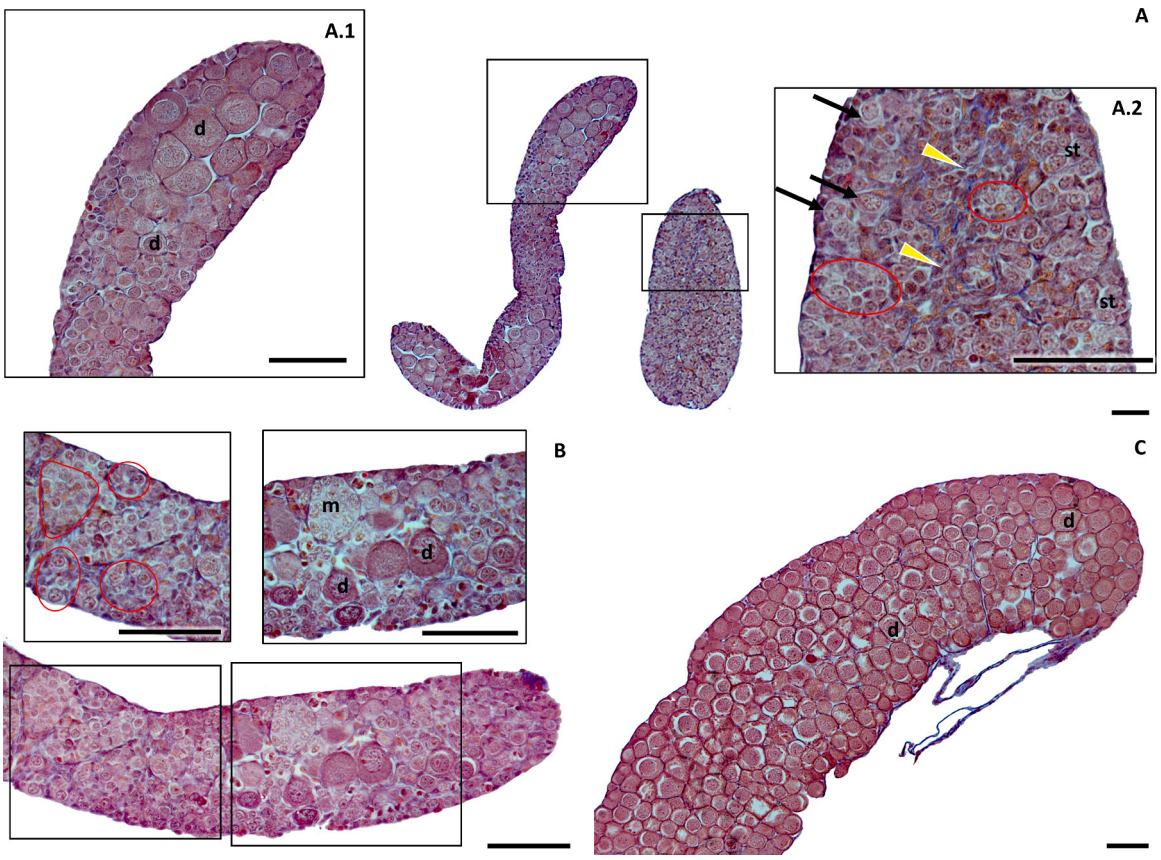

Fig. 3. Gonads of individuals from EE2 exposure groups. A: Intersex individual with one ovary (A.1) and a contralateral, abnormal gonad (A.2). The ovary (A.1) exhibits a typical structure corresponding to developmental stage VII, with diplotene oocytes (d) of various sizes and a thick cortical layer composed of younger germ cell classes. The contralateral gonad (A.2) has an abnormal structure, containing residual tubular elements (outlined in red) typical of testes and a central cluster of somatic cells, possibly indicating a regressing or developing rete testis (yellow arrowheads). Notably, few diplotene oocytes (black arrows) are also present. B: Mixed-sex gonad. One region shows ovarian features, including diplotene oocytes (d), primary oogonia (p), and clusters of early meiocytes (m), while the other region resembles testicular tissue with seminiferous tubules (st). C: Normal ovary at developmental stage IX, entirely filled with diplotene oocytes (d). Please note, that cytoplasmic shrinkage in the central region of some diplotene oocytes suggests suboptimal fixation, rather than abnormality.

X. laevis and *Hyla arborea*, and no impact on SVL or weight in *Bufotes viridis*.

Our findings of BPA's negative impact on somatic condition are indirectly supported by other studies, that report growth impairments caused by BPA exposure in amphibians such as *X. laevis*, likely caused by disruption of thyroid functions (Heimeier and Shi, 2010).

For EE2, studies reported no significant effects on SVL or weight in *X. laevis* (Cevasco et al., 2008; Tamschick et al., 2016a), *Xenopus tropicalis* (Pettersson et al., 2006) and in *Lithobates pipiens* (Hogan et al., 2008; Mackenzie et al., 2003). However, increases in SVL were observed in *Lithobates sylvaticus* (Mackenzie et al., 2003) and in both SVL and weight in *B. viridis* (Tamschick et al., 2016a), while *H. arborea* exhibited reduced SVL but unchanged weight under EE2 exposure (Tamschick et al., 2016a). These inconsistencies likely reflect species-specific endocrine sensitivity and exposure timing.

4.3. Sex ratio and histology of gonads: impact of EE2

Our histological evaluation of gonads of frogs exposed to ethinylestradiol (EE2) confirmed its profound effect on phenotypic sex, consistent with previous studies. The EE2 group produced a markedly female-biased sex ratio. These findings align with a growing body of literature reporting estrogen-induced sex reversal in amphibians. EE2 was shown to induce significantly female-biased sex ratios in *X. laevis* (10^{-9}–10^{-10} M, Pettersson et al., 2007), *X. tropicalis* (10^{-7}– 10^{-9} M, Pettersson et al., 2006; Gyllenhammar et al., 2009), *Rana temporaria* (10^{-10} M, Pettersson et al., 2007), *L. pipiens* (3×10^{-9} – 3×10^{-8} M, Mackenzie et al., 2003), *L. sylvaticus* (3.22×10^{-8} M, Tompsett et al., 2013), and *Bufo bufo* (3×10^{-6} M, Ujhegyi and Bókony, 2020). Complete sex reversal of males, confirmed by genetic testing, caused by EE2 was shown in studies of *X. laevis* (10^{-8} – 10^{-9} M, Tamschick et al., 2016b), *X. tropicalis* (2×10^{-11} – 10^{-10} M, (Hirakawa et al., 2013)), *H. arborea* (10^{-8} – 10^{-9} M, Tamschick et al., 2016b), and *B. viridis* (10^{-8} – 10^{-9} M, Tamschick et al., 2016b).

