Entwicklung einer Tierversuchsersatzmethode für endokrine Disruptoren – Grundlagenuntersuchungen am Hühnerembryo

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Jessica Scheider
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Dekan: **Prof. Dr. Sven Klimpel**
Goethe-Universität Frankfurt am Main
Institut für Ökologie, Evolution und Diversität
Integrative Parasitologie und Zoophysiologie
Max-von-Laue-Str. 13, D-60438 Frankfurt am Main

Gutachter: Prof. Dr. Jörg Oehlmann
Johann Wolfgang Goethe-Universität Frankfurt am Main
Institut für Ökologie, Evolution und Diversität
Aquatische Ökotoxikologie
Max-von-Laue-Str. 13, D-60438 Frankfurt am Main

Prof. Dr. Sven Klimpel
Johann Wolfgang Goethe-Universität Frankfurt am Main
Institut für Ökologie, Evolution und Diversität
Integrative Parasitologie und Zoophysiologie
Max-von-Laue-Str. 13, D-60438 Frankfurt am Main

Datum der Disputation: _____

Entwicklung einer Tierversuchsersatzmethode für endokrine Disruptoren – Grundlagenuntersuchungen am Hühnerembryo

Jessica Scheider

November 19

Niemals werden wir mit der Erforschung
des Lebens endgültig abschließen,
und wenn wir einen vorläufigen Abschluss
zeitweise versuchen, so wissen wir doch sehr wohl,
dass auch das Beste, was wir geben können, nicht mehr
bedeutet als eine Stufe zum Besseren.

August Weismann in: "Vorträge einer Deszendenztheorie",
1904 gehalten in Freiburg i.Br.

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Abkürzungsverzeichnis & Glossar

Amniota	(griech. amnion: Embryonalhülle) "Nabeltiere"/höhere Wirbeltiere: Reptilien, Vögel, Säugetiere
Antagonist	(griech. antagonistés: Gegner, Widersacher) Gegenspieler
BPA	Bisphenol A
Carnivor	Fleischfressend
DDT	Dichlordiphenyltrichlorethan, ein Organochlor-Insektizid
E_2	17β-Estradiol
EE_2	17α-Ethinylestradiol
ED	Endokriner Disruptor (engl. disrupt: stören)
Endokrin	(griech. Endo = innen, krinein = ausscheiden) im Inneren des Körpers hergestellte Stoffe, meist Signal- oder Botenstoffe (bspw. Hormone)
Exprimiert	(lat. expressio: [der] Ausdruck) Realisation der Information, die in der DNA eines Gens gespeichert ist
Endpunkt	In einer toxikologischen Untersuchung erfasstes Wirkkriterium
Fekundität	Tatsächliche Fruchtbarkeit, „Outcome“
Fertilität	biologische Fähigkeit zur Reproduktion
Gametenviabilität	Überlebensfähigkeit von Eizellen/Spermien
Gonaden	(griech. gone: Geschlecht/Erzeugung/Same, aden: Drüse) Keimdrüse
Inkubation	Bebrütung
Integument	(lat. integumentum: Decke/Hülle/äußere Haut) gegenüber dem übrigen Gewebe differenzierte äußere Körperhülle bei allen Gewebetieren. Impliziert keine anatomische, sondern eine funktionale Betrachtung.
Metabolismus	(griech. metabolismós mit lateinischer Endung -us) Stoffwechsel, chemische und physikalische Vorgänge der Umwandlung von Stoffen in Zwischenprodukte und Endprodukte im Organismus von Lebewesen.
OECD	Organisation für wirtschaftliche Zusammenarbeit und Entwicklung (engl. Organization for Economic Co-Operation and Development)
Oocyte	Eizelle
Ovar	Eierstock
Ovariektomie	Entfernung der Ovarien = Kastration bei Weibchen
Phänotypisch	(griech. phaíno: ich erscheine und týpos: Gestalt) Äußeres Erscheinungsbild
Piscivor	Fischfressend
Sex reversal	Geschlechtsumkehr
Subkutan	(lat. sub: unter, cutis: Haut) unter der Haut
SuperSAGE	Serielle Genexpressionsanalyse
Testes	Hoden

TierSchG	Tierschutzgesetz
Tubuli seminiferi	Hodenkanälchen
Utilitarismus	(lat. utilitas: Nutzen, Vorteil) Nützlichkeitsprinzip, teleologische/zweckorientierte Ethikauffassung: Beurteilung einer individuellen Handlung am aggregierten Gesamtnutzen
Vertebrata	Wirbeltiere
Virilisierung	(Synonym: Virilismus; von lat. vir: „Mann“) Vermännlichung
Xenohormon	Fremdes, nicht körpereigenes Hormon = Endokriner Disruptor

Abstract

In recent decades, the number of animals used in research and medical technology has increased due to increasing regulatory requirements and new legislations for chemicals. This is because newly developed chemicals possess additional mode of action properties and therefore need to be tested in animal systems for human and environmental risk assessment. The chemicals enter the environment in different ways and finally might negatively affect human and animal health.

In the methods used so far for environmental risk assessment, juvenile or adult animals are mainly taken for the assessment of substances, partially even in complete generation tests with whole animals. It is known that the development of reproductive disorders occurs in the embryonic phase of the respective individuals, when all properties of the later individual are being formed. In order to reduce animal consumption, some in vitro test systems are used. However, these tests mostly indicate a specific cell type at a certain stage of development, which severely limits the significance of the actual effect compared to a complex tissue, if not to speak of the complete organism and its development. Thus, the power of this method must be seen within certain restrictions.

In the presented work, an alternative replacement method is presented with the aim of increasing the significance of toxicologically and ecotoxicologically relevant endpoints. The focus is on the effects of androgenic and estrogenic substances on the sexual development of chicken embryos (*Gallus gallus domesticus*) at the level of whole mRNA expression of gonads, combined with effects at the level of organ histology and morphology compared to the normal conditions of untreated individuals. The method can be used in the context of human and environmental risk assessment of substances and is a suitable tool to carry out the necessary studies with the required high quality of assessment. At the same time, this animal test replacement method with high potency might reduce the consumption of higher experimental animals, which also corresponds to a socio-ethical need.

Zusammenfassung

Auf den Einsatz von Tieren im Rahmen der (Umwelt-)Risikobewertung von Stoffen kann nach wie vor nicht verzichtet werden. Dabei führen die Überprüfungen einer zunehmenden Anzahl neu entwickelter Stoffe, aber auch die gestiegenen Anforderungen der Gesetzgebungen zu einem hohen Verbrauch von Versuchstieren. Diese Untersuchungen sind wichtig, da viele der in Gebrauch befindlichen und in allen Bereichen genutzten Chemikalien potentiell endokrin wirksam sind, auf unterschiedlichen Wegen in die Umwelt gelangen und sich potentiell negativ auf die Gesundheit von Mensch und Tier auswirken können.

Bei den bisher verwendeten Methoden werden vor allem juvenile oder adulte Tiere, aber auch Tiere zur Untersuchung des kompletten Lebenszyklus über eine oder mehrere Generationen für die Beurteilung von Substanzen eingesetzt. Dabei ist bekannt, dass die Entstehung reproduktiver Störungen in der Embryonalphase der jeweiligen Individuen auftritt. Um den Tierverbrauch zu reduzieren, werden teilweise In-vitro-Testsysteme angewendet. Es zeigt sich aber, dass diese Tests lediglich einen bestimmten Zelltyp in einem bestimmten Entwicklungsstadium abbilden können, was die Aussagekraft über die tatsächliche Wirkung auf ein komplexes Gewebe und dessen Entwicklung, erst Recht für den kompletten Organismus, stark einschränkt. Die Aussagekraft dieser Methoden ist daher in bestimmten eingeschränkten Grenzen zu sehen.

In der vorliegenden Arbeit wird eine alternative Ersatzmethode vorgestellt mit dem Ziel einer stärkeren Aussagekraft bei toxikologisch und ökotoxikologisch relevanten Endpunkten. Im Fokus stehen hierbei die Effekte von androgenen und estrogenen Substanzen auf die Geschlechtsentwicklung von Hühnerembryonen (*Gallus gallus domesticus*) auf Ebene der Expression der mRNA, vereint mit Effekten auf Ebene der Organhistologie und –morphologie, verglichen mit den Normalzuständen unbehandelter Individuen.

Die neu entwickelte Methode zur Beurteilung solcher Substanzen kann im Rahmen der human- und umwelttoxikologischen Risikobewertung von Stoffen eingesetzt werden und ist ein geeignetes Werkzeug, um die notwendigen Untersuchungen mit der gefordert hohen Beurteilungsqualität durchzuführen.

Gleichzeitig kann mit dieser Tierversuchsersatzmethode bei hoher Aussagekraft auch der Verbrauch an weiter und höher entwickelten Versuchstieren verringert werden, was auch einem gesellschaftlich-ethischen Bedürfnis gerecht wird.

1 Einleitung

Auch wenn heute schon viele wissenschaftliche Fragestellungen durch den Einsatz von Zellkulturen, computergestützten Verfahren und weiteren Alternativmethoden beantwortet werden können, kann auf den Einsatz von Tieren für wissenschaftliche und regulatorische Zwecke, namentlich für die Stoffzulassung, (noch) nicht verzichtet werden. Ein Ziel ist es daher, weitere Ersatzmöglichkeiten zu entwickeln, um die tatsächlich benötigte Anzahl von Tieren für die Versuche zu verringern. Für die Zulassung und die damit verbundene Überprüfung der Verträglichkeit von neu entwickelten Stoffen für Mensch und Umwelt ist es bisher notwendig, neben den bekannten produktspezifischen Effekten alle andere relevanten zu erkennen. Hierzu werden im Allgemeinen verschiedene (Tierversuchs-)Tests angewendet, die heute standardisiert und gesetzlich vorgeschrieben sind. Die Geschlechtsentwicklung und –differenzierung von Embryonen stellt dabei eine besonders sensible Lebensphase dar. Dies vor allem, weil Entwicklungsprobleme in dieser Phase sich negativ auf die Fortpflanzung des Individuums und damit auf den Weiterbestand der Spezies auswirken können.

1.1 Ausgangsbasis

Die vorrangigen Ziele der Toxikologie sind der Erhalt der uneingeschränkten Gesundheit vor allem des Menschen, aber auch von Tier und Pflanze sowie der unbehelligte Zustand der sie umgebenden Umwelt. Dabei interessiert vor allem die Auswirkung einzelner chemischer und pharmakologischer Substanzen auf die Gesundheit und die Art und das Ausmaß von Schadwirkungen auf den Organismus. Auf diese Weise sollen schädliche Wirkungen erkannt werden, um eine Gefährdung durch potentiell gefährliche Substanzen und Substanzgemische vorherzusagen und deren Risiken bei einer gegebenen oder angenommenen Exposition abzuschätzen.

In der klassischen Toxikologie wird davon ausgegangen, dass es für jeden Stoff eine individuelle Dosis oder Konzentration gibt, ab der ein toxischer Effekt festgestellt werden kann. Die Mortalität von Lebewesen bei Exposition dient dabei oft als Endpunkt zur Festlegung der Effektschwelle oder der Risikoschwelle, ab der Vergiftungserscheinungen auftreten.

Die Anwendung dieser Resultate und Erkenntnisse auf den Menschen nimmt einen wesentlichen Platz ein, da dessen Schutz oft im Fokus steht. Um zu diesen Informationen zu gelangen, werden oft Tierversuche unter standardisierten Bedingungen durchgeführt. Dabei stehen in der Regel ganz spezifische Wirkmechanismen mit den Endpunkten Mortalität oder Reizung des Organismus im Zentrum.

Im Unterschied zur Toxikologie geht die Ökotoxikologie einen Schritt weiter. Sie untersucht Effekte auf Lebewesen in der Umwelt (Luft, Wasser, Boden), wobei neben der klassischerweise betrachteten Mortalität auch weitere Endpunkte hinzugezogen werden. Diese ordnen sich nach den Organisationsebenen der betrachteten Systeme: von messbaren Effekten auf der endokrinen Ebene über Effekte in Organen und der Fortpflanzungsfähigkeit von Individuen bis hin zu Auswirkungen auf Stufe der Populationen.

Die vorliegende Arbeit bewegt sich sowohl im Themenfeld der Ökotoxikologie als auch der Toxikologie. So werden aus ökotoxikologischer Sicht Parameter untersucht, die einerseits das Individuum beeinflussen, andererseits auch die folgenden Generationen (F1, F2). Gleichzeitig soll die Möglichkeit der Übertragbarkeit der Ergebnisse auf den Menschen berücksichtigt werden, indem ähnliche endokrine Endpunkte beschrieben werden. Es ergeben sich daraus zusätzlich ethische Fragestellungen, inwieweit Untersuchungsmethoden an Embryonen einer Wirbeltierart durch Methoden an einer anderen Wirbeltierart ersetzt werden sollen und können. Dieser Aspekt hat in den letzten Jahren mit Blick auf die Anwendbarkeit von Tierversuchsersatzmethoden an Bedeutung gewonnen.

1.2 Endokrine Disruptoren (ED)

Endokrine Disruption bezeichnet den negativen Effekt von Chemikalien auf das Hormonsystem von Lebewesen. Das endokrine System (vom griech. endo: innen, krinein: ausscheiden) ist wesentlich für viele Funktionen im Körper eines Individuums. Selbst produzierte Hormone sind für praktisch alle Regulationen im Organismus verantwortlich und werden in der Hauptsache von Drüsengeweben produziert. Sie führen in den Zielorganen zu besonderen Wirkungen, indem sie an spezifische zelluläre Rezeptoren binden und dort entsprechende Reaktionen auslösen. Primäre endokrine Organe der Wirbeltiere sind die Nebenniere, Hypophyse, Schilddrüse, Nebenschilddrüse, Bauchspeicheldrüse und Gonaden (Ovarien bzw. Testes). Letztere spielen eine zentrale Rolle bei der Geschlechtsausprägung eines sich entwickelnden Individuums und der anschließenden Funktion dieser Geschlechtsorgane für die Reproduktion und damit für den Fortbestand der Spezies.

Die vorliegende Arbeit verwendet die Bezeichnung Endokriner Disruptor: „ED“ nach der Definition der WHO (2002) [1, 2]:

> **„Ein ED ist eine exogene Substanz oder Mischung, die die Funktion(en) des endokrinen Systems verändert und in der Konsequenz negative gesundheitliche Effekte in einem intakten Organismus, dessen Nachkommen oder (Sub-)Populationen hervorruft.“**

Dabei werden **potentielle** ED wie folgt definiert:

> **„Eine exogene Substanz oder Mischung, die Eigenschaften besitzt, von denen erwartet werden kann, eine endokrine Disruption in einem intakten Organismus, dessen Nachkommen oder (Sub-)Populationen hervorzurufen.“**

In den letzten Jahren haben sich die Hinweise gehäuft, dass Chemikalien, die durch Müll oder Abwässer in die Umwelt gelangen, sich auf das endokrine System von Tieren auswirken.

In diesem Zusammenhang werden auch reproduktive Anomalien festgestellt, wobei sich insbesondere bei der Embryonalentwicklung und im reproduktiven Trakt negative Effekte und Schädigungen zeigen. Diese Effekte auf Stufe der sich entwickelnden Organe werden durch ein gestörtes Hormonsystem ausgelöst und können in wirbellosen Tieren, wie Muscheln und Schnecken, genauso gefunden werden wie in Fischen, Reptilien, Vögeln und Säugern [3-8]. Solche Effekte werden auch in freier Wildbahn beobachtet, wobei die meisten Untersuchungen bei aquatischen Lebewesen durchgeführt werden [1, 9-11]. Der Fokus auf aquatischen Organismen ist unter anderem dem Umstand geschuldet, dass aufgrund menschlichen Einflusses (durch z.B. Kläranlagenabflüsse) in den natürlichen Gewässern eine Vielzahl verschiedener Substanzen in relevanten Konzentrationen festgestellt wird. Gleichzeitig kann angekommen werden, dass in vielen Fällen die Exposition hier sehr hoch ist, weil Substanzen im umgebenden Wasser durch das Integument und besonders über respiratorische Organe, wie die Kiemen, aufgenommen werden [12-16].

Dabei werden bestimmte aufgenommene Stoffe über die Nahrungskette von Prädatoren, wie z.B. höheren Fischen, Meeressäugern und Seevögeln, aufgenommen, so in den Organismen weiter angereichert und schließlich auch im Menschen integriert und akkumuliert [17-20]. Eines der prominentesten Beispiele von endokriner Disruption aufgrund solcher Akkumulationsprozesse über die Nahrungskette findet sich in der Verdünnung der Eierschalen bei Greifvögeln Mitte des letzten Jahrhunderts. Ursache dafür war das Insektizid Dichlordiphenyltrichlorethan (DDT), wodurch eine Bebrütung der Eier unmöglich wurde, da diese zerbrachen, sobald sich ein Elterntier zum Brüten auf sie legte. Weltweit kam es zu massiven Populationseinbrüchen verschiedener Greifvogelarten und anderer carni- und piscivorer Vogelarten [21-23].

Allgemein kann bei einer endokrinen Störung (ED) gesagt werden (nach [24]):

1.: Eine Exposition gegenüber einem ED im adulten Stadium wird in vielen Fällen durch den Organismus kompensiert.

2.: Eine Exposition während der Embryonalentwicklung kann eine permanente Verschiebung der (genetischen) Funktion oder eine erhöhte Sensitivität gegenüber Stimulantien (ED, andere Umwelteinflüsse) verursachen.

3.: Eine Exposition gegenüber der gleichen Dosis eines endokrin wirkenden Stoffs (Hormon, ED) in verschiedenen Lebens- und Entwicklungsphasen kann unterschiedliche Effekte hervorrufen.

4.: Durch (hormonelle) Querverbindungen („cross talk“) zwischen verschiedenen endokrinen Systemen können ED auch unvorhergesehene Effekte in anderen Systemen hervorrufen.

5.: In Bezug auf 4. ist es besonders wichtig, bei der Extrapolation hormoneller Aktivitäten von In-vitro-Messungen mögliche nicht erkannte Effekte *in vivo* in Betracht zu ziehen.

Die vorliegende Arbeit konzentriert sich an dieser Stelle vor allem auf Effekte, die während der Embryonalentwicklung durch potentielle ED hervorgerufen werden (Punkt 2).

1.3 Gonadendifferenzierung bei Vögeln

Im Unterschied zur Situation bei den Säugetieren ist bei Vögeln das genetische Männchen homozygot (ZZ), während das Weibchen heterozygot (ZW) ist. Am Anfang der Bebrütung sind alle Embryonen mit der gleichen ungeschlechtlichen Gonadenbasis ausgestattet. Durch die Zirkulation verschiedener, in ihrer Konzentration geschlechtsabhängig genetisch festgelegter Steroidhormone differenzieren sich die Gonaden in männliche oder weibliche Organe. Ohne äußeren Einfluss entwickelt sich die undifferenzierte Vogelgonade beim Männchen in einen Hoden. Bei genetischen Weibchen hingegen wirkt die W-chromosomal gesteuerte Synthese der CytochromP450-Aromatase (auch CYP19A1 genannt) zur Umwandlung der Androgene in Estrogen mit, was letztlich zur Ausbildung des weiblichen Ovars führt [25, 26]. Die Rolle der weiteren natürlichen Hormone in der Gonadendifferenzierung der Vögel ist nur in Teilen bekannt. Bei Vögeln sind die Geschlechtshormone bei einer ganzen Reihe verschiedener, weiterer reproduktiver Funktionen involviert, inklusive der sekundären Geschlechtsmerkmale, wie Federfarbe und –form, dem Gesangsrepertoire und phänotypischen Geschlechtsunterschieden, wie Oviduktentwicklung, Follikelreifung und Eischalenausbildung.

Es ist bereits bekannt, dass Xenohormone in der Embryonalphase irreversible Fehlbildungen der Gonaden von Vögeln hervorrufen oder das geschlechtsspezifische Verhalten verändern können, wohingegen in einem weniger sensitiven Adultstadium nur leichte, oft reversible Effekte beobachtet werden ([24, 27, 28] und Kap. 1.2).

Die morphologische Differenzierung der embryonalen Gonaden beginnt im Wesentlichen nach dem siebten Tag der Inkubation (Abb. 1). Hierbei wird in weiblichen Gonaden die Umwandlung von Testosteron in Estradiol durch das Enzym Aromatase vorangetrieben, während bei männlichen Gonaden das Anti-Müller-Hormon (AMH) in den Vordergrund tritt (Abb. 2). Dadurch wird die Differenzierung der rechten weiblichen Gonade zum Ovar angeregt, während bei den männlicher Gonaden testikuläres Gewebe ausgebildet wird. Die linke weibliche Gonade bleibt undifferenziert, sie kann aber im Fall des Fehlens der linken Gonade zu einem funktionsfähigen Ovar ausgebildet werden [29].

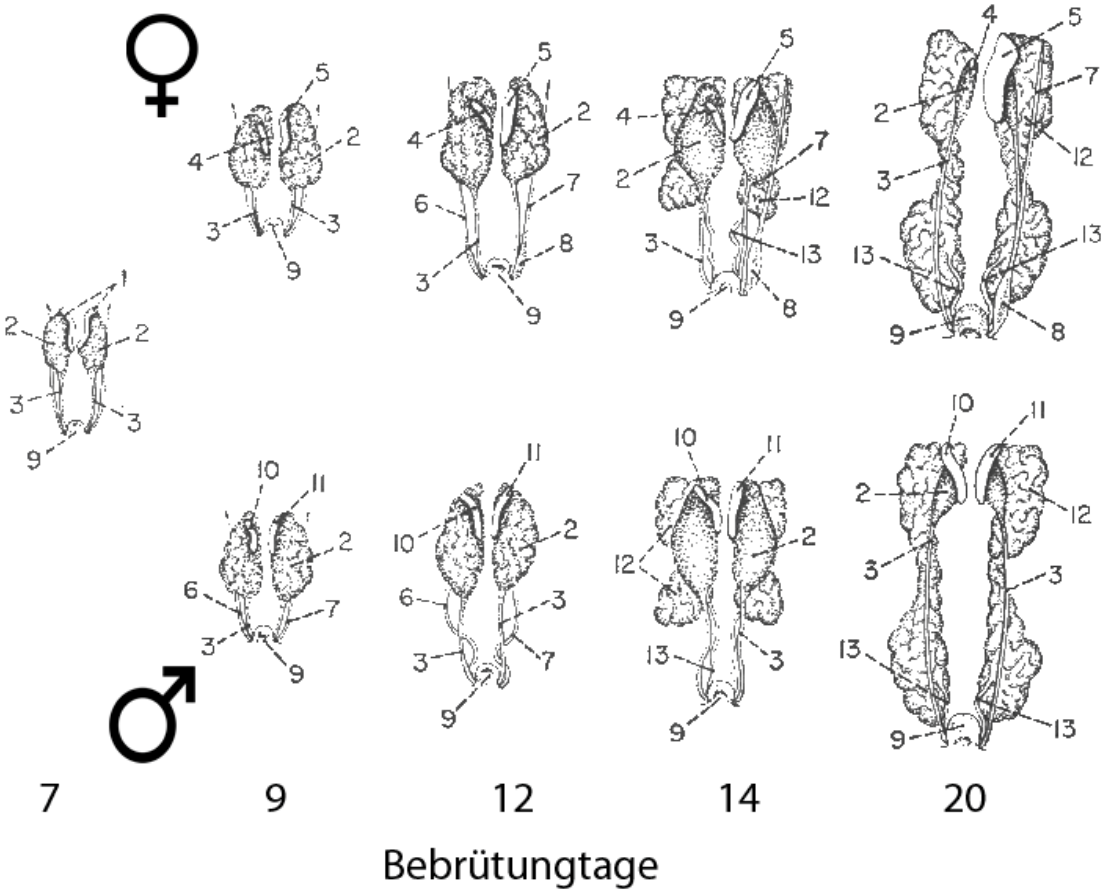

Abbildung 1: Entwicklung des Urogenitalsystems von männlichen (unten) und weiblichen (oben) Hühnerembryonen vom Tag 7 bis 20 der Inkubation.
Bebrütungstag 7: sexuell indifferentes Stadium; 1: undifferenzierte Gonade; 2: Wolff'sche Körper; 3: Wolff'sche Gänge; 4: rechtes Ovar; 5: Linkes Ovar; 6: rechter Müller'scher Gang; 7: linker Müller'scher Gang; 9: Kloake; 10: rechter Hoden; 11: linker Hoden; 12: Metanephros; 13 Ureter (Harnröhre) (nach [30], verändert).

Der Estrogenrezeptor wird in männlichen Gonaden zwischen Tag 7 und 10 exprimiert. Gerade in dieser Phase sind männliche Embryonen gegenüber Änderungen der Hormonspiegel (durch bspw. ED) empfindlich, was zu einem Wechsel des morphologischen Geschlechts (engl.: sex reversal) führen kann. Eine äußere Beeinflussung der Hormonspiegel, vor allem des Estrogenspiegels, wirkt sich daher besonders auf die Gonadenausprägung des männlichen Geschlechts aus (Abb. 2).

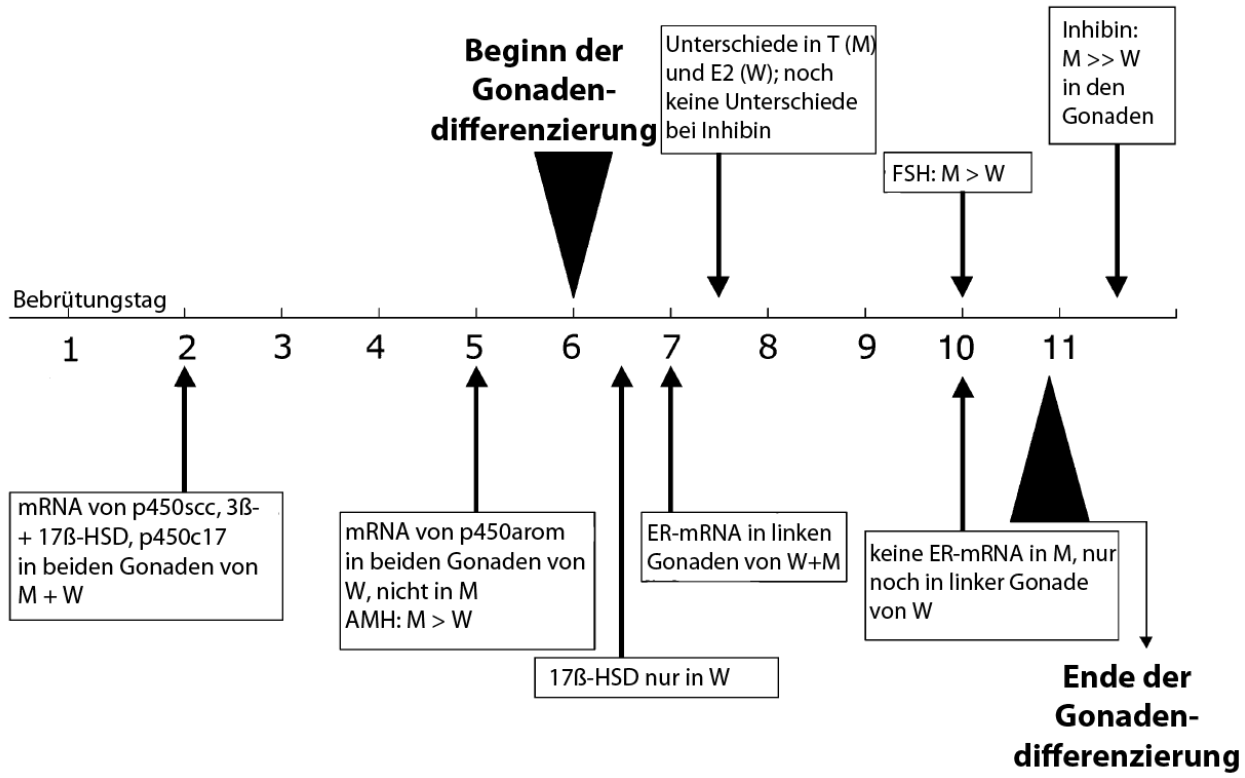

Abbildung 2: Zeitachse des Auftretens von Hormonen im Plasma und Beginn der Expression der wichtigsten Schlüsselenzyme, die in die Steroidgenese involviert sind, innerhalb der ersten 12 Bebrütungstage von männlichen (M) und weiblichen (F) Hühnerembryonen.
P450 arom: p450 Aromatase; p450scc: p450 SideCchain Cleavage Enzym; 3β-and 17β-HSD: 3β-and 17β-Hydroxysteroid-Dehydrogenase; p450c17: p450 17Anydroxysteroid-Dehydrogenase; AMH: Anti-Müller-Hormon; ER: Estrogenrezeptor; T: Testosteron; E2: 17β-Estradiol. Nach: [31], verändert.

1.4 Disruption der Gonadendifferenzierung

Durch die Injektion von Chemikalien, v.a. Hormonen in Vogeleiern, ist es möglich, die Entwicklung der Gonaden zu beeinflussen [32-38]. Dabei untersuchten C. Berg und Kollegen Eier-legende Hennen, die *in ovo* **Ethinylestradiol** (EE_2, 20 ng/g Ei) appliziert bekommen hatten [39]. Die so behandelten Hennen besaßen weniger Kapillaren in ihrer Eischalendrüse, die zudem eine geringere Aktivität der Carboanhydrase aufwiesen. Dabei konnte gezeigt werden, dass die Tiere bei der Reproduktion unnatürlich dünne Eierschalen produzierten, was die Problematik einer hormonellen Störung bei der Reproduktion besonders gut veranschaulicht [40, 41]. Andere Arbeiten untersuchten die Wirkung von **Aromataseinhibitoren** auf die Gonaden und konnten in 50% der Fälle die Bildung von Ovotestes bei den genetisch weiblichen Gonaden auslösen [33]. **Estrogen** hingegen bewirkt bei Männchen die Ausbildung von Ovotestes, während bei Weibchen die Müller‘schen Gänge und der Ovidukt in ihrer Entwicklung beeinträchtigt werden [42].