Moreover, the histological results in our study demonstrated that two individuals developed as intersexes, each displaying one normal ovary at the early developmental stage (VI) and a contralateral, abnormal testis. Additionally, one individual exhibited a gonad with mixed-sex tissue and a contralateral abnormal testis. Similar findings were noted in other species. Mixed-sex gonads or intersex individuals were reported in *X. laevis* (10^{-8} – 10^{-10} M, Tompsett et al., 2012, 2013), *X. tropicalis* (10^{-11} M, (Hirakawa et al., 2013)), *R. temporaria* (10^{-10} M, Pettersson et al., 2007), *Rana septentrionalis* ($\sim 10^{-11}$ M, Park and Kidd, 2005), and *L. pipiens* (5×10^{-9} M, Hogan et al., 2008). Presence of oocytes within testicular tissue after exposure to EE2 was also shown in *X. laevis* (10^{-8} M, Cevasco et al., 2008) and *L. pipiens* (5×10^{-9} M, Hogan et al., 2008). Moreover, EE2 exposure resulted in retarded and abnormal gonadal development in *H. arborea* (10^{-8} M, Tamschick et al., 2016c) and *B. viridis* (10^{-8} –10^{-9} M, Tamschick et al., 2016c).

Together, these studies confirm that EE2 induces profound, species-specific effects on amphibian gonadal differentiation, including sex reversal, intersex formation and mixed-sex gonads. These findings are of considerable ecological importance. A marked female bias in amphibian populations may alter mating dynamics in amphibians by increasing

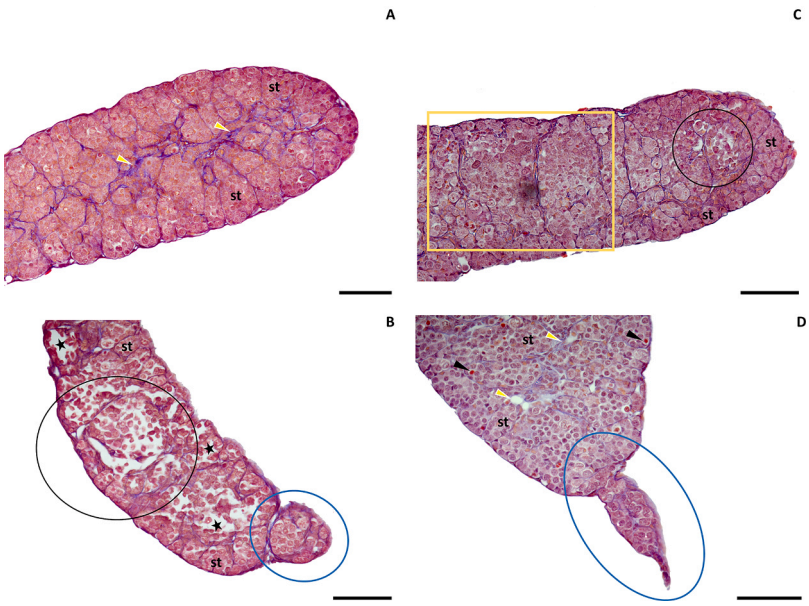

Fig. 4. Histological sections of testes from different exposure groups. A: Control group. Testis appears normal and corresponds to developmental stage VII, with compact and well-organized seminiferous tubules (st) filled with gonocytes. A developing rete testis is visible (yellow arrowheads). B: BPA7 group. Testis shows seminiferous tubules with abnormally large lumens (indicated by a star). In a large part of the gonad, germ cells are detached from the tubule walls and Sertoli cells, suggesting degeneration (circled in black). A rudimentary metamer is present in the distal region (circled in blue). C: BPA8 group. Most of the gonad lacks typical seminiferous tubules. A large structure filled with germ cells (framed in yellow) suggests disintegration of tubule walls and disrupted testicular architecture, possibly indicating atrophy. In one area, germ cells are also detached from the tubule walls (circled in black). D: BPA9 group. Testis at developmental stage VII. Rete testis is well-defined (yellow arrowheads). A few degenerating germ cells are present (black arrowheads). A rudimentary metamer (circled in blue) is visible in the distal part.

competition among females for access to males and shifting the intensity of sexual selection, as shown for *Oophaga pumilio* (Pröhl, 2002), formerly *Dendrobates pumilio*.

Moreover, in *Pelophylax* species complex in particular, a strongly female-skewed sex ratio could elevate the risk of hybridization in systems with *Pelophylax esculentus*. *P. esculentus* reproduces via hybridogenesis — a hemiclonal process where the hybrid discards the genome of one parental species during gametogenesis and relies on backcrossing with the other parental species to regenerate the hybrid lineage (Berger, 1977; Dedukh et al., 2019). In a theory, a deficit of *P. ridibundus* males in hybrid complexes could increase the probability of matings involving *P. esculentus* males, thereby destabilizing the balance between parental species and hybrids. Over time, this may alter gene flow, the persistence of pure species, and the stability of hybrid complexes.

4.4. Sex ratio and histology of gonads: impact of BPA

Regarding the impact of BPA on phenotypic sex and gonadal development, studies have reported varying effects depending on species and experimental conditions. In *X. laevis*, Kloas et al. (1999) and Levy et al. (2004) demonstrated that BPA exposure at the concentration of 10^{-7} M led to feminization of gonads and a female-biased sex ratio, although morphological irregularities were rare, with 1.1 % of testes showing mixed-sex conditions (Levy et al., 2004). Histological analyses confirmed that most gonads appeared well-developed without structural abnormalities.