Diese Arbeiten an Vogeleiern zeigen beispielhaft, wie die morphologische Beurteilung der Gonaden genutzt werden kann, um die Auswirkung bestimmter ED zu bestimmen. Dabei handelt es sich um eine sensitive Testmethode, welche auch für andere Umweltchemikalien genutzt werden könnte. Trotzdem wurde diese Methode bisher nicht in einem größeren Umfang angewendet.

Die vorliegende Arbeit möchte diese Lücke schließen und setzt daher die Beurteilung der Gonadenentwicklung in Vogeleiern systematisch ein, um die zu untersuchenden Substanzen auf ihren Effekt hin zu beurteilen. Zusätzlich soll dabei auch die Auswirkung von **Androgenen** auf die Gonadenausprägung von vor allem Weibchen überprüft werden. Inwiefern weibliche Individuen für einen erhöhten Testosteronlevel empfänglich sind, ist noch nicht hinreichend bekannt, außer, dass mithilfe der Inhibition der Estrogenprodution durch Aromataseinhibitoren ein Estrogenmangel erreicht werden kann – was aber nicht zwingend zu einem Testosteron-überschuss führt [43].

1.5 Regulatorischer Kontext

Chemikalien können die Ausbildung von Organen insbesondere in der Embryonalentwicklung stören. Tests, die diesen Lebensabschnitt nicht berücksichtigen, sind daher nicht in der Lage, allfällige Effekte in dieser sensiblen Lebensphase abzubilden und nachzuweisen. Beispielhaft dafür ist die Verwendung des Schlaf- und Beruhigungsmittels Contergan (Wirkstoff Thalidomid, 1957-1962) das, während der Schwangerschaft von Müttern eingenommen, eine teratogene Wirkung auf die Embryonalentwicklung ihrer Kinder hatte. Diese zeigten starke Missbildungen an den Gliedmaßen (Dysmelie-Syndrom [44]). Seit der Neufassung des Arzneimittelgesetzes von 1978 sind in Deutschland daher umfassendere Prüfungen der Arzneimittelsicherheit rechtlich vorgeschrieben.

Die teratogene Wirkung von Thalidomid konnte damals im Tierversuch nicht festgestellt werden, was von Tierversuchsgegnern gelegentlich herangezogen wird, um die Unzuverlässigkeit von Sicherheitsprüfungen am Tier aufzuzeigen. Tatsächlich jedoch wurde die schädigende Wirkung von Contergan deshalb im Tierversuch nicht festgestellt, weil zu jener Zeit noch keine Versuche an trächtigen Tieren - und dadurch ebenfalls nicht an Embryonen - durchgeführt wurden. Durch diese Vorfälle, aber auch angestoßen durch das etwa zeitgleich auftretende Buch von Rachel Carson „Silent spring“ [45] und viele weitere folgende Untersuchungen, verlangen inzwischen gesetzliche Regulatorien entsprechende Tests, die auch die Effekte auf die Embryonalentwicklung mit berücksichtigen.

1.5.1 REACh und POP-Verordnung

Durch die technische Entwicklung können zunehmend neue chemische Stoffe für Anwendungen in verschiedenen Bereichen entwickelt und hergestellt werden. Diese neuen Stoffe müssen vor der Marktzulassung (Bspw. Pflanzenschutz- und Arzneimittel) hinsichtlich ihrer toxischen und ökotoxischen Eigenschaften geprüft werden. Dabei gelten internationale Vorschriften. Gestützt auf diese Untersuchungen kann entschieden werden, ob bei den vorgesehenen Anwendungen ein Risiko für Mensch oder Umwelt besteht und ob eine Zulassung möglich ist.

Die hierfür gelten Vorgaben stützen sich auf die europäische Chemikalienverordnung zur Registrierung, Bewertung, Zulassung und Beschränkung chemischer Stoffe REACh

(Registration, Evaluation, Authorisation and Restriction of Chemicals, Verordnung (EG) Nr. 1907/2006 [46, 47]), die CLP-Verordnung (Classification, Labelling and Packaging; Verordnung(EG) Nr. 1272/2008) sowie die die POP-Verordnung (Persistent Organic Pollutants; Verordnung (EG) Nr. 850/2004).

Dabei sind die Hersteller und Importeure verantwortlich, den Nachweis zu erbringen, dass mit der vorgesehenen Anwendung dieser Stoffe die menschliche Gesundheit und die Umwelt nicht gefährdet werden. Dazu werden verschiedene, meist international standardisierte Tests nach den Richtlinien der Organisation für wirtschaftliche Zusammenarbeit und Entwicklung (Organization for Economic Co-Operation and Development, OECD) durchgeführt. Die Ergebnisse dieser Tests gehen in die Risikobewertung jedes einzelnen Stoffs ein. Im Rahmen der Umweltrisikobewertung werden u.a. Stoffe identifiziert und untersucht, um Hinweise auf persistierende, bioakkumulierende und toxische Eigenschaften (PBT-Stoffe) zu erhalten, respektive Eigenschaften über besonders besorgniserregende Stoffe (substances of very high concern, SVHC) zu kennen, wie beispielweise endokrine Disruptoren.

Laut EU Biozid-Verordnung (EU 528/2012) und EU Pestizid-Verordnung (EG 1107/2009) dürfen endokrine Disruptoren aufgrund ihrer Problematik nur in Ausnahmefällen zugelassen, also vermarktet werden. Dabei werden toxikologisch und verwaltungsrechtlich begründete Grenzwerte festgelegt.

1.5.2 Tierversuche und Ersatzmethoden

Die Vorgaben der verschiedenen oben erwähnten Richtlinien verlangen nach entsprechenden standardisierten Tierversuchen, um die Risikobeurteilung sicherzustellen. Dabei haben die Anforderungen des EU-Rechts Vorrang vor nationalem Recht. Dies führt dazu, dass Produktanbieter durch die EU-Gesetzgebung zu Tierversuchen in Sicherheitsprüfverfahren verpflichtet sind, obwohl dies aufgrund des nationalen Gesetzes nicht notwendig wäre. Dadurch steigt allgemein auch die Anzahl der dafür notwendigen Versuchstiere.

In der öffentlichen und politischen Diskussion ist dadurch die Tierhaltung und vor allem deren Verbrauch bei Versuchen in den Fokus gerückt. Untersuchungen, welche

beispielsweise Anlass zur öffentlichen Diskussion boten, waren das Einbringen von Tumorzellen in Mäusen, die Implantation von Gelenkprothesen bei Pavianen oder die Exposition mit Dieselabgasen von Affen. Hier stellt sich die Frage, inwieweit die hohen Anforderungen (vgl. REACh, Kap. 1.4.1) durch Alternativen sichergestellt werden können.

Die Entwicklung neuer Methoden mit dem Ziel der Verbesserung des Tierwohls bis hin zum vollständigen Ersatz dieser Tierversuche ist ein Weg, diesem Anspruch entgegen zu treten. Sie sind auch gesetzlich vorgeschrieben, wann immer das möglich ist (TierSchG §7a(2)2). Dabei kommt systematisch das 3R-Prinzip nach Russel und Burch zur Anwendung [48]. Dieses Prinzip besagt, dass Tierversuche entweder vollständig ersetzt (Replacement), in deren Anzahl reduziert werden (Reduction) oder zumindest eine Minderung des Belastungsgrades der Tiere (Refinement) erreicht wird.

Das 2015 neu gegründete Deutsche Zentrum zum Schutz von Versuchstieren (Bf3R) sowie das European Union Reference Laboratory for Alternatives to Animal Testing (EURL-ECVAM) geben die entsprechenden Alternativmethoden vor.

1.6 Der Hühnerembryo als Tierversuchsersatzmethode

Der Hühnerembryo ist verhältnismäßig gut untersucht und aufgrund seiner Komplexität mit dem menschlichen Organismus vergleichbar. Er ist seit Anbeginn der Wissenschaft ein Modell in der Entwicklungsbiologie und Embryologie, weil er sehr einfach zu untersuchen und zu beobachten ist. Er wurde als Modellorganismus für Vertebrata und vor allem Amniota (höhere Wirbeltiere: Reptilien, Vögel, Säugetiere) verwendet, weil sein großes Ei die Beobachtung der Embryogenese bereits nach zwei Bebrütungstagen mit bloßem Auge ermöglicht; vorher ist dies mit der Lupe oder dem Mikroskop durchführbar. Manipulationen sind so sehr einfach durchzuführen.

Aus diesem Grund wird er auch heute zu vielen Grundlagenuntersuchungen in Forschung und Lehre sowie in der pharmakologischen Wirtschaft eingesetzt. So werden befruchtete und angebrütete Hühnereier meist zur Vermehrung viraler Impfstoffe, z.B. Influenza-Impfseren verwendet.

Die Besonderheit des Vogeleis besteht darin, dass es im Gegensatz zum Säugerkeim für Beobachtungen und Manipulationen relativ leicht zugänglich ist: Der Embryo entwickelt sich nicht innerhalb, sondern außerhalb des Körpers des Muttertieres. Dadurch erscheint der Hühnerembryo als Modellorganismus für Tierversuche im Rahmen der Untersuchungen für ED geeignet. Testsubstanzen können einzeln oder in Kombination zu dem sich entwickelnden Embryo direkt in das Ei gegeben werden. So können vom mütterlichen Metabolismus unabhängig die Testsubstanz sowie auch ihre Metabolite einzeln appliziert werden. Basierend auf ihrem hormonellen System kann die Antwort männlicher und weiblicher Hühnerembryonen auf verschiedene Substanzen sehr unterschiedlich ausfallen (vgl. Kap.1.3.2). So sind die Ergebnisse der Geschlechterverhältnisse und der Mortalität bereits inkludiert. Da sich genetisches und phänotypisches Geschlecht ggf. unterscheiden, muss das genetische Geschlecht eindeutig und schnell feststellbar sein, was per PCR gegeben ist [49].

Verschiedene Hühner-Embryonen-Tests (HET) an gering entwickelten Hühnerembryonen, wie der Test an der Chorioallantoismembran (HET-CAM) zum Ersatz des Draize-Tests am Kaninchenauge [50] oder auch der Test zur Micronucleus-Induktion (HET-MN) zum Ersatz von In-vivo-Mikrokernprüfungen an Nagern [51], zeigen, dass sich das Hühnerei grundsätzlich für toxikologische Tests eignet. Das Modell Hühnerei ist effizient und sensitiv.

Die Eignung der verschiedenen, vorhandenen Tier**ersatz**modelle der OECD für endokrine Disruptoren auf Ebene der Amniota ist unterschiedlich und wird nachfolgend kurz beschrieben:

- Test Nr. 206: Avian Reproduction Test [52]:

  Hierbei wird Wachteln (*Coturnix coturnix japonica*) über das Futter die Testsubstanz verabreicht (> 20 Wochen). Untersucht wird die Auswirkung auf die Elterntiere und die Nachkommen. Endpunkte sind Mortalität der Elterntiere, Eiproduktion, Anteil zerbrochener Eier, Eischalendicke, Überleben, Schlupfrate und Effekte auf die Jungvögel. Da dieser Test nur über eine Generation ausgeführt wird und keine genaueren Effekte auf die Nachkommen untersucht werden, ist dieser Test nur bedingt geeignet, endokrine Disruption zu untersuchen, zumal keine genaueren „Effekte auf die Jungvögel“ untersucht werden und die betrachtete Entwicklungszeit der Jungvögel bei diesem Test mit 14 Tagen zu kurz ist.

- Test Nr. 440: Uterotropher Bioassay mit Nagern [53]:

  Ein In-vivo-Kurzzeit-Screeningtest, basierend auf der Gewichtszunahme und anderen Reaktionen des Uterus. Hierbei werden zwei estrogen-sensitive Lebensphasen der Tiere unterschieden: i) immature Weibchen nach dem Abstillen und vor der Pubertät und ii) jungadulte Weibchen nach Ovariektomie (Entfernung der Eierstöcke) mit adäquater Erholungszeit zur Rückbildung des uterinen Gewebes. Die Testsubstanz wird über das Futter verabreicht oder subkutan injiziert. Vierundzwanzig Stunden nach der letzten Verabreichung werden die Tiere untersucht. Für Estrogen-Agonisten wird das mittlere Uterusgewicht der behandelten Tiere mit der Kontrollgruppe verglichen. Eine statistisch signifikante Zunahme des Gewichts gilt als positive Antwort des Bioassays. Weitere untersuchte Endpunkte sind die tägliche Futteraufnahme der Tiere, die Gewichtszunahme (täglich gemessen) und das Verhalten.

- Test Nr. 441: Hershberger Bioassay bei Ratten [54]:

  Dieser In-vivo-Kurzzeit-Screeningtest betrachtet die biologische Aktivität von Androgen-Agonisten und -Antagonisten sowie die Aktivität der 5α-Reduktase-Inhibitoren. Endpunkte sind die Gewichtsveränderungen in den androgen-abhängigen Geweben bei peripubertär kastrierten Männchen: Prostata, Samenblasendrüse *Glandula vesicularis*, Heber des Afters *Musculus levator ani-bulbocavernosus*, Bulbourethraldrüse *Glandula bulbourethralis,* und die Peniseichel *Glans penis.* Die androgene oder antiandrogene Substanz wird über das Futter oder eine subkutane Injektion verabreicht. Die Untersuchung des Frischgewichts der Gewebe erfolgt 24 Stunden nach der letzten Gabe. Eine statistisch signifikante Zunahme oder Abnahme des Gewichts der Gewebe wird als androgene respektive anti-androgene Wirkung der Testsubstanz gewertet.

- Detailed Review Paper für den Zwei-Generationentest bei Vögeln [55, 56]:

  Bemühungen vor allem der US EPA, den Test Nr. 206 zu verbessern, resultierten in der Entwicklung dieses Zwei-Generationstests [55]. Bei diesem Test sind Wachteln in der Elterngeneration (P1) einer Testsubstanz ausgesetzt. Die Untersuchung der Effekte erstreckt sich bis zur zweiten Filialgeneration (F2). Somit können Auswirkungen über zwei Generationen erkannt werden. Endpunkte sind Fitness (Wachstum, Fressverhalten, Fekundität, Gametenviabilität, Brutverhalten) und Physiologie (Organwachstum und morphologische Veränderungen, Geschlechtscharakteristika, -entwicklungen und –verhältnisse, Entwicklung von Gonaden und zugehörigen Strukturen - auch nichtreproduktiver Gewebe, Histopathologie von juvenilen und adulten Tieren, biochemische Marker). Dieser Test dauert mindestens 38 Wochen und verbraucht (je nach Anzahl der untersuchten Konzentrationen der untersuchten Chemikalie) mehrere hundert Tiere. Zudem zeigt der Test Schwierigkeiten bei der Robustheit und Reproduzierbarkeit. Die OECD entschied daher 2014, die Weiterentwicklung dieses standardisierten Zwei-Generationen-Tests einzustellen.

Aufgrund der Komplexität und Variabilität, die der Zwei-Generationentest bei Vögeln mit sich bringt, liegt die Überlegung nahe, die ökotoxikologischen Untersuchungen in mehrere

kleine Abschnitte aufzuteilen und sich auf bestimmte Schlüsselphasen zu konzentrieren. Die Untersuchung der Entwicklung der Gonaden und anderer, der Fortpflanzung dienender Gewebe und Strukturen ist ein solcher Ansatz. Gleichzeitig kann in Betracht gezogen werden, ob im Rahmen toxikologischer Untersuchungen der Hershberger und der Uterotrophe Assay durch die tiefergehende Untersuchungsmethode (ggf. komplett) ersetzt werden können.