In contrast, in our study, we did not see a feminizing effect of BPA. The sex ratio in BPA groups was approximately balanced or slightly male-biased, but without genetic sex identification, it is not possible to confirm the presence or lack of events of sex reversals. It was clear, however, that BPA induced both degenerative and morphogenetic abnormalities in amphibian gonads. We found widespread degeneration of gonadal tissue, with the loss of germ cells, in the testes of a number of males in the BPA exposure groups (Table 1.) indicating potential sterility. This finding is paralleling with the observation of significantly reduced germ cell counts and abnormal testicular structures with enlarged lumens within seminiferous tubules observed in *X. laevis* under exposure to 10^{-6}, 10^{-7}, and 10^{-8} M of BPA (Tamschick et al., 2016a).

Additionally, in our study, several males from the BPA exposure groups had separated, distal segments containing testicular tissue, which can be interpreted as rudimentary metamers that failed to degenerate properly at earlier developmental stages (Piprek et al., 2015; Tamschick et al., 2016a). Observed in our specimens, rudimentary metamers were always located at the distal end of the gonads, were consistently smaller and less developed compared to the main body of the gonad and were separated from it by a visible gap or constriction. In all cases, only single, isolated metamers were present, lacking the typical organization and size seen in normally developing gonadal tissue. Similar observations were made by Tamschick et al. (2016a), who found segmented testes in *H. arborea* and *B. viridis* in groups exposed to BPA. Under normal testicular differentiation, the gonad is initially metameric (segmented), and the testes develop from only the proximal (anterior) metameres, while the distal (posterior) metameres degenerate – a process starting at gonadal developmental Stage IV (Haczkiewicz and Ogielska, 2013).

Interestingly, we observed separated distal segments also in ovaries of three females from BPA exposure groups, which can similarly be interpreted as remnants of metameres that, in this case, failed to develop into fully differentiated ovarian lobes. Normally, ovarian metameres are formed at gonadal Stage III and then differentiate into ovarian lobes (sacs) (Ogielska and Kotusz, 2004; Piprek et al., 2015). The presence of such rudimentary metamers in our female specimens at later developmental stages suggests arrested or retarded lobe development, indicating impaired ovarian morphogenesis.

Taken together, our results indicate that BPA primarily disrupts gonadal development and tissue integrity rather than altering phenotypic sex determination. Data from the literature suggests, that BPA can significantly impact estrogenic pathways, but it does not always function as a typical estrogen. It can bind to estrogen receptors, but it acts either as an agonist or antagonist depending on dose and receptor subtype (Li et al., 2012). It was also shown to modulate estrogen-responsive endpoints, including expression of estrogen receptor genes in *Andrias*

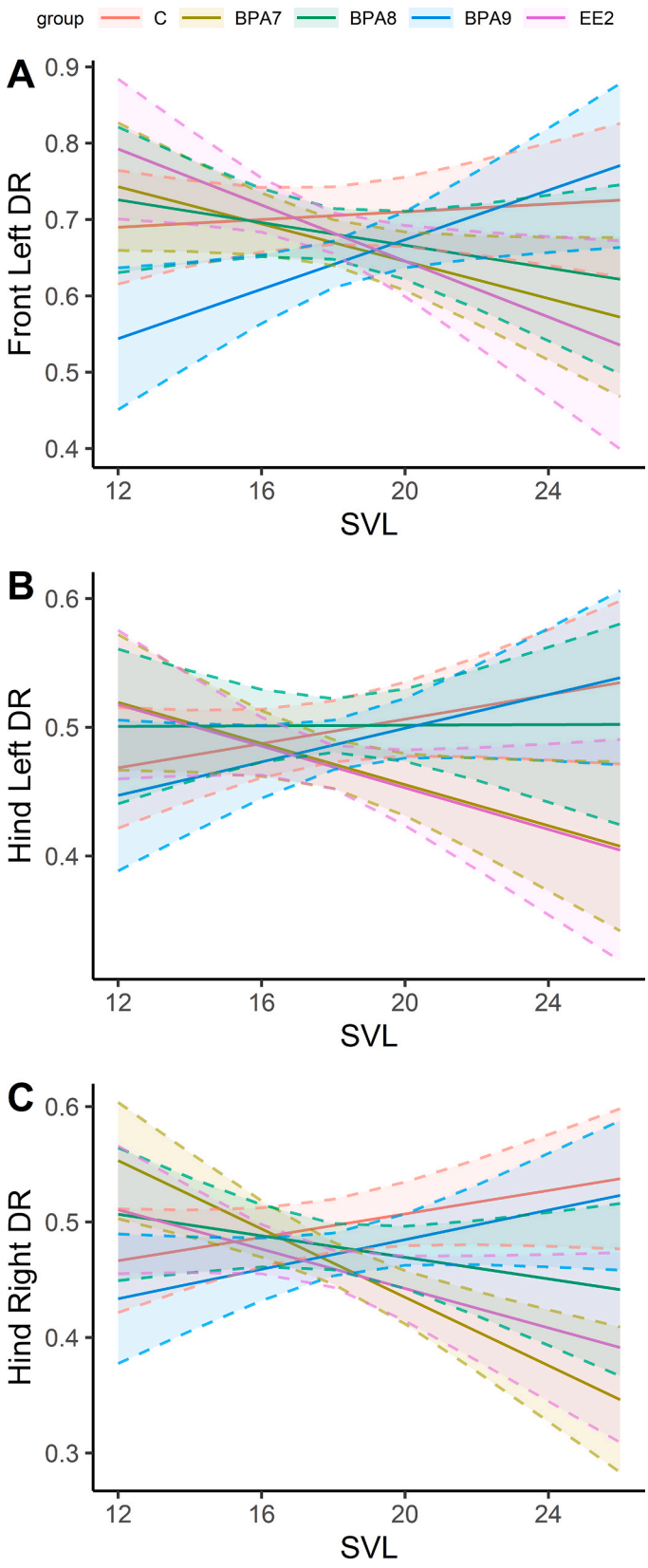

Fig. 5. Size-adjusted 2D:4D digit ratio (DR) of fore- and hind-limbs in the marsh frogs *Pelophylax ridibundus* exposed to endocrine-disrupting compounds. Mean ± 95 % CI of the DR are plotted against snout-to-vent length (SVL) for each treatment group: *C* = control; BPA7 = 10^{-7} M bisphenol A, BPA8 = 10^{-8} M bisphenol A, BPA9 = 10^{-9} M bisphenol A, EE2 = 10^{-9} M 17-α-ethinylestradiol.

davidianus (Gao et al., 2019) and *X. laevis* (Levy et al., 2004), egg-yolk protein genes in *Bombina orientalis* (Gye and Kim, 2005) and *X. laevis* (Kloas et al., 1999) as well as to reduce activity of aromatase, a key enzyme in steroidogenesis in *B. orientalis* (Lee et al., 2010). BPA was also observed to interfere with thyroid hormone pathways in *X. laevis in vivo* (Heimeier et al., 2009; Iwamuro et al., 2003) and *in vitro* (Iwamuro et al., 2003; 2006), which could be crucial in the process of gonadal development. In contrast to a potent estrogen EE2, BPA exposure in our study did not result in detectable feminization at the population level, but instead caused pronounced degenerative abnormalities in both testes and ovaries. This distinction suggests that BPA acts through mechanisms that impair gonadal differentiation and maintenance without necessarily changing the sex-determining pathway, highlighting a mode of endocrine disruption that is developmentally disruptive but not obligatorily sex-reversing.

4.5. Digit ratio

In this study, we found no significant differences in 2D:4D digit ratio between males and females in *P. ridibundus*. This is consistent with previous findings in some amphibians, such as *Leptodactylus fuscus* (Lofeu et al., 2017), *Leiopelma pakeka* (Germano et al., 2011) and *Rhinella marina* (Beaty et al., 2016).

Nevertheless, while DR may not reflect phenotypic sex in *P. ridibundus*, it could still potentially serve as a sensitive bioindicator of environmental influences. As such, our results demonstrate that exposure to both EE2 and bisphenol A BPA affected adjusted DR, with effects dependent on body size (SVL). Significant treatment × SVL interactions were observed in both hindlimbs (EE2, BPA7) and the left forelimb (EE2), where larger individuals tended to have lower adjusted DR. Interestingly, in the control group and lowest BPA concentration (10^{-9} M), larger individuals showed increased adjusted DR, whereas in higher-dose groups (BPA7, BPA8, EE2), the trend reversed.

The direction of these changes is somewhat counterintuitive. In mammals, higher DR ratios are typically associated with feminization, due to reduced androgen or increased estrogen exposure during development (Auger et al., 2013; Hönekopp and Watson, 2010). In amphibians, Lofeu et al. (2017) showed that individuals of *L. fuscus* exposed to testosterone as tadpoles have decreased DR, indicating a masculinizing effect following a similar pattern. In our study, however, decreased adjusted DR was observed after exposure to estrogenic EDCs (EE2 and BPA).

Estrogenic compounds would be expected to increase DR if amphibians mirrored mammalian patterns. In the study on mammals, Auger et al. (2013) found that BPA acted as a typical estrogen and strongly increased DR (feminized it) in male rats. Such differences emphasize that DR responses to endocrine disruptors are not universal and require careful, taxon-specific interpretation. Our results suggest that in *P. ridibundus*, estrogenic compounds may interfere with endogenous hormone balance or act via non-classical pathways, producing DR shifts comparable to androgen-induced changes observed in other species. Regardless of their direction, such alterations in DR provide evidence of endocrine disruption.

Importantly, our previous research on allometrically controlled digit ratios in *R. arvalis* and *R. temporaria* showed that males exhibited significantly lower adjusted DR values across all limbs (Frątczak et al., 2025c), a pattern consistent with that observed in mammals. In contrast, in *Pelobates fuscus* males had significantly higher adjusted DR values across all limbs, which is the opposite of the typical mammalian pattern (Frątczak et al., 2025a). Worth noting, we also observed limb- and side-specific effects of EDCs on DR. In mammals the right forelimb is considered more sensitive to prenatal hormones (Hönekopp and Watson, 2010). Accordingly, in rats, the estrogenic compound BPA affected DR more strongly on the right forelimb, increasing it (Auger et al., 2013). In our study, however, EE2 exposure led to decreased adjusted DR primarily in the left forelimb, suggesting that amphibians may differ from

mammals in lateralization patterns of hormonal sensitivity and digit development.

Importantly, the timing of limb development in Anuran tadpoles significantly differs from the one in mammals and other amniotes, as it does not start during embryogenesis, but is initiated much later, just before the metamorphosis (Nieuwkoop and Faber, 2020; Royle et al., 2021), independently of action of thyroid hormones (Rot-Nikcevic and Wassersug, 2004; Royle et al., 2021). Additionally, the timing of forelimb and hindlimb bud emergence varies among anuran taxa, which may contribute to interspecific differences in DR pattern. Independently of bud emergence, hindlimbs have been reported to undergo faster development (including toe differentiation) than forelimbs (McDiarmid and Altig, 2000), which could be consistent with the more pronounced DR responses to EDC exposure observed in hindlimbs in our study.

The emergence of left and right forelimbs is also typically asynchronous, with the direction of asymmetry being taxon-specific and often associated with the spiracle (opening from the gill chamber) position (Malashichev, 2006; Zechini et al., 2017). Although there is currently no evidence that such asymmetry is present at the stage of early limb bud formation or digit differentiation, asymmetrical forelimb development may nonetheless contribute to differences in sensitivity of forelimb DR to EDCs exposure. Taken together, the literature and our data highlight that DR formation in anurans follows trajectories distinct from those of other vertebrates, underscoring the taxon-specific nature of DR variation.

Interpretation of our results is further complicated by methodological limitations in previous amphibian studies. Anuran DR patterns appear to differ significantly between species, and even population-level variation has been reported (for the review see: Kaczmarski et al., 2021). Such discrepancies may reflect both intrinsic and environmental factors, but also methodological issues, including incorrect digit numbering in forelimbs (Kaczmarski et al., 2021). The problem is also lack of allometric correction in most studies (Frątczak et al., 2025a). Because digit ratios are calculated from absolute digit lengths, individuals with generally longer or shorter digits may show different DR values even in the absence of true sex-related differences (Beaty et al., 2016; Lofeu et al., 2017). Without accounting for this effect, DR analyses may overestimate or mask sexual dimorphism (Lolli et al., 2017). Therefore, considering allometric effect allowed us to achieve a clearer assessment of sex-related patterns.

Overall, while DR did not differ between sexes in *P. ridibundus*, its responsiveness to estrogenic endocrine disruptors in a size- and limb-dependent manner suggests a potential link with endocrine disruption; however, given the currently limited empirical evidence in amphibians, these findings should be considered exploratory. Future studies should investigate the developmental mechanisms underlying DR variation, the role of lateralization, and the applicability of DR as a bioindicator across diverse amphibian taxa.