1.7 Integration in den aktuellen Stand der Wissenschaft

1.7.1 Hintergrund der Arbeit

Die vorliegende Arbeit entstand im Rahmen des Kooperationsprojekts „GenOvotox – Entwicklung und Evaluierung eines sensitiven und kostengünstigen Tierersatzsystems für die Abschätzung des Hormon-toxischen Potenzials von Chemikalien als Disruptoren der embryonalen Gonadenentwicklung“. Das Projekt begann 2008 als Kooperation zwischen der Universität Frankfurt und der GenXPro GmbH als KMU-Partner. Die Fragestellungen aus dem histologischen Bereich sowie alle Bebrütungen, die Präparationen und die Bereitstellung der Proben für die GenXPro wurden an der Universität Frankfurt durchgeführt. Die Erstellung der mRNA-Daten aus den Proben mittels SuperSAGE [57, 58] oblag der GenXPro. Die statistische Datenaufbereitung der genetischen Daten wurde seitens der GenXPro durchgeführt, während die Auswertung dieser Daten Teil der vorliegenden Arbeit war.

1.7.2 Stand der Forschung zu Testverfahren bei endokrinen Disruptoren für Vögel

Tests zur Wirkung von ED wurden bereits an verschiedenen Vogelspezies durchgeführt und zeigten deren Anfälligkeit für exogene Stoffe. Hierbei wurden häufig Verhaltenstests durchgeführt. DDT als Estrogen-Antagonist (auch **Anti-Estrogene** genannt) stört beispielsweise die Bildung von Hirnregionen bei Männchen der amerikanischen Wanderdrossel (*Turdus migratorius*), die für den Gesang zuständig sind. Der Gesang verarmt und die Fortpflanzungserfolge stagnieren [59]. Im Umkehrschluss liegt die Vermutung nahe, dass Estrogenrezeptor-Agonisten (**Estrogene**) wie 17β-Estradiol (E_2), Bisphenol A (BPA) oder Phthalate eine gegenteilige Wirkung erzielen könnten. Tatsächlich wurden bei männlichen Staren, die diese ED in umweltrelevanten Konzentrationen zu fressen bekamen, die Strophen länger, das Gesangsrepertoire vergrößerte sich und die Fortpflanzungschancen der Tiere stieg an, da der Gesang bei Staren ein zentrales Kriterium bei der Partnerwahl ist. Allerdings haben die ED gleichzeitig eine suppressive Wirkung auf das Immunsystem [60].

Ein anderes Beispiel ist die Verzögerung der Geschlechtsentwicklung aufgrund einer direkten Inhibition durch ED bei Zugvögeln. Da die saisonale Befruchtung und der Zeitpunkt der Reproduktion bei Zugvögeln entscheidend ist, kann dieser Effekt dazu führen, dass die Tiere die Geschlechtsreife zu spät erreichen und dadurch eine Nachwuchs-Generation verloren geht [16].

Histologische Untersuchungen der Effekte von Chemikalien auf die Entwicklung der Gonaden bei Hühnern und Wachteln (*Coturnix coturnix japonica*) betrafen in erster Linie die Wirkung von **Estrogenen** wie EE_2, BPA, Tetrabrombisphenol A (TBBPA), DDT (mit seinen Abbauprodukten DDD, DDE) oder Nonylphenol (NP) (u.a. [33, 38, 61-66]). Den Publikationen ist zu entnehmen, dass die verweiblichenden Effekte der unterschiedlichen Substanzen einheitliche Wirkungen zeigten, indem sie die männlichen Gonaden entweder in einen Ovotestes oder bei hoher Dosis oder Potenz der Substanz in ein funktionierendes Testes-Paar entwickeln lassen.

Bezüglich der Wirkung von **Androgenen** hingegen wurden nur wenige Untersuchungen veröffentlicht. Hier wurde entweder das Testosteronderivat Methyltestosteron oder verestertes Testosteron, wie bspw. Testosteronpropionat, verwendet [67, 68]. Eine Vermännlichung der Embryonen durch **indirekt androgen** wirkende Stoffe, wie die **Aromatase-Inhibitoren,** ist eine weitere Wirkung, die in mehreren Studien mittels des potenten Aromatase-Inhibitors Fadrozol herbeigeführt wurde [33, 69-72]. Bei diesem Prozess wird die Umwandlung des vorhandenen Testosterons in Estrogen verhindert, was dazu führt, dass bei den genetischen Weibchen zugleich ein Testosteron-Überschuss sowie ein Estrogen-Mangel auftritt. Da Estrogen bei weiblichen Vögeln die ursprünglich angelegten männlichen Gonaden in weibliche umwandelt, hat der Mangel von Estrogen einen relevanten Effekt auf die Geschlechtsdifferenzierung [73]. Die Kompensation des Mangels durch eine zusätzliche Gabe von Estrogen hebt diesen Effekt wieder auf, was beweist, dass spezifisch das Estrogen selbst den Schlüssel im Wirkmechanismus darstellt [31].

Weitere Studien konzentrieren sich auf die Genregulation oder Proteinexpression. Dabei sind heute Wirkungen von ED auf folgende Endpunkte bekannt [64, 74-79]:

• Estrogenrezeptor α und β (ER α/β)	Rezeptor
• Androgenrezeptor (AR)	Rezeptor
• Retinoid X-Rezeptor (RXR)	Rezeptor
• Aromatase (CYP19A1)	Proteinsynthese
• Anti-Müller-Hormon (AMH)	Proteinsynthese
• Vitellogenin	Proteinsynthese
• Steroidogenic Factor-1 (SF1)	Transkriptionsfaktor
• Sry-related box gene 9 (SOX9)	Transkriptionsfaktor
• Doublesex and the Mab-3 related transcription factor 1 (DMRT1, Z-Chromosom)	Transkriptionsfaktor
• PKC inhibitorisches Protein WPKCI (W-Chromosom)	Proteinkinase

Die vorgeschriebenen Testverfahren der OECD wurden in Kap. 1.5 bereits aufgezeigt. Nichtsdestotrotz gab es bereits Bestrebungen, diese zu verbessern. Die Gruppe um C. Berg publizierte bereits 1998 erste Tests, in denen sie die Beobachtung xenoestrogener Effekte in Vögeln beschreiben und histologische Methoden für die methodische Erfassung dieser Wirkung vorschlagen [61]. Weitere Arbeiten konzentrierten sich dabei vor allem auf die Effektbeschreibung weiterer Xenoestrogene auf die Morphologie und histologisch beurteilbare Effekte bei Gonaden von Hühnern und Wachteln [37-39, 62, 63, 80, 81]. Eine Verbindung der oben beschriebenen genetischen mit den dargestellten histologischen Methoden zum Erhalt eines Gesamtbilds wurde bisher noch nicht durchgeführt.

Hier schließt die vorliegende Arbeit an und führt sie in diesem Sinne weiter aus, indem sie eine Methode anstrebt, zum Ersatz und zur Verfeinerung der bestehenden Vorschriften und Guidelines für estrogen, aber auch zusätzlich androgen wirkende Substanzen beizutragen. Die Arbeit möchte dabei die histologischen Befunde mit den genetischen Grundlagen verbinden, indem sie untersucht:

(1) ob die gefundenen histologischen Ergebnisse konstant sind und auch für weitere Modellsubstanzen reproduzierbar bleiben, und

(2) ob auch bei den genetischen Ergebnissen eine entsprechende Konstanz und Reproduzierbarkeit festgestellt werden kann mit dem Ziel, einen größeren genetischen Basisdatensatz aufzubauen, der standardmäßig für alle estrogen und androgen wirkenden Substanzen eingesetzt werden kann.

1.8 Abschätzung des Potentials der zu entwickelnden Methode

Vorteile dieser Versuchsmethode sind, dass im Gegensatz zu anderen Versuchen und OECD-Richtlinien:

- auf In-vivo-Tierversuche verzichtet werden kann (Anwendung der 3R-Regel Replacement, Reduction und Refinement nach RUSSEL & BURCH 1959 [48]),
- eine juristische Beurteilung von Küken bis vor dem Schlupf als Tierversuch in der EU in Zukunft nicht absehbar und nicht wahrscheinlich ist (vgl. Kap. 2.6),
- kein Tierversuchsantrag notwendig ist und damit – im Gegensatz zu Säugerversuchen – für den Anwender eine erhebliche Zeit- und Kostenersparnis besteht (vgl. Kap. 2.6),
- jedes Tier individuell getestet und ausgewertet werden kann, wodurch individuelle Unterschiede herausgearbeitet und beachtet werden: jedes Tier ist ein einzelnes Replikat,
- bereits in frühen Stadien morphologische Veränderungen beobachtet werden können,
- ein schnelles, effektives Screening für neu entwickelte Substanzen entwickelt wird, um zu prüfen, ob - und wenn ja, wie - diese in die Geschlechtsentwicklung eines Individuums eingreifen,
- der personelle Aufwand zwischen den Testphasen relativ gering ist, da befruchtete Eier jederzeit bestellt werden können und keine Tierhälterung stattfindet.
- die zu erhebenden Genaktivitätsbestimmungen sich in Datenbanken einpflegen lassen, wodurch sie die quantitative Bestimmung von Grenzwerten ermöglichen, die ggf. als Grundlage für die Eingruppierung von Stoffen in Teratogenitätsklassen und damit als rationale Grundlage für die Risikobeurteilung dienen können.

Die zu klärenden Fragen für die vorliegende Arbeit sind die folgenden:

- Die Größe und Form der Gonaden weisen natürlicherweise eine große Variabilität auf. Ist in dem zu untersuchenden Entwicklungsstadium der Embryonen eine eindeutige morphologische Einteilung in die Kategorien Männlich – Weiblich – Hermaphrodit möglich?
- Es gibt unzählige Gene, die bei der Entstehung eines Individuums eine Rolle spielen und sich gegenseitig beeinflussen. Ist es möglich, wenige Markergene zu identifizieren, deren Regulierung ausreicht, um die Ausbildung der Gonaden zu beeinflussen?

Diese beiden Fragen stellen eine große Herausforderung dar, die es im Rahmen der vorliegenden Arbeit zu bearbeiten galt.

1.9 Ziele und Hypothesen der vorliegenden Arbeit, Vorgehensweise

Diese Arbeit untersucht, inwiefern sich Hühnerembryonen als Modellorganismen für endokrine Substanzen eignen und ob sie mit Blick auf die gewählten Endpunkte auf androgen und estrogen wirkende Substanzen reagieren. Es galt zu klären, ob die histologischen und genetischen Veränderungen bei bebrüteten Embryonen (19 Tage) sich zur Untersuchung der Wirkung von Chemikalien eignen.

Im Detail sollten hierbei folgende Hypothesen geprüft werden:

1. Die Gonaden von unbehandelten Hühnerembryonen sind am 19. Bebrütungstag morphologisch und auf Grund ihres Genexpressionsprofils gut differenzierbar, und es können klare Kriterien zur Unterscheidung von männlichen und weiblichen Gonaden formuliert werden.
2. Die Testsubstanzen lassen sich einfach lösen und injizieren.
3. Die gewählten Lösemittel haben keinen Einfluss auf die Gonadenentwicklung.
4. Androgene und estrogene Substanzen haben klare und jeweils spezifische Auswirkungen auf die morphologische und histologische Gonadenausprägung.
5. ED haben signifikante Auswirkungen auf das Genexpressionsprofil der Gonaden.

Diese Hypothesen wurden im Rahmen dieser Dissertation in drei Veröffentlichungen, welche einen Peer-Review-Prozess mit internationalen Experten durchliefen, überprüft.

In einem ersten Schritt wurden die Gonaden unbehandelter Embryonen morphologisch, histologisch und parallel auf der Ebene der Genexpression untersucht. Dies war notwendig, um eine Basisebene für die natürlichen Varianzen der Genexpression innerhalb der Gonaden sowie der Formentwicklung der Gonaden zu erhalten. Hierfür wurden die Eier von unbehandelten Hühnerembryonen verwendet. Die Bebrütung wurde nach 19 Tagen beendet und die Gonaden isoliert. Zur Analyse der Genexpression in den Gonaden wurde die mRNA isoliert und mittels SuperSAGE analysiert, eine Technik zur qualitativen und quantitativen Analyse exprimierter mRNA [57, 58].

Mittels dieser Transkriptionsanalyse wurden von Gonaden der Embryonen getrennte Expressionsprofile (Männchen/Weibchen, rechts/links) hergestellt und die Profile miteinander verglichen. Hierbei konnten klare Unterschiede zwischen männlichen und weiblichen Gonaden festgestellt werden. Zudem wurden starke Unterschiede zwischen der rechten und linken Gonade bei weiblichen Embryonen festgestellt.

Diese Studie wurde als erster Bestandteil dieser Dissertation veröffentlicht (Annex 1.1):

Gene expression of chicken gonads is side-and sex-specific.

Scheider J, Afonso-Grunz F, Hoffmeier K, Horres R, Groher F, Rycak L, Oehlmann J, Winter P (2014): Sexual Development 8, 178-191.

Für eine standardisierte Untersuchungsmethode im Rahmen von OECD-Guidelines werden robuste Bezugswerte benötigt, die eine Angabe zur Veränderung der gemessenen Werte im Vergleich zu den Normwerten ermöglichen. Um einen Überblick über die Variabilität der natürlichen Gonadenformen und -größen und deren histologischem Aufbau wie die Dicke des Cortex und der Aufbau der Medulla bei Weibchen sowie die Anzahl der sich im Aufbau befindlichen Tubuli seminiferi zu bekommen, wurden aus einer großen Reihe identisch bebrüteter, unbehandelter Embryonen genau diese Werte standardisiert vermessen. Hierzu kam zu jeder Bebrütungsreihe der unbehandelten Embryonen ebenfalls eine parallel dazu bebrütete Reihe von Individuen, die mit Dimethylsulfoxid (DMSO) behandelt wurden. DMSO ist eine natürlich vorkommende Schwefelverbindung, ist fett- und wasserlöslich und ein vielseitiges Lösungsmittel. Es wird häufig in der Ökotoxikologie als Lösungsmittel eingesetzt [82-86], auf Grund seiner Eigenschaft, Zellwände durchdringen zu können und so gelöste Stoffe in die Zelle einzubringen. Zur ersten Überprüfung der Normwerte wurden Versuche durchgeführt, bei denen die Embryonen verschiedene Dosierungen von BPA oder EE_2, gelöst in DMSO, injiziert bekamen.

Diese Studie wurde als zweiter Bestandteil dieser Dissertation veröffentlicht (Annex 1.2):

The domestic fowl (*Gallus gallus domesticus*) embryo as an alternative for mammalian experiments – validation of a test method for the detection of endocrine disrupting chemicals.

Jessl L, Scheider S, Oehlmann J (2018): Chemosphere 196: 502-513.