4.6. Study limitations

For ecological relevance, we applied a weekly static-renewal with repeated pulse exposure, serving as a practical compromise. It should be noted, that some decline in EDCs concentrations may have occurred between renewals through sorption, biodegradation and photodegradation, although likely moderate (Bai and Acharya, 2019; Ji et al., 2014). The tanks were made of food-grade HDPE with very low sorption potential for hydrophobic compounds such as BPA and EE2 (Uber et al., 2019; Wisniewski et al., 2025). Moreover, mesocosms contained drinking-quality tap water, additionally carbon-filtered, with no initial microbial biomass, preventing significant biodegradation.

Under freshwater conditions, photodegradation and algal-mediated transformation have been reported to result in approximately 10–15 % BPA loss over 10 days and EE2 half-lives of 11–20 days (Bai and Acharya, 2019; Ji et al., 2014). BPA biodegradation may lead to the formation of multiple products (Ike et al., 2002; Im and Löffler, 2016), most of which exhibit markedly lower biological activity (Ike et al., 2002; Suzuki et al., 2004). Also photodegradation of BPA can generate a diverse compounds, with uncertain estrogenic activity (Han et al., 2023). Microbial biotransformation of EE2 may involve initial conversion to less potent estrogens (Wang et al., 2019; Yu et al., 2013a), and intermediate metabolites whose biological activity is not clear (Hom-Diaz et al., 2015; Wang et al., 2019). Similarly, EE2 photolysis can yield multiple derivatives (Śliwka-Kaszyńska et al., 2019), however, under natural conditions, photochemical reactions of EE2 are believed to result in biologically inactive products (Whidbey et al., 2012). While such products were not identified or quantified in the present study, their contribution to the observed biological responses cannot be excluded. Importantly, fluctuating concentrations and co-occurrence of transformation products are characteristic of natural freshwater systems, supporting the ecological relevance of the applied exposure scenario (Kot-Wasik et al., 2016; Yu et al., 2013b).

Another limitation concerns survival, which differed among treatments groups (Fig. S5), and the binomial GLM detected a treatment effect. This represents a limitation, as selective mortality could in principle bias trait distributions among survivors. However, background mortality was also high in the control group, and routine measurements showed no between-tank differences in temperature, pH, conductivity, salinity or TDS, nor any contamination of control tanks with BPA/EE2, arguing against group-specific husbandry artefacts. The strongest biological signals (female bias with EE2) were internally consistent and in line with previous findings in anurans, suggesting that sex-specific mortality is unlikely to explain the reported effects. EE2, chosen by us as a positive control is a potent estrogen, well known to induce feminization and sex reversal in amphibians (Mackenzie et al., 2003; Pettersson and Berg, 2007;Tamschick et al., 2016b). Moreover, our main outcomes were estimated from individual-level models with confidence intervals, so unequal survival mainly reduced precision rather than produced false treatment effects.

An important limitation of the present study is also the lack of genetic sex identification, which restricts the interpretation of treatment effects. Current knowledge of sex-determining genes in amphibians remains limited (for the review, see: Bullejos et al., 2024) and genetic tools for sex identification are available only for a few species. To our best knowledge, no such methods are established for *P. ridibundus*. Accordingly, sex was assessed based on gonadal morphology. This approach allows reliable detection of strong hormonal effects, however, excludes confirmation of subtle sex reversal events. Future studies, integrating histological analysis with genetic sex markers (once such tools become available for *P. ridibundus*) would enable a more precise analysis of sex ratios and would further strengthen data on the effects of EDCs in this species.

5. Conclusions

This study demonstrates that exposure to two common endocrine-disrupting compounds, BPA and EE2, can affect sexual development, sex ratios and somatic condition in *P. ridibundus*, a widespread and non-model amphibian. These findings suggest that even low, environmentally relevant concentrations may impair traits essential for population stability, with potential consequences for biodiversity in contaminated habitats. Although the 2D:4D digit ratio did not reliably differentiate sexes in this species, its sensitivity to exposure points to possible use as a non-invasive biomarker of endocrine disruption, warranting further validation. Despite the logistical challenges of outdoor mesocosms, this approach provides an ecologically realistic exposure scenario and yields insights beyond those obtainable in laboratory assays. Incorporating non-model amphibians and semi-natural systems into ecotoxicological research is crucial for improving environmental risk assessment and for designing monitoring strategies that better capture the threats faced by wildlife.

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CRediT authorship contribution statement

Martyna Frątczak: Writing – review & editing, Writing – original draft, Methodology, Investigation, Funding acquisition, Conceptualization. **Katarzyna Szkudelska:** Writing – review & editing, Supervision, Methodology, Investigation, Conceptualization. **Marta Grobelna:** Writing – review & editing, Investigation. **Adam Hermaniuk:** Writing – review & editing, Methodology, Conceptualization. **Łukasz Jankowiak:** Writing – review & editing, Visualization, Formal analysis. **Mikołaj Kaczmarski:** Writing – review & editing, Methodology, Conceptualization. **Łukasz Myczko:** Writing – review & editing, Methodology, Investigation, Conceptualization. **Beata Rozenblut-Kościsty:** Writing – review & editing, Methodology, Investigation, Conceptualization. **Piotr Tryjanowski:** Writing – review & editing, Supervision, Methodology, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Martyna Fratczak reports financial support was provided by National Science Centre Poland. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.aquatox.2026.107788.

Data availability

Additional data and statistical models used in the study are available in the Supplementary Materials. The raw dataset analysed in this study is available from the corresponding author on reasonable request.

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Oświadczenia autorów

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Oświadczenie o współautorstwie

Niniejszym oświadczam, że w pracy: Frątczak, M., Kaczmarski, M., Szkudelska, K., & Tryjanowski, P. (2025). Assessing species bias in amphibian research on endocrine disruptors: beyond *Xenopus laevis*. Frontiers in Environmental Science, 13: 1556788. mój indywidualny udział w jej powstaniu polegał na: współudziale w opracowaniu koncepcji pracy i metod badawczych, samodzielnym zebraniu danych (przeprowadzeniu krytycznego przeglądu literatury, sformułowaniu wniosków), samodzielnym przygotowaniu pierwszej wersji manuskryptu, współudziale w korekcie manuskryptu i przygotowaniu odpowiedzi na recenzje.

Data ..20.04.2026.....

Podpis .....Martyna Frątczak.....

Imię i nazwisko: dr inż. Mikołaj Kaczmarski
Afiliacja:
Katedra Zoologii, Uniwersytet Przyrodniczy w Poznaniu

Oświadczenie o współautorstwie

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Data 20.05.2026 Podpis .....

Imię i nazwisko: prof. UPP dr hab. Katarzyna Szkudelska
Afiliacja:
Katedra Fizjologii i Biochemii Zwierząt, Uniwersytet Przyrodniczy w Poznaniu

Oświadczenie o współautorstwie

Niniejszym oświadczam, że w pracy: Frątczak, M., Kaczmarski, M., Szkudelska, K., & Tryjanowski, P. (2025). Assessing species bias in amphibian research on endocrine disruptors: beyond *Xenopus laevis*. Frontiers in Environmental Science, 13: 1556788. mój indywidualny udział w jej powstaniu polegał na: współudziale w nadzorze merytorycznym pracy, współudziale w opracowaniu koncepcji pracy i metod badawczych, współudziale w korekcie manuskryptu i przygotowaniu odpowiedzi na recenzje.

Data 2026.04.13 Podpis Szkudelska

Imię i nazwisko: prof. dr hab. Piotr Tryjanowski
Afiliacja:
Katedra Zoologii, Uniwersytet Przyrodniczy w Poznaniu

Oświadczenie o współautorstwie

Niniejszym oświadczam, że w pracy: Frątczak, M., Kaczmarski, M., Szkudelska, K., & Tryjanowski, P. (2025). Assessing species bias in amphibian research on endocrine disruptors: beyond *Xenopus laevis*. Frontiers in Environmental Science, 13: 1556788. mój indywidualny udział w jej powstaniu polegał na: współudziale w nadzorze merytorycznym pracy, współudziale w opracowaniu koncepcji pracy i metod badawczych, współudziale w korekcie manuskryptu i przygotowaniu odpowiedzi na recenzje.

Data ..20.04.2026..... Podpis .....

Imię i nazwisko: lek. wet. Martyna Frątczak
Afiliacja:
Katedra Zoologii, Uniwersytet Przyrodniczy w Poznaniu

Oświadczenie o współautorstwie

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Data ..20.04.2026.....

Podpis .....Martyna Frątczak.....

Imię i nazwisko: dr inż. Mikołaj Kaczmarski
Afiliacja:
Katedra Zoologii, Uniwersytet Przyrodniczy w Poznaniu

Oświadczenie o współautorstwie

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Data 20.04.2026 Podpis .....

Imię i nazwisko: prof. UPP dr hab. Katarzyna Szkudelska
Afiliacja:
Katedra Fizjologii i Biochemii Zwierząt, Uniwersytet Przyrodniczy w Poznaniu

Oświadczenie o współautorstwie

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Data 2026.04.13 Podpis Szkudelska

Imię i nazwisko: prof. dr hab. Piotr Tryjanowski
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Oświadczenie o współautorstwie

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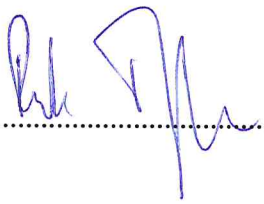
Data 20.04.2026

Podpis 

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Oświadczenie o współautorstwie

Niniejszym oświadczam, że w pracy: Frątczak, M., Kaczmarski, M., Jankowiak, Ł., Klessa, J., Bielicki, K., Lyskov, B., & Tryjanowski, P. (2025). Digit ratio in the common spadefoot toad Pelobates fuscus (Anura: Mesobatrachia: Pelobatidae): patterns and correlations. The European Zoological Journal, 92(1), 203-209. mój indywidualny udział w jej powstaniu polegał na: samodzielnym wykonaniu krytycznego przeglądu literatury, współudziale w opracowaniu koncepcji badań, współudziale w opracowaniu metodyki badań, współudziale w zebraniu danych (wykonanie sekcji osobników, współudział w wykonaniu pomiarów morfometrycznych i wykonaniu zdjęć osobników, współudział w wykonaniu pomiarów digit ratio), współudziale w analizie statystycznej wyników i ich interpretacji, samodzielnym przygotowaniu pierwszej wersji manuskryptu, współudziale w korekcie manuskryptu i przygotowaniu odpowiedzi na recenzje.

Data 20.04.2026 Podpis Martyna Frątczak

Imię i nazwisko: dr inż. Mikołaj Kaczmarski
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Oświadczenie o współautorstwie

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Data ...20.04.2026..

Podpis .....

Imię i nazwisko: Prof. USz. dr hab. Łukasz Jankowiak
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Katedra Ekologii i Antropologii, Uniwersytet Szczeciński

Oświadczenie o współautorstwie

Niniejszym oświadczam, że w pracy: Frątczak, M., Kaczmarski, M., Jankowiak, Ł., Klessa, J., Bielicki, K., Lyskov, B., & Tryjanowski, P. (2025). Digit ratio in the common spadefoot toad Pelobates fuscus (Anura: Mesobatrachia: Pelobatidae): patterns and correlations. The European Zoological Journal, 92(1), 203-209. mój indywidualny udział w jej powstaniu polegał na: współudziale w opracowaniu metod badawczych, wykonaniu analizy statystycznej danych i interpretacji wyników oraz współudziale w korekcie manuskryptu i przygotowaniu odpowiedzi na recenzje.

Data 02.04.2026 Podpis .....

Imię i nazwisko: Jacek Klessa
Afiliacja:
Katedra Zoologii, Uniwersytet Przyrodniczy w Poznaniu

Oświadczenie o współautorstwie

Niniejszym oświadczam, że w pracy: Frątczak, M., Kaczmarski, M., Jankowiak, Ł., Klessa, J., Bielicki, K., Lyskov, B., & Tryjanowski, P. (2025). Digit ratio in the common spadefoot toad Pelobates fuscus (Anura: Mesobatrachia: Pelobatidae): patterns and correlations. The European Zoological Journal, 92(1), 203-209. mój indywidualny udział w jej powstaniu polegał na: współudziale w zbieraniu danych (współudział w wykonaniu pomiarów digit ratio) oraz współudziale w korekcie manuskryptu.