Zur Überprüfung der Normwerte und der Auswirkungen von Androgenen und indirekten Androgenen auf die Gonaden wurden Tributylzinn (TBT) und Methyltestosteron (MT) injiziert. Hierfür wurden den Hühnereiern mehrere Konzentrationen TBT (0,5, 3, 10, 30 pg/g KG) und MT (30 pg/g KG) injiziert und die Veränderungen auf histologischer Seite vermessen. Parallel dazu wurde ebenfalls eine molekulare Untersuchung mittels SuperSAGE (s.o.) durchgeführt zur quantitativen Bestimmung der mRNA in den Gonaden. Die untersuchten Konzentrationen lagen für TBT bei 10 pg/g KG und für MT 30 pg/g KG. Die phänotypisch deutlich sichtbare Virilisierung wurde durch veränderte Expressionsprofile geschlechtsabhängiger Gene widergespiegelt. Hierbei sind mehrere Transkriptions- und Wachstumsfaktoren (z.B. FGF12, CTCF, NFIB) zu nennen, deren geänderte Expression als Markersatz dienen könnte für eine frühe Identifizierung endokrin aktiver Chemikalien, die die embryonale Entwicklung beeinflussen.

Diese Studie wurde als dritter Bestandteil dieser Dissertation veröffentlicht (Annex 1.3):

Morphological and transcriptomic effects of endocrine modulators on the gonadal differentiation of chicken embryos: the case of tributyltin (TBT).

Scheider J, Afonso-Grunz F, Jessl L, Hoffmeier K, Winter P, Oehlmann J (2018): Toxicology letters 284: 143-151.

2 Diskussion

2.1 Wichtigste Ergebnisse der vorliegenden Arbeit

Im Folgenden werden die wichtigsten Ergebnisse der Dissertation dargestellt. Die ausführlichen Beschreibungen der Experimente, deren Ergebnisse und eine detaillierte Diskussion können den Artikeln im Annex 1 entnommen werden.

2.1.1 Unterscheidbarkeit der Geschlechter *in situ*

Die Resultate der ersten sowie der dritten Publikation über die natürliche Unterscheidbarkeit der unbehandelten Embryonen können wie folgt zusammengefasst werden:

- Die Geschlechter sind – unbeeinflusst jeglicher äußerer Manipulation – zuverlässig nach dem Öffnen des Bauchraumes zu unterscheiden. Wie in Annex A.1.1 und A.1.2 beschrieben, ist jedes unbehandelte Tier trotz individueller Varianzen anhand der Gonaden klar als Männchen oder Weibchen identifizierbar. Zusätzlich sind bei den weiblichen Gonaden klare laterale Unterschiede festzustellen, da die rechte Gonade nur etwa 20% der Oberflächengröße der linken Gonade besitzt.
- In histologischen Schnitten sind die Gonadengewebe von 19 Tage lang bebrüteten Hühnerembryonen ebenfalls eindeutig differenzierbar. Wie ebenfalls in Annex A.1.2 und A.1.3 gezeigt und beschrieben, sind die Abschnitte der weiblichen Gonaden schnell und einfach in Medulla und Cortex zu klassifizieren sowie bei den männlichen Gonaden die Anlagen der *Tubuli seminiferi* zu erkennen.

- Wie in Annex A.1.1 beschrieben, zeigt die mRNA-Analyse der Gewebe, dass sich die Genexpressionsprofile der männlichen und weiblichen Gonaden stark unterscheiden. Zusätzlich unterscheidet sich die Genexpression in den weiblichen Gonaden auf der jeweiligen rechten und linken Seite auffallend (Annex A.1.1). Auch bei den männlichen Gonaden gab es Unterschiede zwischen den rechten und linken Gonaden. Diese waren aber deutlich geringer.

2.1.2 Wirksamkeit von Lösungsmitteln

In ökotoxikologischen Studien ist besonders bei der Testung apolarer und damit schlecht in wässrigen Medien löslicher Substanzen ein Lösungsmittel unverzichtbar, die Wahl des Lösungsmittels für die jeweilige zu prüfende Substanz aber von zentraler Bedeutung. Idealerweise sollte das Lösungsmittel ausschließlich die Substanz lösen und als Carrier dienen, selbst aber keinen Effekt auf das zu prüfende Individuum haben. Zudem sollte keine Kombination mehrerer Lösungsmittel verwendet werden, um den ggf. trotzdem vorliegenden genetischen Effekt der Stoffe in Summe so gering wie möglich zu halten. Durch die unterschiedliche Löslichkeit der Prüfsubstanzen müssen dennoch verschiedene Lösemittel verwendet werden. Dabei haben der hohe Fettgehalt des Dotters und des Embryos, aber auch das Löslichkeitsverhalten der Testsubstanz, einen wesentlichen Einfluss auf die richtige Wahl.

TBT ist lipophil, daher wurde in der Studie mit dieser Organozinnverbindung Sesamöl als Lösemittel für fettlösliche Substanzen verwendet. Sesamöl wurde bereits in verschiedenen Vogelstudien getestet, ohne dass morphologische Effekte gefunden wurden [87-89].

BPA ist löslich in organischen Lösungsmitteln und nur schlecht wasserlöslich, daher wurde Dimethylsulfoxid (DMSO) als Trägersubstanz ausgewählt. DMSO ist ein hygroskopisches, organisches Lösungsmittel. Aufgrund seiner Membranpermeabilität dient es oft als Transportvermittler für Arzneimittel in Salben, Gelen, Pflastern und Tinkturen und findet auch oft in ökotoxikologischen Untersuchungen Verwendung [85, 86, 90, 91].

- Auf morphologischer Ebene wurde bei Sesamöl (8 µL) ein marginaler, nicht signifikanter androgener Effekt (leicht kleinere linke Gonade) bei den weiblichen Gonaden festgestellt, ebenso eine leichte Verringerung der Cortexdicke (Annex A.1.3). Dies war bisher in keiner Veröffentlichung festgestellt worden, wobei allerdings in früheren Studien nur anatomische Untersuchungen und keine feingewebliche Histologie eingesetzt wurde.
- Bei beiden Lösungsmitteln wurde kein signifikanter Effekt auf die Mortalität festgestellt. Zudem konnte auch kein Einfluss bei verschiedenen Injektionsvolumina (DMSO: 15 – 60 µL) festgestellt werden (Annex A.1.2 und A.1.3).
- Bei DMSO verkleinerte sich die Oberfläche der weiblichen linken Gonade signifikant, während die Cortexdicke in einigen Versuchsreihen bei männlichen und weiblichen linken Gonaden vergrößert war. Dieser Effekt wurde durch höhere Injektionsmengen (15 µL / 60 µL, Annex A.1.2) verstärkt.
- Auf genetischer Ebene zeigte Sesamöl einen Effekt auf die Expression unterschiedlichster Gene. Für die Berechnung des Effekts der Testsubstanz musste daher dieser Effekt durch das Lösungsmittel berücksichtigt werden. (Annex A.1.3).
- Die Auswertung der genetischen Daten zur Wirkung von DMSO wurde noch nicht ausgewertet und veröffentlicht.

Fazit: Beide Lösungsmittel haben einen unterschiedlichen Einfluss auf die Gonaden, der jedoch relativ gering und somit vernachlässigbar war. Der Effekt auf die Genexpression war stärker ausgeprägt, konnte aber entsprechend differenziert und berücksichtigt werden.

2.1.3 Effekte von Androgenen

Tribultylzinn und Methyltestosteron sind als indirekt (TBT) bzw. direkt androgen (MT) wirkende Substanzen u.a. dafür verantwortlich, dass sich kein Cortex bildet und stattdessen Tubuli seminiferi ausbilden.

- Bei der Zugabe von TBT und ebenfalls von MT wurde bei weiblichen Gonaden ein signifikant dünnerer Cortex im Vergleich zur Kontrolle festgestellt sowie die Ausbildung einzelner Tubuli seminiferi diagnostiziert (Annex A.1.3).
- Es wurde kein Effekt auf die Histologie oder Morphologie bei einem Überschuss an Androgenen auf die männlichen Gonaden festgestellt (Annex A.1.3).
- MT und TBT beeinflussen die Expression einer großen Anzahl von Genen, die für die Entwicklung der Gonaden verantwortlich sind (Annex A.1.3). Beispielsweise werden einige Pluripotenzmarker bei MT sehr stark bei den Weibchen herunterreguliert, während andere sehr stark heraufreguliert werden. TBT hatte einen unspezifischeren, nicht so starken Effekt wie MT auf die untersuchten Gene (Annex A.1.3, Abb. 4)

2.1.4 Effekte von Estrogenen

Bisphenol A und EE_2 sind als estrogene Disruptoren weitreichend für einen verweiblichenden Effekt bei vielen Organismen bekannt. Bei den Hühnerembryonen wurden die folgenden Wirkungen hervorgerufen:

- Beide Estrogene riefen eine signifikante Reduktion der Cortexdicke bei Weibchen hervor (Annex A.1.2).
- In Männchen verursachten beide Substanzen einen signifikant verdickten Cortex mit oocytenartigen Zellen und eine mit weiblichen Strukturen vergleichbare äußere Gonadenform, was auch in anderen veröffentlichten Studien beschrieben wird (Annex A1.2, [37, 61-63].

- Es zeigte sich bei beiden Testsubstanzen eine deutlich geringere Anzahl und weniger weit entwickelte *Tubuli seminiferi* sowie typisch weibliche Strukturen wie Lacunae in den männlichen Gonaden. Während BPA das Auftreten von *Tubuli seminiferi* leicht unterband, war das testikuläre Gewebe von EE_2-behandelten Tieren stark verändert (Annex A.1.2).
- Im direkten Vergleich ruft EE_2 erheblich ausgeprägtere Effekte hervor als BPA, was auf eine höhere estrogene Potenz von EE_2 hinweist. Dies wurde auch bereits in anderen Untersuchungen mit Wirbeltieren, darunter mit dem Reiskärpfling (*Oryzias latipes*) und Zebrabärbling (*Danio rerio*), aber auch in verschiedenen In-vitro-Test festgestellt (Annex A1.2, [92, 93]).

2.2 Histologische Veränderungen und Genexpressionsanalyse unter Substanzexposition im Vergleich

Wie in Kap. 2.1.2 beschrieben, ist die richtige Wahl des Lösungsmittels wesentlich, um Störeffekte auszuschließen. Wie erwartet hatten die gewählten Lösungsmittel auf die Anatomie der Gonadengewebe und die histologischen Untersuchungen keinen oder einen vernachlässigbaren Einfluss. Umso erstaunlicher war der doch deutliche Einfluss auf die Genexpression. Besonders auffällig war der Effekt von Sesamöl (Annex A.1.3). Aufgrund der Resultate muss davon ausgegangen werden, dass Sesamöl tatsächlich die Genexpression der Individuen signifikant beeinflusst. Auf der anderen Seite zeigten die histologischen Befunde, dass auf morphologischer Ebene keine negativen Auswirkungen von Lösungsmitteln festgestellt werden können. Die messbaren Effekte auf Stufe der Genexpression haben offenbar keinen relevanten Einfluss auf die Organe. Dem gegenüber zeigten jedoch die getesteten ED einen deutlichen Effekt.

Es scheint, dass die Effekte auf Stufe der Genexpression, die durch das Lösungsmittel verursacht wurden, durch den Organismus gut kompensiert werden konnten, was schließlich zu einer ungestörten Entwicklung der Organe führte. Auf der anderen Seite können unterschiedliche Effekte durch weitere, problematischere Substanzen durch den Organismus nicht mehr kompensiert werden. Leider war es mit den vorliegenden Daten auf Stufe der mRNA-Untersuchungen im Alter von ausschließlich 19 Tagen Bebrütung nicht möglich zu unterscheiden, welche Veränderungen genau für den Organismus letztlich relevant und somit schädlich waren und welche nicht.

Interessant ist aber der Befund, dass die Genexpression hoch sensibel reagieren kann und so eine sehr hohe Variabilität, aber auch eine gewisse Plastizität aufweist. Möglicherweise werden einige Einflüsse auf die Expression einzelner Gene durch eine Veränderung der Genexpression an anderer Stelle kompensiert. Andere Einflüsse hingegen können offenbar nicht kompensiert werden und pflanzen sich bis in die höheren Organisationsstufen der Lebewesen fort (Genexpression, Organ, Fortpflanzung, Population).

Dabei zeigte sich in den Resultaten, dass die Varianz der Genexpression bei den Kontrolltieren mit und ohne Lösungsmittel erheblich größer war als die Varianz bei den Tieren, die mit MT behandelt wurden (Annex A.1.3). Offenbar führte ein für den Organismus schädlicher Stoff mit negativen Wirkungen auf der Organstufe zu einer geringen Varianz bei der Genexpression. Oder anders gesagt könnte diese geringe Varianz auf Stufe der Genexpression auch ein Ausdruck einer schädlichen Wirkung sein, welche letztlich nicht mehr durch die Expression anderer Gene kompensiert wird oder nicht mehr kompensiert werden kann.

Dieses auf den ersten Blick widersprüchliche Verhalten auf Stufe der Genexpression im Vergleich zu den Resultaten auf der Stufe der Organe kann offenbar zu Fehlinterpretationen führen, wenn ausschließlich eine Untersuchungsmethode betrachtet wird. In Unkenntnis der tatsächlichen Wirkmechanismen auf Genexpressionsebene ist möglicherweise gerade eine geringe Varianz in der Genexpression Ausdruck eines negativen Effekts für den Organismus. Inwieweit ein solcher Effekt tatsächlich besteht und ob dieser für die Beurteilung für den Nachweis relevanter Effekte auf genetischer Stufe genutzt werden könnte, sollte noch genauer untersucht werden.

2.3 Eignung der verwendeten Methoden und Empfehlungen für die Praxis

Bereits historische Untersuchungen an Hühnern und Wachteln zeigten eine phänotypische Verweiblichung männlicher Embryonen, sobald diese mit Estrogenen oder Estrogen-aktiven Substanzen behandelt wurden [30, 37, 61-63, 94-97]. Diesen Effekt für die Entwicklung eines neuartigen, standardisierten Testsystems zu nutzen, war ein Ziel der vorliegenden Arbeit. Ein neuer Aspekt dieser Methode war die parallele, vergleichende Gabe von androgen wirkenden Stoffen, um eine Vergleichbarkeit zu ermöglichen und die Untersuchungszeit zu verkürzen. Die Auswirkungen der Gabe von Androgenen waren bisher nur in wenigen Studien beschrieben worden [70, 71, 98-100].

Die vorliegenden Untersuchungen zeigen in Übereinstimmung mit den Ergebnissen der Arbeitsgruppe von C. Berg, dass estrogen sowie auch androgen wirkende Stoffe auf histologischer Ebene reproduzierbare Effekte verursachen. Somit bestätigt sich, dass diese Methode grundsätzlich in der Lage ist, entsprechende Wirkungen auch von anderen, ähnlich wirkenden Substanzen zu quantifizieren. Insofern könnte die Methode auch für die systematische Effekterfassung von Stoffen eingesetzt werden.

Auf der anderen Seite besteht in Verbindung mit den histologischen Resultaten auch ein wertvoller Datensatz über die Auswirkungen auf genetischer Ebene. Die Resultate der SuperSAGE-Analyse erlaubten die Identifizierung von Einflüssen auf die Expression von Genen, welche bislang nicht bekannt waren, die die Liste der bisherigen bekannten reagierenden Gene (vgl. Kap. 1.7.2) erweitern kann. Bei den Androgenen wurden beispielsweise zusätzlich zu den bisher in anderen Studien identifizierten Genen, welche auf die Behandlung mit MT bzw. TBT reagierten, neue weitere Gene gefunden (Annex A.1.3). Aufgrund der vollständigen Erfassung der Expression aller Gene jeweils für männliche und weibliche Gonaden (Annex A.1.1). müsste in einem nächsten Schritt das Genexpressionsmuster aufgrund der Wirkung von BPA und EE_2 untersucht und mit dem Expressionsmuster der Androgen-Effekte verglichen werden. Auf diese Weise scheint es möglich, viele Gene, welche bei der Geschlechtsentwicklung involviert sind, bestimmen zu können. Einschränkend ist, dass während der Embryonalentwicklung bestimmte Gene nur zu verschiedenen Zeitpunkten exprimiert oder eben nicht exprimiert werden [101, 102].

Da die Untersuchungen am 19. Bebrütungstag ausgeführt wurden, gibt es in eben diesem Punkt eine gewisse Unsicherheit. Gleichzeitig können auf diese Weise eventuelle Ersatzgene identifiziert werden, um dem oben angesprochenen Problem der Relevanz der kompensatorischen Kräfte eines Organismus Rechnung zu tragen.

Auf der anderen Seite antworten die Endpunkte der Histologie, wie Cortexdicke und prozentualer Anteil der *Tubuli seminiferi*, ebenso zuverlässig auf die Modellestrogene BPA und EE_2 wie auch auf die Modellandrogene MT und TBT. Die präsentierten Referenzwerte aus Annex A.1.2 und A.1.3 liefern auf histologischer Ebene bereits einen guten Bezugspunkt für die Beurteilung schädlicher Substanzen. Zudem wurden in der Zwischenzeit weitere Antiestrogene, wie Tamoxifen und Fulvestrant, mit diesen histologischen Methoden überprüft. Es zeigte sich, dass die Methode auch in diesen Untersuchungen verlässliche Resultate liefert [103].

2.4 Relevanz der gewonnenen Erkenntnisse

Die Ergebnisse bilden einen guten Basisdatensatz, denn hier sind bei androgen und estrogen wirkenden Stoffen Hinweise auf Effekte bei den betroffenen Individuen identifizierbar. Dieses Modell hat daher bereits ein großes Potential, Tierversuche zu ergänzen und zu ersetzen, indem es die 3R-Regel in mehrfacher Hinsicht erfüllt:

- Replacement:

- Juvenile Säuger werden durch nicht geschlüpfte Hühnerembryonen ersetzt, sodass rechtlich gesehen kein Tierversuch vorliegt.

- Reduction:

- Die Versuchsdauer ist erheblich kürzer.
- Die Versuche können jederzeit sofort begonnen werden, es muss nicht langfristig geplant werden.
- Es ist keine Gonadektomie notwendig, die beim Hershberger- und Uterotrophen Assay bereits mehrere Wochen vor der eigentlichen Applikation der Testsubstanzen erforderlich ist und als operativer Eingriff mit zusätzlichem Leiden für die Versuchstiere verbunden ist.
- Eine Tierhälterung entfällt.

- Refinement:

- Es werden Auswirkungen auf beide Geschlechter gleichzeitig registriert, nicht nur auf ein Geschlecht.
- Die Schmerznozizeption und -perzeption der E19-Embryonen ist geringer als die bereits adulter und sichtbar schmerzempfindlicher juveniler Säuger (vgl. [104] und Kap. 2.6).

2.5 Überprüfung der Hypothesen

In Bezug auf die zu untersuchenden Hypothesen (Kap. 1.9) können aufgrund der erzielten Ergebnisse folgende Aussagen getroffen werden:

1. Die Gonaden von unbehandelten Hühnerembryonen sind am 19. Bebrütungstag morphologisch und auf Grund ihres Genexpressionsprofils gut differenzierbar, und es können klare Kriterien zur Unterscheidung von männlichen und weiblichen Gonaden formuliert werden.

➔ Diese Hypothese wird durch die vorliegenden Ergebnisse unterstützt.

2. Die Testsubstanzen lassen sich einfach lösen und injizieren.

Unter Beachtung des maximal injizierbaren Volumens (<100µL) ist dies bei den getesteten Substanzen möglich. Weiter muss die Löslichkeit (lipophil/lipophob) der Prüfsubstanz beachtet werden, um die minimal injizierbare Menge zu erreichen.

➔ Diese Hypothese wird durch die vorliegenden Ergebnisse unterstützt.

3. Die gewählten Lösemittel haben keinen Einfluss auf die Gonadenentwicklung.

Auf histologischer Ebene sind die Auswirkungen von Sesamöl und DMSO marginal. Es sind nicht signifikante Veränderungen bei Sesamöl und DMSO vorhanden, diese können also vernachlässigt werden.

➔ Diese Hypothese wird durch die vorliegenden histologischen Ergebnisse unterstützt.

Bei der Genexpression zeigte sich bei Sesamöl dagegen ein sichtbarer Einfluss, der für die Berechnung des Effekts der Prüfsubstanz herausgerechnet werden sollte. Auf genetischer Ebene geht dies nur bedingt, da die tatsächlichen physiologischen Auswirkungen der sichtbaren genetischen Veränderungen noch nicht absehbar sind, ebenso wie die kompensatorische Wirkung der Gene, die zwar auf die Behandlung reagieren, was aber als indirekte Reaktion anzusehen ist.

➔ Die Hypothese muss also auf genetischer Ebene aufgrund der vorliegenden Ergebnisse abgelehnt werden.

4. Androgene und estrogene Substanzen haben klare und jeweils spezifische Auswirkungen auf die morphologische und histologische Gonadenausprägung.

➔ Ungeachtet der unterschiedlichen Potenz der Hormone EE_2 und MT im Vergleich zu BPA und TBT wird diese Hypothese wird durch die vorliegenden Ergebnisse unterstützt.

5. ED haben signifikante Auswirkungen auf das Genexpressionsprofil der Gonaden.

In den veränderten Expressionsmustern der Gene ist ein Vielfaches an Veränderung zu erkennen im Vergleich zu den optisch erkennbaren Veränderungen in den histologischen Gewebeschnitten. Die beiden (indirekt) androgenen Substanzen rufen vergleichbare histologische Veränderungen hervor, die Muster der von den beiden Substanzen hervorgerufenen veränderten Genexpression sind aber nicht identisch. Mit dem bisherigen Stand der Dinge ist nur ersichtlich, dass eine stark veränderte Genexpression stattfindet.

➔ Insofern wird die Hypothese für androgene Substanzen durch die vorliegenden Ergebnisse unterstützt.

2.6 Rechtliche Aspekte vs. ethische Perspektive – wo liegt das Problem?

In Deutschland hat der Tierschutz einen hohen Stellenwert. Dies wird nicht zuletzt durch seine Verankerung im Jahr 2002 im Grundgesetz, Art. 20a deutlich. Vor diesem Hintergrund sind auch Tierversuchen in Deutschland rechtlich enge Grenzen gesetzt. Der zentrale Grundsatz des Tierschutzgesetzes (TierSchG) lautet: "Niemand darf einem Tier ohne vernünftigen Grund Schmerzen, Leiden oder Schaden zufügen" (§ 1 TierSchG) [105]. Die meisten der im Zuge der Novelle des TierSchG von 2013 für den Bereich der Tierversuche vorgenommen Änderungen wurden durch die 2010 vom Europäischen Parlament verabschiedete Tierversuchsrichtlinie RL 2010/63/EU veranlasst.

Tierversuche im Sinne des deutschen Tierschutzgesetztes (TierSchG) sind Versuche an lebenden Tieren. Die Tötung eines Tieres, z.B. für Versuche an Organen und Geweben, ist also kein Tierversuch im gesetzlichen Sinne. Nach § 8 TierSchG sind Tierversuche bei Wirbeltieren generell genehmigungspflichtig, außer die Durchführung geschieht aufgrund von Gesetzen, Verordnungen, richterlicher Anordnung, zu Zwecken der Impfung, der Blutentnahme oder sonstiger diagnostischer Maßnahmen. Nach § 8a müssen diese ausgenommenen Fälle, wie auch Tierversuche an Dekapoden und Cephalopoden, der zuständigen Landesbehörde nur angezeigt werden. Versuche an Embryonen, Feten, bebrüteten Eiern sind dagegen nach § 1/§ 4/§ 17 TierSchG weder anzeige- noch genehmigungspflichtig. Dies wurde auch in einschlägigen Kommentaren zum Tierschutzgesetz noch einmal präzisiert [106-108]. Auch in der Tierschutzversuchsverordnung (TierSchVersVO) vom 12. August 2013 werden „Wirbeltiere und Kopffüßer“ sowie in § 39 „Zehnfußkrebse“ genannt, aber Embryonen, Feten oder bebrütete Eier werden nicht erwähnt.

Die vorliegende Arbeit geht daher an dieser Stelle davon aus, dass es sich bei den Untersuchungen bei bebrüteten Eiern nicht um Tierversuche handelt. Durch den Einsatz der 3R-Regel [48] könnte eine Verbesserung erreicht werden, indem mit der Methode Experimente mit Ratten, Mäusen oder ganzen Wachtelgenerationen ersetzt werden. In diesem Sinn würde in jedem Fall das Leiden von Tieren reduziert, indem einerseits ein anderes Modell genutzt, andererseits aber auch eine bessere Effizienz erzielt wird, indem mit der beschriebenen Technik eine Verfeinerung der vorgeschriebenen Versuche möglich ist (vgl. Kap. 2.4).

In den erzielten Ergebnissen der neuen Methode findet nicht ausschließlich eine Beschreibung der (finalen) Symptome statt, sondern es soll zusätzlich eine Ursachendarstellung durch die Veränderung der Umsetzung der Information der DNA in das Embryonalsystem stattfinden: Die Kaskaden spezifischer Gene und ihrer Produkte in Wechselwirkung mit den zugesetzten Substanzen. Dadurch kann die Effizienz der Nachweismethode zusammen mit den molekularen Techniken weiter verbessert werden, was wiederum den Verbrauch von Versuchstieren reduzieren kann - sobald die in Kap. 2.3 geforderten Grundlagenuntersuchungen abgeschlossen sind.

Gleichzeitig gibt es aber immer noch eine ethisch-moralische Perspektive, wenn betrachtet wird, dass in den vorliegenden Versuchen schlupffähige Tiere aus den Eischalen geholt werden, um sie abzutöten, obwohl sie bereits Schmerzempfindlichkeit besitzen. Untersuchungen zur Schmerzperzeption und Nozizeption von Hühnerembryonen zeigen, dass die Fähigkeit hierzu sich schrittweise entwickelt, beginnend am Tag sieben der Inkubation. Es ist wichtig, zwischen bewusstem Schmerzempfinden und einer unbewussten Reizverarbeitung durch Nozizeption zu unterscheiden. Zudem ist bei Vögeln zu beachten, dass durch das Fehlen einer ausgeprägten Hirnrinde andere Hirnareale eine Rolle in der Schmerzwahrnehmung spielen könnten. Prinzipiell gilt es als erwiesen, dass Vogelembryonen zur In-ovo-Nozizeption in der Lage sind [104, 109]. Aufgrund fehlender Kenntnisse ist der genaue Zeitpunkt, an dem der Hühnerembryo Schmerzen empfinden kann, nicht bekannt, wobei der Hühnerembryo ab Tag 13 über ein funktionell entwickeltes Gehirn verfügt, denn ab diesem Tag ist das Neuralrohr als voll entwickelt zu betrachten [109].

Es scheint auf den ersten Blick anmaßend, Vogelembryonen mit dem Embryonenschutzgesetz, das ausschließlich für den Menschen gedacht ist, in Beziehung zu bringen, aber Bedenken beim Anblick eines eigentlich lebensfähigen, aber kurz davor getöteten Kükens sind nicht unangebracht, ebenso wie der Gedanke um das Recht der Wertigkeit des Lebens unterschiedlicher Taxa. Es stellt sich die Frage, ob und inwiefern der menschliche Nutzen tierisches Leiden und Sterben rechtfertigt. So gesehen erscheint es möglich, dass sich die gesetzliche Regelung nicht vollständig mit dem moralischen Empfinden vieler Menschen in unserer Gesellschaft deckt. Dieser Teil der Gesellschaft würde vermutlich in diesem Punkt einen Schritt weiter gehen. Andererseits muss festgestellt werden, dass bei der großbetrieblichen Hühnerproduktion männliche Küken in großer Anzahl tagtäglich gekeult werden [110]. Offenbar ist in diesem Punkt die gesellschaftliche Toleranz größer.

In letzter Konsequenz aber steht der Schutz des menschlichen Lebens moralisch an oberster Stelle. Das Benutzen anderer Lebewesen, um diesen Schutz zu gewährleisten, muss daher auch als moralisch angesehen werden. Dabei soll aber auf untergeordneter Stufe das Tierwohl verringert werden. Im Sinne des Utilitarismus müsste dabei der aggregierte Gesamtnutzen die Summe des Wohlergehens aller Betroffenen maximieren [111, 112]. Somit stehen die beiden Faktoren Anzahl der verbrauchten Versuchstiere und Leidensdruck der Versuchstiere in einem bestimmten Verhältnis. Aus Sicht der Ethik müsste dieses Verhältnis optimiert werden.

Für diese Optimierung der Tierversuche ist derzeit die Betrachtung der Nutzen-Risiko-Aspekte unverzichtbar, lassen sich doch zunehmend mehr spezielle wissenschaftliche Fragen ohne Tierversuche durch Modellierung, durch In-vitro- oder auch In-silico-Versuche bearbeiten. Die vollständige Translation von Grundlagen- und Forschungserkenntnissen auf unterschiedliche Taxa bei der Neuentwicklung von Stoffen ohne eine Zwischenbewertung und Weiterentwicklung im Tierversuch ist aber (immer noch) nicht denkbar. Es ist mit dem bisherigen Wissen zurzeit nicht möglich, sie vollständig zu ersetzen, ohne substanzielle Verluste an Chemikaliensicherheit und Forschungsqualität in Kauf zu nehmen.

Es gibt sehr viele Homologien und Analogien bei den Taxa, ihrem Aufbau, den Organfunktionen und Hormonen. Aber die vielen kleinen und großen Unterschiede zwischen Menschen und anderen Säugern, Vögeln, Reptilien und Amphibien bis hin zu den Wirbellosen lassen es nicht zu, dass eine toxikologische oder gar eine ökotoxikologische Bewertung neu entwickelter Stoffe nur in seiner Wirkung auf die Zielorganismen untersucht wird. Es müssen bei der Bewertung von Stoffen die Effekte und Wirkungen auf Nichtzielorganismen erfasst werden, weil Letztere beim (ggf. unabsichtlichen) Freisetzen einer Substanz in die Umwelt von dieser kontaminiert werden. Der Mensch als denkendes, höheres Wesen trägt auch eine ethische Verantwortung, die Umwelt und andere Tiere und Pflanzen zu schützen, indem er die Umweltrisiken der von ihm hergestellten Substanzen beurteilt und die Freisetzung dieser Stoffe in letzter Konsequenz minimiert und reglementiert. Und dies geht bisher nicht ohne Tierversuche.