Data 20.04.2026 Podpis Jacek Klessa

Imię i nazwisko: Kajetan Bielicki
Afiliacja:
Katedra Zoologii, Uniwersytet Przyrodniczy w Poznaniu

Oświadczenie o współautorstwie

Niniejszym oświadczam, że w pracy: Frątczak, M., Kaczmarski, M., Jankowiak, Ł., Klessa, J., Bielicki, K., Lyskov, B., & Tryjanowski, P. (2025). Digit ratio in the common spadefoot toad Pelobates fuscus (Anura: Mesobatrachia: Pelobatidae): patterns and correlations. The European Zoological Journal, 92(1), 203-209. mój indywidualny udział w jej powstaniu polegał na: współudziale w zbieraniu danych (współudział w wykonaniu pomiarów morfometrycznych osobników i wykonaniu zdjęć osobników) oraz współudziale w korekcie manuskryptu.

Data ..8..04..2026...

Podpis ...Kajetan Bielicki...

Imię i nazwisko: lek. wet. Borys Lyskov
Afiliacja:
Katedra Zoologii, Uniwersytet Przyrodniczy w Poznaniu

Oświadczenie o współautorstwie

Niniejszym oświadczam, że w pracy: Frątczak, M., Kaczmarski, M., Jankowiak, Ł., Klessa, J., Bielicki, K., Lyskov, B., & Tryjanowski, P. (2025). Digit ratio in the common spadefoot toad Pelobates fuscus (Anura: Mesobatrachia: Pelobatidae): patterns and correlations. The European Zoological Journal, 92(1), 203-209. mój indywidualny udział w jej powstaniu polegał na:
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Data 10/09/2026 Podpis Borys Lyskov

Imię i nazwisko: prof. dr hab. Piotr Tryjanowski
Afiliacja:
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Oświadczenie o współautorstwie

Niniejszym oświadczam, że w pracy: Frątczak, M., Kaczmarski, M., Jankowiak, Ł., Klessa, J., Bielicki, K., Lyskov, B., & Tryjanowski, P. (2025). Digit ratio in the common spadefoot toad Pelobates fuscus (Anura: Mesobatrachia: Pelobatidae): patterns and correlations. The European Zoological Journal, 92(1), 203-209. mój indywidualny udział w jej powstaniu polegał na: współudziale w nadzorze merytorycznym pracy, współudziale w opracowaniu koncepcji pracy i opracowaniu metod badawczych oraz współudziale w korekcie manuskryptu.

Data ...20.04.2026.....

Podpis .....

Imię i nazwisko: lek. wet. Martyna Frątczak
Afiliacja:
Katedra Zoologii, Uniwersytet Przyrodniczy w Poznaniu

Oświadczenie o współautorstwie

Niniejszym oświadczam, że w pracy: Frątczak, M., Szkudelska, K., Grobelna, M., Hermaniuk, A., Jankowiak, Ł., Kaczmarski, M., Myczko, Ł., Rozenblut-Kościsty, B, Tryjanowski, P. (2026). Impact of bisphenol A and ethinyloestradiol on sex, body condition and digit ratio of the marsh frog *Pelophylax ridibundus* in the mesocosm exposure system. Aquatic Toxicology, 107788. mój indywidualny udział w jej powstaniu polegał na: samodzielnym wykonaniu krytycznego przeglądu literatury, współudziale w opracowaniu koncepcji badań, współudziale w opracowaniu metodyki badań, współudziale w przeprowadzeniu eksperymentu (współudział w przygotowywaniu zbiorników eksperymentalnych i odchowie osobników, kontrola warunków w zbiornikach, pobieranie prób do analizy chemicznej wody) i zebraniu danych (samodzielne wykonanie pomiarów morfometrycznych i zdjęć osobników, współudział w wykonaniu pomiarów digit ratio, samodzielne wykonanie sekcji osobników, pobranie materiału do analizy histologicznej, współudział w analizie histologicznej próbek), współudziale w analizie statystycznej wyników i ich interpretacji, samodzielnym przygotowaniu pierwszej wersji manuskryptu, współudziale w korekcie manuskryptu i przygotowaniu odpowiedzi na recenzje.

Data 20.04.2026 Podpis Martyna Frątczak

Imię i nazwisko: prof. UPP dr hab. Katarzyna Szkudelska
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Oświadczenie o współautorstwie

Niniejszym oświadczam, że w pracy: Frątczak, M., Szkudelska, K., Grobelna, M., Hermaniuk, A., Jankowiak, Ł., Kaczmarski, M., Myczko, Ł., Rozenblut-Kościsty, B, Tryjanowski, P. (2026). Impact of bisphenol A and ethinyloestradiol on sex, body condition and digit ratio of the marsh frog *Pelophylax ridibundus* in the mesocosm exposure system. Aquatic Toxicology, 107788. mój indywidualny udział w jej powstaniu polegał na: współudziale w nadzorze merytorycznym pracy, współudziale w opracowaniu koncepcji pracy i metod badawczych, współudziale w realizacji eksperymentu (przygotowywanie roztworów do ekspozycji), współudziale w korekcie manuskryptu i przygotowaniu odpowiedzi na recenzje.

Data 2026.09.13 Podpis Szkudelska

Imię i nazwisko: mgr Marta Grobelna
Afiliacja:
Katedra Zoologii, Uniwersytet Przyrodniczy w Poznaniu

Oświadczenie o współautorstwie

Niniejszym oświadczam, że w pracy: Frątczak, M., Szkudelska, K., Grobelna, M., Hermaniuk, A., Jankowiak, Ł., Kaczmarski, M., Myczko, Ł., Rozenblut-Kościsty, B, Tryjanowski, P. (2026). Impact of bisphenol A and ethinyloestradiol on sex, body condition and digit ratio of the marsh frog *Pelophylax ridibundus* in the mesocosm exposure system. Aquatic Toxicology, 107788. mój indywidualny udział w jej powstaniu polegał na: współudziale w przeprowadzeniu eksperymentu (współudział w przygotowywaniu zbiorników eksperymentalnych i odchowie osobników), zbieraniu danych (współudział w wykonaniu pomiarów digit ratio) oraz współudziale w korekcie manuskryptu.