3 Der Hühnerembryo als Tierversuchsersatzmethode - Schlussfolgerungen und Ausblick

Abschließend lässt sich feststellen, dass die im Rahmen der Dissertation durchgeführten Experimente geeignet waren, um die aufgestellten Hypothesen (Kap. 1.8 und 2.5) zu untersuchen. Der Nachweis für die Genaktivität als Grundlage für die histologischen Befunde mit vermännlichend wirkenden Substanzen wurde geführt. Es wurden in den Untersuchungen Gengruppen identifiziert, die herangezogen werden können, um einen Grund-Datensatz für die Identifikation typischer Effekte von androgenen und estrogen wirkenden Stoffen zu erstellen. Die Ergebnisse der Versuche belegen die prinzipielle Eignung der entwickelten Methode, um eine Effektbeurteilung für neu entwickelte Stoffe durchzuführen.

Zur Weiterentwicklung des Tests auf genetischer Ebene ist noch erheblicher Arbeitsaufwand notwendig. Es sollte einerseits – nach dem Vergleich der gefundenen Gene bei androgen wirkenden Stoffen mit denen der estrogen wirkenden Stoffe – dieser Datensatz verwendet werden, um ein geeignetes Alter der Embryonen herauszufinden, das möglichst weit unterhalb der 19 Tage Bebrütung (Tag 8-12, vgl. Abb. 1 und 2 und Kap. 2.6) liegt. Bei diesem Unterfangen ist es wichtig, ein Untergehen der gesuchten Gene im Rauschen der anderen vorhandenen Gene zu verhindern: eine Präparation der Gonaden bei kleineren Embryonen wird zunehmend schwieriger und andere Gewebeabschnitte des Embryos rund um die sich bildenden Gonaden (Mesonephron, Nieren) müssen ggf. mit in die Untersuchung mit einfließen, ergo deren Genexpression zur Weiterentwicklung des Tests dann ebenfalls beachtet werden. Vorliegende Untersuchungen zu 3, 5 und 6 Tage bebrüteten, ausschließlich dekapitierten und anschließend homogenisierten Embryonen zeigten, dass die Daten der gesuchten Gene im Rauschen untergehen und nicht mehr aufgefunden werden können, ebenso deren Veränderung – der Anteil der Gonaden im gesamten Embryo ist zu klein (GenXPro, pers. comm). Zusätzlich sollte ebenfalls die kompensatorische Wirkung der Gene nicht außer Acht gelassen werden. All dies erfordert allerdings noch sehr viel Grundlagenforschung zu den einzelnen Funktionen der gefundenen Gene und damit eine Findung der Balance zwischen der Tiefe der Untersuchungsmethode und deren praktischer Anwendbarkeit.

Da der Test auf genetischer Ebene noch sehr viel Grundlagenforschung erfordert, ist die primäre Weiterverfolgung der erfolgreich verlaufenen histologischen Arbeiten sinnvoll. Diese Weiterentwicklung ist bereits in Angriff genommen worden, indem an anderer Stelle mit dem Aromatasehemmer Fadrozol diese Untersuchungstechnik weitergeführt wird [113, 114]. Zusätzlich wird ebenfalls mit den histologischen Techniken weitergearbeitet, um die Anwendbarkeit auf weitere Wirkmechanismen zu überprüfen und die volle Bandbreite potentieller Effekte endokriner Disruptoren auf den Organismus untersuchen und bewerten zu können. Dies ist bereits mit den Antiestrogenen Tamoxifen und Fulvestrant geschehen [103], während aktuell die Daten für die Antiandrogene Flutamid, DDE (ein DDT-Derivat) und Cyproteronacetat noch ausgewertet werden [115].

Die Entwicklung einer Methode, bei der die histologischen Entwicklungen geklärt werden können und die verhältnismäßig schmerzarm, schneller und effektiver vonstattengeht, ist also bereits in toxikologischer Hinsicht ein sinnvoller Ersatz für den Uterotrophen (OECD-Test Nr. 440) und Hershberger Bioassay (OECD-Test Nr. 441) in allen drei Anforderungen der 3R-Regel: replacement, reduction und refinement. Zusätzlich ist sie in ökotoxikologischer Hinsicht ein effektiver Ersatz oder zumindest eine Ergänzung für den Avian Reproduction Test (OECD-Test Nr. 206) sowie den abgebrochenen, nicht fertig gestellten zwei-Generationsassay im Rahmen der Stoffprüfung auf der Suche nach potentiellen endokrinen Disruptoren.

Referenzen

1. IPCS (2002). Global assessment of the state-of-the science of endocrine disruptors. In International Programme on Chemical Safety, Damstra, T, S. Barlow, A. Bergman, R. Kavlock and G. Ven der Kraak, eds. (World Health Organisation).
2. WHO/UNEP (2013). State of the science of endocrine disrupting chemicals - 2012. An assessment of the state of the science of endocrine disruptors prepared by a group of experts for the United Nations Environment Programme (UNEP) and WHO. J.J.H. Åke Bergman, Susan Jobling, Karen A Kidd and R Thomas Zoeller, ed.
3. Oehlmann, J., Fioroni, P., Stroben, E., and Markert, B. (1996). Tributyltin (TBT) effects on *Ocinebrina aciculata* (Gastropoda: Muricidae): Imposex development, sterilization, sex change and population decline. Science of the Total Environment *188*, 205-223.
4. Alzieu, C. (2000). Impact of tributyltin on marine invertebrates. Ecotoxicology *9*, 71-76.
5. Allner, B., von der Goenna, S., Griebeler, E.-M., Nikutowski, N., Weltin, A., and Stahlschmidt-Allner, P. (2010). Reproductive functions of wild fish as bioindicators of reproductive toxicants in the aquatic environment. Environmental Science and Pollution Research *17*, 505-518.
6. Ankley, G., Mihaich, E., Stahl, R., Tillitt, D., Colborn, T., McMaster, S., Miller, R., Bantle, J., Campbell, P., Denslow, N., et al. (1998). Overview of a workshop on screening methods for detecting potential (anti-) estrogenic/androgenic chemicals in wildlife. Environmental Toxicology and Chemistry *17*, 68-87.
7. Iguchi, T., Watanabe, H., Katsu, Y., Mizutani, T., Miyagawa, S., Suzuki, A., Kohno, S., Sone, K., and Kato, H. (2002). Developmental toxicity of estrogenic chemicals on rodents and other species. Congenital Anomalies *42*, 94-105.
8. Watts, M.M., Pascoe, D., and Carroll, K. (2002). Population responses of the freshwater amphipod *Gammarus pulex* (L.) to an environmental estrogen, 17 α-ethinylestradiol. Environmental Toxicology and Chemistry *21*, 445-450.
9. Ankley, G.T., and Giesy, J.P. (1998). Endocrine disruptors in wildlife: a weight-of-evidence perspective. In Principles and processes for evaluating endocrine disruption in wildlife. ''Proceedings from Principles and Processes for Evaluating Endocrine Disruption in Wildlife; March 1996; KiawahIsland, South Carolina, R.J. Kendall, R.L. Dickerson, J.P. Giesy and W.P. Suk, eds. (Pensacola, Florida: Society of Environmental Toxicology and Chemistry (SETAC) Press), pp. 349-367.
10. Tyler, C.R., Jobling, S., and Sumpter, J.P. (1998). Endocrine disruption in wildlife: A critical review of the evidence. Critical Reviews in Toxicology *28*, 319-361.
11. Kortenkamp, A., Martin, O., Faust, M., Evans, R., McKinlay, R., Orton, F., and Rosivatz, E. (2012). State of the Art Assessment of Endocrine Disruptors, Final Report. Project Contract Number 070307/2009/550687/SER/D3.
12. McKim, J.M., and Erickson, R.J. (1991). Environmental impacts on the physiological mechanisms controlling xenobiotic transfer across fish gills. Physiological Zoology *64*, 39-67.
13. Kime, D.E. (1999). A strategy for assessing the effects of xenobiotics on fish reproduction. Science of the Total Environment *225*, 3-11.
14. Schäfers, C. (2003). Auswirkungen von Arzneimitteln und wirksamen Substanzen auf die aquatische Lebensgemeinschaft. In Spurenstoffe in Gewässern. Pharmazeutische Reststoffe und endokrin wirksame Substanzen, T. Track and G. Kreysa, eds. (Weinheim, Germany: Wiley-VCH), pp. 47–60.

15. Schäfers, C. (2010). Potential criteria for exclusion of active substances in plant protection products from registration due to endocrine effects under the new regulation 1107/2009/EC. Expert opinion prepared for the German Federal agency for consumer protection and food safety (BVL). (Schmallenberg, Germany: Fraunhofer-Institute for Molecular Biology and Applied Ecotoxicology).
16. Crain, D.A., Eriksen, M., Iguchi, T., Jobling, S., Laufer, H., LeBlanc, G.A., and Guillette, L.J. (2007). An ecological assessment of bisphenol A: Evidence from comparative biology. Reproductive Toxicology *24*, 225-239.
17. Fossi, M.C., Casini, S., and Marsili, L. (2006). Endocrine disruptors in Mediterranean top marine predators. Environmental science and pollution research international *13*, 204-207.
18. Veltman, K., Huijbregts, M.A.J., van den Heuvel-Greve, M.J., Vethaak, A.D., and Hendriks, A.J. (2006). Organotin accumulation in an estuarine food chain: Comparing field measurements with model estimations. Marine Environmental Research *61*, 511-530.
19. Markey, C.M., Rubin, B.S., Soto, A.M., and Sonnenschein, C. (2002). Endocrine disruptors: from Wingspread to environmental developmental biology. Journal of Steroid Biochemistry and Molecular Biology *83*, 235-244.
20. EFSA (2004). Opinion of the scientific panel on contaminants in the food chain on a request from the commission to as sess the health risks to consumers associated with exposure to organotins in foodstuffs (Question N° EFSA-Q-2003-110)
21. Fry, D.M. (1995). Reproductive effects in birds exposed to pesticides and industrial chemicals. Environmental Health Perspectives *103*, 165-171.
22. Ratcliffe, D.A. (1967). Decrease in eggshell weight in certain birds of prey. Nature *215*, 208-210.
23. Holm, L., Blomqvist, A., Brandt, I., Brunstrom, B., Ridderstrale, Y., and Berg, C. (2006). Embryonic exposure to o,p'-DDT causes eggshell thinning and altered shell gland carbonic anhydrase expression in the domestic hen. Environmental Toxicology and Chemistry *25*, 2787-2793.
24. Duis, K., Scheider, J., Warnecke, D., Van der Veen, A., Coors, A., Knacker, T., and Schäfers, C. (2014). Substances of very high concern under REACh – an evaluation of uncertainties in the environmental risk assessment of endocrine active substances. (Final report UBA-project FKZ 3710 63 416).
25. Kagami, H., and Hanada, H. (1997). Current knowledge of sexual differentiation in domestic fowl. Worlds Poultry Science Journal *53*, 111-123.
26. Scheider, J., Afonso-Grunz, F., Hoffmeier, K., Horres, R., Groher, F., Rycak, L., Oehlmann, J., and Winter, P. (2014). Gene expression of chicken gonads is sex- and side-specific. Sexual Development *8*, 178-191.
27. Adkins-Regan, E. (1990). Hormonal basis of sexual differentiation in birds. Hormones, Brain and Behavior in Vertebrates *8*: 1-14.
28. Ottinger, M.A., and Abdelnabi, M.A. (1997). Neuroendocrine systems and avian sexual differentiation. American Zoologist *37*, 514-523.
29. Hutson, J.M., Donahoe, P.K., and MacLaughlin, D.T. (1985). Steroid modulation of Mullerian duct regression in the chick embryo. General and Comparative Endocrinology *57*, 88-102.
30. Romanoff, A. (1960). The avian embryo., (Galt, Ontario: The Macmillan Company).
31. Bruggeman, V., Van As, P., and Decuypere, E. (2002). Developmental endocrinology of the reproductive axis in the chicken embryo. Comparative Biochemistry and Physiology A *131*, 839-846.
32. Vaillant, S., Guemene, D., Dorizzi, M., Pieau, C., Richard-Mercier, N., and Brillard, J.P. (2003). Degree of sex reversal as related to plasma steroid levels in genetic female chickens (*Gallus domesticus*) treated with fadrozole. Molecular Reproduction and Development *65*, 420-428.

33. Abinawanto, Shimada, K., Yoshida, K., and Saito, N. (1996). Effects of aromatase inhibitor on sex differentiation and levels of P450 (17α) and P450 arom messenger ribonucleic acid of gonads in chicken embryos. General and Comparative Endocrinology *102*, 241-246.
34. Elf, P.K., and Fivizzani, A.J. (2002). Changes in sex steroid levels in yolks of the leghorn chicken, *Gallus domesticus*, during embryonic development. Journal of Experimental Zoology *293*, 594-600.
35. Sechman, A., Hrabia, A., Lis, M.W., and Niedziolka, J. (2011). Effect of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) on steroid concentrations in blood and gonads of chicken embryo. Toxicology Letters *205*, 190-195.
36. Wartenberg, H., Lenz, E., and Schweikert, H.U. (1992). Sexual differentiation and the germ cell in sex reversed gonads after aromatase inhibition in the chicken embryo. Andrologia *24*, 1-6.
37. Berg, C., Halldin, K., and Brunstrom, B. (2001). Effects of bisphenol A and tetrabromobisphenol A on sex organ development in quail and chicken embryos. Environmental Toxicology and Chemistry *20*, 2836-2840.
38. Halldin, K., Berg, C., Bergman, A., Brandt, I., and Brunstrom, B. (2001). Distribution of bisphenol A and tetrabromobisphenol A in quail eggs, embryos and laying birds and studies on reproduction variables in adults following in ovo exposure. Archives of Toxicology *75*, 597-603.
39. Berg, C., Blomqvist, A., Holm, L., Brandt, I., Brunstrom, B., and Ridderstrale, Y. (2004). Embryonic exposure to oestrogen causes eggshell thinning and altered shell gland carbonic anhydrase expression in the domestic hen. Reproduction *128*, 455-461.
40. von Engelhardt, W., Breves, G., Diener, M., and Gäbel, G. (2015). Physiologie der Haustiere, 4. Auflage, (Stuttgart, Germany: Georg Thieme Verlag).
41. Krampitz, G., Engels, J., and Helfgen, I. (1974). Über das Vorkommen von Carboanhydratase in der Eischale des Huhnes. Experientia *30*, 228-229.
42. Brunstrom, B., Axelsson, J., Mattsson, A., and Halldin, K. (2009). Effects of estrogens on sex differentiation in Japanese quail and chicken. General and Comparative Endocrinology *163*, 97-103.
43. Dewil, E., Buyse, J., Veldhuis, J.D., Mast, J., De Coster, R., and Decuypere, E. (1998). In ovo treatment with an aromatase inhibitor masculinizes postnatal hormone levels, abdominal fat pad content, and GH pulsatility in broiler chickens. Domestic Animal Endocrinology *15*, 115-127.
44. Nowack, E. (1965). Die sensible Phase bei der Thalidomid-Embryopathie. Humangenetik *1*, 516-136.
45. Carson, R., Darling, L., Darling, L., Houghton Mifflin, C., and Riverside, P. (1962). Silent spring, (Boston; Cambridge, [Mass.]: Houghton Mifflin Company ; The Riverside Press).
46. Verordnung (EG) Nr. 1907/2006 (2007). Registration, Evaluation, Authorisation and Restriction of Chemicals.
47. European Commission (2007). Verordnung (EG) Nr. 1907/2006 des Europäischen Parlaments und des Rates zur Registrierung, Bewertung, Zulassung und Beschränkung chemischer Stoffe (REACh).
48. Russel, W., and Burch, R. (1959). The principles of humane experimental technique, Reprinted by UFAW, 1992: 8 Hamilton Close, South Mimms, Potters Bar, Herts EN6 3QD England Edition, (London).
49. Fridolfsson, A.K., and Ellegren, H. (1999). A simple and universal method for molecular sexing of non-ratite birds. Journal of Avian Biology *30*, 116-121.
50. Luepke, N.P. (1985). Hen's egg chorioallantoic membrane test for irritation potential. Food and Chemical Toxicology *23*, 287-291.