Data 20.04.2026 Podpis Grobelna

Imię i nazwisko: dr Adam Hermaniuk

Afiliacja:

1. Zespół badawczy Ewolucja Strategii Życiowych, Wydział Biologii, Uniwersytet Jagielloński

2. Katedra Ekologii Ewolucyjnej i Fizjologicznej, Wydział Biologii, Uniwersytet w Białymstoku

Oświadczenie o współautorstwie

Niniejszym oświadczam, że w pracy: Frątczak, M., Szkudelska, K., Grobelna, M., Hermaniuk, A., Jankowiak, Ł., Kaczmarski, M., Myczko, Ł., Rozenblut-Kościsty, B, Tryjanowski, P. (2026). Impact of bisphenol A and ethinyloestradiol on sex, body condition and digit ratio of the marsh frog *Pelophylax ridibundus* in the mesocosm exposure system. Aquatic Toxicology, 107788. mój indywidualny udział w jej powstaniu polegał na: współudziale w opracowaniu koncepcji pracy i opracowaniu metod badawczych oraz współudziale w korekcie manuskryptu.

Data ....09.04.2026 r.

Podpis ....Adam Hermaniuk....

Imię i nazwisko: Prof. USz. dr hab. Łukasz Jankowiak
Afiliacja:
Katedra Ekologii i Antropologii, Uniwersytet Szczeciński

Oświadczenie o współautorstwie

Niniejszym oświadczam, że w pracy: Frątczak, M., Szkudelska, K., Grobelna, M., Hermaniuk, A., Jankowiak, Ł., Kaczmarski, M., Myczko, Ł., Rozenblut-Kościsty, B, Tryjanowski, P. (2026). Impact of bisphenol A and ethinyloestradiol on sex, body condition and digit ratio of the marsh frog *Pelophylax ridibundus* in the mesocosm exposure system. Aquatic Toxicology, 107788. mój indywidualny udział w jej powstaniu polegał na: współudziale w opracowaniu metod badawczych, wykonaniu analizy statystycznej danych i interpretacji wyników oraz współudziale w korekcie manuskryptu i przygotowaniu odpowiedzi na recenzje.

Data 07.04.2026 Podpis .....

Imię i nazwisko: dr inż. Mikołaj Kaczmarski
Afiliacja:
Katedra Zoologii, Uniwersytet Przyrodniczy w Poznaniu

Oświadczenie o współautorstwie

Niniejszym oświadczam, że w pracy: Frątczak, M., Szkudelska, K., Grobelna, M., Hermaniuk, A., Jankowiak, Ł., Kaczmarski, M., Myczko, Ł., Rozenblut-Kościsty, B, Tryjanowski, P. (2026). Impact of bisphenol A and ethinyloestradiol on sex, body condition and digit ratio of the marsh frog *Pelophylax ridibundus* in the mesocosm exposure system. Aquatic Toxicology, 107788. mój indywidualny udział w jej powstaniu polegał na: współudziale w opracowaniu koncepcji pracy i opracowaniu metod badawczych, współudziale w korekcie manuskryptu i przygotowaniu odpowiedzi na recenzje.

Data ..20.04.2026..

Podpis .....

Imię i nazwisko: dr hab. Łukasz Myczko prof. UPP
Afiliacja: Katedra Zoologii, Uniwersytet Przyrodniczy w Poznaniu

Oświadczenie o współautorstwie

Niniejszym oświadczam, że w pracy:Frątczak, M., Szkudelska, K., Grobelna, M., Hermaniuk, A., Jankowiak, Ł., Kaczmarski, M., Myczko, Ł., Rozenblut-Kościsty, B, Tryjanowski, P. (2026). Impact of bisphenol A and ethinyloestradiol on sex, body condition and digit ratio of the marsh frog *Pelophylax ridibundus* in the mesocosm exposure system. AquaticToxicology, 107788. mój indywidualny udział w jej powstaniu polegał na: współudziale w opracowaniu koncepcji pracy i opracowaniu metod badawczych, współudziale w realizacji eksperymentu (przygotowywanie zbiorników eksperymentalnych) oraz współudziale w korekcie manuskryptu.

Data 16.04.2026.....

Podpis .....

Imię i nazwisko: dr Beata Rozenblut-Kościsty

Afiliacja: Zakład Biologii Ewolucyjnej i Ochrony Kręgowców,
Wydział nauk Biologicznych,
Uniwersytet Wrocławski

Oświadczenie o współautorstwie

Niniejszym oświadczam, że w pracy: Frątczak, M., Szkudelska, K., Grobelna, M., Hermaniuk, A., Jankowiak, Ł., Kaczmarski, M., Myczko, Ł., Rozenblut-Kościsty, B, Tryjanowski, P. (2026). Impact of bisphenol A and ethinyloestradiol on sex, body condition and digit ratio of the marsh frog *Pelophylax ridibundus* in the mesocosm exposure system. Aquatic Toxicology, 107788. mój indywidualny udział w jej powstaniu polegał na: współudziale w opracowaniu koncepcji pracy i opracowaniu metod badawczych, współudziale w zbieraniu danych (współudział w analizie histologicznej) oraz współudziale w korekcie manuskryptu.

Data 08.04.2026 Podpis Beata Rozenblut-Kościsty

Imię i nazwisko: prof. dr hab. Piotr Tryjanowski
Afiliacja:
Katedra Zoologii, Uniwersytet Przyrodniczy w Poznaniu

Oświadczenie o współautorstwie

Niniejszym oświadczam, że w pracy: Frątczak, M., Szkudelska, K., Grobelna, M., Hermaniuk, A., Jankowiak, Ł., Kaczmarski, M., Myczko, Ł., Rozenblut-Kościsty, B, Tryjanowski, P. (2026). Impact of bisphenol A and ethinyloestradiol on sex, body condition and digit ratio of the marsh frog *Pelophylax ridibundus* in the mesocosm exposure system. Aquatic Toxicology, 107788. mój indywidualny udział w jej powstaniu polegał na: współudziale w nadzorze merytorycznym pracy, współudziale w opracowaniu koncepcji pracy i opracowaniu metod badawczych, współudziale w korekcie manuskryptu i przygotowaniu odpowiedzi na recenzje.

Data ....20.04.2026....

Podpis .....