51. Wolf, T., Niehaus-Rolf, C., Banduhn, N., Eschrich, D., Scheel, J., and Luepke, N.-P. (2008). The hen's egg test for micronucleus induction (HET-MN): Novel analyses with a series of well-characterized substances support the further evaluation of the test system. Mutation Research/Genetic Toxicology and Environmental Mutagenesis *650*, 150-164.
52. OECD (1984). Test No. 206: Avian Reproduction Test. OECD Guidelines for the Testing of Chemicals, Section 2. (OECD Publishing, Paris, France: Organisation for Economic Co-Operation and Development).
53. OECD (2007). Test No. 440: Uterotrophic Bioassay in Rodents: A short-term screening test for oestrogenic properties, OECD Guidelines for the Testing of Chemicals, Section 4. (OECD Publishing, Paris, France: Organisation for Economic Co-Operation and Development.).
54. OECD (2009). Test No. 441: Hershberger Bioassay in Rats. OECD Guidelines for the Testing of Chemicals, Section 4. (OECD Publishing, Paris, France: Organisation for Economic Co-Operation and Development).
55. OECD (2007). Detailed review paper for avian two-generation toxicity test. OECD series on testing and assessment No. 74. ENV/JM/MONO(2007)21. (Paris, France: Organisation for Economic Co-Operation and Development).
56. Touart, L.W. (2005). Final detailed review paper for avian two-generation toxicity test. (The U.S. environmental protection agency).
57. Matsumura, H., Ito, A., Saitoh, H., Winter, P., Kahl, G., Reuter, M., Kruger, D.H., and Terauchi, R. (2005). SuperSAGE. Cellular Microbiology *7*, 11-18.
58. Matsumura H., Reuter M., Krüger D.H., Winter P., Kahl G., Terauchi R. (2008) SuperSAGE. In: Nielsen K.L. (eds) Serial Analysis of Gene Expression (SAGE). Methods in Molecular Biology™, vol 387, 55-70 Humana Press
59. Iwaniuk, A.N., Koperski, D.T., Cheng, K.M., Elliott, J.E., Smith, L.K., Wilson, L.K., and Wylie, D.R.W. (2006). The effects of environmental exposure to DDT on the brain of a songbird: Changes in structures associated with mating and song. Behavioural Brain Research *173*, 1-10.
60. Markman, S., Leitner, S., Catchpole, C., Barnsley, S., Müller, C.T., Pascoe, D., and Buchanan, K.L. (2008). Pollutants increase song complexity and the volume of the brain area HVC in a songbird. PLOS ONE *3*, e1674.
61. Berg, C., Halldin, K., Brunstrom, B., and Brandt, I. (1998). Methods for studying xenoestrogenic effects in birds. Toxicology Letters *103*, 671-676.
62. Berg, C., Halldin, K., Fridolfsson, A.K., Brandt, I., and Brunstrom, B. (1999). The avian egg as a test system for endocrine disrupters: Effects of diethylstilbestrol and ethynylestradiol on sex organ development. Science of the Total Environment *233*, 57-66.
63. Berg, C., Holm, L., Brandt, I., and Brunstrom, B. (2001). Anatomical and histological changes in the oviducts of Japanese quail, *Coturnix japonica*, after embryonic exposure to ethynyloestradiol. Reproduction *121*, 155-165.
64. Elbrecht, A., and Smith, R. (1992). Aromatase enzyme activity and sex determination in chickens. Science *255*, 467-470.
65. Sakimura, M., Tsukada, A., Usami, M., Hanzawa, S., Saito, N., Ohno, Y., and Shimada, K. (2002). Effect of estradiol and nonylphenol on mRNA expression of estrogen receptors α and β, and cytochrome P450 aromatase in the gonad of chicken embryos. Journal of Poultry Science *39*, 302-309.
66. Blomqvist, A., Berg, C., Holm, L., Brandt, I., Ridderstrale, Y., and Brunstrom, B. (2006). Defective reproductive organ morphology and function in domestic rooster embryonically exposed to o,p'-DDT or ethynylestradiol. Biology of Reproduction *74*, 481-486.
67. Adkins, E.K. (1979). Effect of embryonic treatment with estradiol or testosterone on sexual differentiation of the quail brain - critical period and dose-response relationships. Neuroendocrinology *29*, 178-185.

68. Ichas, F., Faucounau, N., Stoll, R., and Maraud, R. (1994). Action of testosterone on the anti-mullerian activity of the chick embryonic testis assayed in vivo by organotypic grafting. Anatomy and Embryology *190*, 297-299.
69. Koba, N., Mori, M., Ha, Y., Mizushima, S., Tsukada, A., Saito, N., Ono, T., and Shimada, K. (2008). Effects of aromatase inhibitor (fadrozole)-induced sex-reversal on gonadal differentiation and mRNA expression of p450arom, AMH and ER α in embryos and growth in posthatching quail. Journal of Poultry Science *45*, 116-124.
70. Smith, C.A., Katz, M., and Sinclair, A.H. (2003). DMRT1 is upregulated in the gonads during female-to-male sex reversal in ZW chicken embryos. Biology of Reproduction *68*, 560-570.
71. Vaillant, S., Dorizzi, M., Pieau, C., and Richard-Mercier, N. (2001). Sex reversal and aromatase in chicken. Journal of Experimental Zoology *290*, 727-740.
72. Zheng, J.-X., and Yang, N. (2007). mRNA expression of genes related to avian sex determination during female-to-male sex reversal in ZW chicken embryos. Yi Chuan *29*, 81-86.
73. Mank, J.E., Hultin-Rosenberg, L., Axelsson, E., and Ellegren, H. (2007). Rapid evolution of female-biased, but not male-biased, genes expressed in the avian brain. Molecular Biology and Evolution *24*, 2698-2706.
74. Andrews, J.E., Smith, C.A., and Sinclair, A.H. (1997). Sites of estrogen receptor and aromatase expression in the chicken embryo. General and Comparative Endocrinology *108*, 182-190.
75. Katoh, H., Ogino, Y., and Yamada, G. (2006). Cloning and expression analysis of androgen receptor gene in chicken embryogenesis. FEBS Letters *580*, 1607-1615.
76. Furuya, M., Sasaki, F., Hassanin, A.M.A., Kuwahara, S., and Tsukamoto, Y. (2003). Effects of bisphenol A on the growth of comb and testes of male chicken. Canadian Journal of Veterinary Research *67*, 68-71.
77. Furuya, M., Adachi, K., Kuwahara, S., Ogawa, K., and Tsukamoto, Y. (2006). Inhibition of male chick phenotypes and spermatogenesis by bisphenol A. Life Sciences *78*, 1767-1776.
78. Nakabayashi, O., Kikuchi, H., Kikuchi, T., and Mizuno, S. (1998). Differential expression of genes for aromatase and estrogen receptor during the gonadal development in chicken embryos. Journal of Molecular Endocrinology *20*, 193-202.
79. Kamata, R., Takahashi, S., and Morita, M. (2004). Gene expression of sex-determining factors and steroidogenic enzymes in the chicken embryo: Influence of xenoestrogens. General and Comparative Endocrinology *138*, 148-156.
80. Berg, C., and Pettersson, I. (2006). Ethynyl estradiol causes female-biased sex ratio in the frog *Xenopus tropicalis*. Marine Environmental Research *62*, S270-S270.
81. Halldin, K., Berg, C., Brandt, I., and Brunstrom, B. (1999). Sexual behavior in Japanese quail as a test end point for endocrine disruption: Effects of in ovo exposure to ethinylestradiol and diethylstilbestrol. Environmental Health Perspectives *107*, 861-866.
82. Chamorro-Garcia, R., Sahu, M., Abbey, R.J., Laude, J., Pham, N., and Blumberg, B. (2013). Transgenerational inheritance of increased fat depot size, stem cell reprogramming, and hepatic steatosis elicited by prenatal exposure to the obesogen tributyltin in mice. Environmental Health Perspectives *121*, 359-366.
83. Hallare, A.V., Köhler, H.R., and Triebskorn, R. (2004). Developmental toxicity and stress protein responses in zebrafish embryos after exposure to diclofenac and its solvent, DMSO. Chemosphere *56*, 659-666.
84. Lazzari, G., Tessaro, I., Crotti, G., Galli, C., Hoffmann, S., Bremer, S., and Pellizzer, C. (2008). Development of an in vitro test battery for assessing chemical effects on bovine germ cells under the ReProTect umbrella. Toxicology and Applied Pharmacology *233*, 360-370.
85. Liu, S., Qin, F., Wang, H., Wu, T., Zhang, Y., Zheng, Y., Li, M., and Wang, Z. (2012). Effects of 17α-ethinylestradiol and bisphenol A on steroidogenic messenger ribonucleic acid levels in the rare minnow gonads. Aquatic Toxicology *122–123*, 19-27.

86. Mortensen, A.S., and Arukwe, A. (2007). Effects of 17α-ethynylestradiol on hormonal responses and xenobiotic biotransformation system of Atlantic salmon (*Salmo salar*). Aquatic Toxicology *85*, 113-123.
87. Eising, C.M., Eikenaar, C., Schwabl, H., and Groothuis, T.G.G. (2001). Maternal androgens in black-headed gull (*Larus ridibundus*) eggs: consequences for chick development. Proceedings of the Royal Society of London B: Biological Sciences *268*, 839-846.
88. Muller, W., Groothuis, T.G.G., Eising, C.M., and Dijkstra, C. (2005). An experimental study on the causes of sex-biased mortality in the black-headed gull - the possible role of testosterone. Journal of Animal Ecology *74*, 735-741.
89. von Engelhardt, N., Carere, C., Dijkstra, C., and Groothuis, T.G.G. (2006). Sex-specific effects of yolk testosterone on survival, begging and growth of zebra finches. Proceedings of the Royal Society B :Biological Sciences *273*, 65-70.
90. Hallare, A., Nagel, K., Köhler, H.-R., and Triebskorn, R. (2006). Comparative embryotoxicity and proteotoxicity of three carrier solvents to zebrafish (*Danio rerio*) embryos. Ecotoxicology and Environmental Safety *63*, 378-388.
91. Silveira, P.C.L., Victor, E.G., Schefer, D., Silva, L.A., Streck, E.L., Paula, M.M., and Pinho, R.A. (2010). Effects of therapeutic pulsed ultrasound and dimethylsulfoxide (DMSO) phonophoresis on parameters of oxidative stress in traumatized muscle. Ultrasound in Medicine and Biology *36*, 44-50.
92. Metcalfe, C.D., Metcalfe, T.L., Kiparissis, Y., Koenig, B.G., Khan, C., Hughes, R.J., Croley, T.R., March, R.E., and Potter, T. (2001). Estrogenic potency of chemicals detected in sewage treatment plant effluents as determined by in vivo assays with Japanese medaka (*Oryzias latipes*). Environmental Toxicology and Chemistry *20*, 297-308.
93. Segner, H., Navas, J.M., Schäfers, C., and Wenzel, A. (2003). Potencies of estrogenic compounds in in vitro screening assays and in life cycle tests with zebrafish in vivo. Ecotoxicology and Environmental Safety *54*, 315-322.
94. Scheib, D., and Baulieu, E.E. (1981). Inhibiting effects of tamoxifen on the female differentiation of the gonads of quail embryos. Comptes Rendus de l'Academie des sciences Serie III-Sciences de la Vie-Life Sciences *293*, 513-518.
95. Scheib, D., Guichard, A., Mignot, T.M., and Cedard, L. (1981). Steroidogenesis by gonads of normal and of diethylstilbestrol-treated quail embryos - radioimmunoassays on organ-cultures General and Comparative Endocrinology *43*, 519-526.
96. Shibuya, K., Mizutani, M., Wada, M., Sato, K., and Nunoya, T. (2004). A new screening model using F1(AWE × WE) japanese quail embryo for evaluating sex reversal effects. Journal of Toxicologic Pathology *17*, 245-252.
97. Xie, M., and Zhang, C. (2004). Estrogenic and toxic effects of polychlorinated biphenyls on cultured ovarian germ cells of embryonic chickens. Reproductive Toxicology *19*, 79-86.
98. Abinawanto, Zhang, C., Saito, N., Matsuda, Y., and Shimada, K. (1998). Identification of sperm-bearing female-specific chromosome in the sex-reversed chicken. Journal of Experimental Zoology *280*, 65-72.
99. Hudson, Q.J., Smith, C.A., and Sinclair, A.H. (2005). Aromatase inhibition reduces expression of FOXL2 in the embryonic chicken ovary. Developmental Dynamics *233*, 1052-1055.
100. Nishikimi, H., Kansaku, N., Saito, N., Usami, M., Ohno, Y., and Shimada, K. (2000). Sex differentiation and mRNA expression of P450c17, P450arom and AMH in gonads of the chicken. Molecular Reproduction and Development *55*, 20-30.
101. Shaikat, A.H., Namekawa, S., Ahmadi, S., Takeda, M., and Ohkubo, T. (2018). Gene expression profiling in embryonic chicken ovary during asymmetric development. Animal Science Journal *89*, 688-694.
102. Wang, Y., Jin, G., Ma, M., and Xiang, X. (2019). Sex differences in serum steroid hormone levels during embryonic development in hen eggs. Poultry Science *0*, 1-10.

103. Jessl, L., Lenz, R., Massing, F.G., Scheider, J., and Oehlmann, J. (2018). Effects of estrogens and antiestrogens on gonadal sex differentiation and embryonic development in the domestic fowl (*Gallus gallus domesticus*). PeerJ *6*, e5094.
104. Aleksandrowicz, E., and Herr, I. (2015). Ethical euthanasia and short-term anesthesia of the chick embryo. ALTEX - Alternatives to Animal Experimentation *32*, 143-147.
105. Verbraucherschutz, B.f.E.L.u. (1972). Tierschutzgesetz (TierschG). Geltung seit: 24.07.1972. Zuletzt geändert durch Art. 141 G v. 29.3.2017 I 626.
106. Hirt, A., Maisack, C., and Moritz, J. (2007). Tierschutzgesetz - Kommentar, (Verlag Franz Vahlen München).
107. Kluge, H.-G. (2002). Tierschutzgesetz - Kommentar, (Verlag W. Kohlhammer).
108. Lorz, A., and Metzger, E. (2008). Tierschutzgesetz - Kommentar, Volume 6, (Verlag C.H.Beck , München).
109. Bjørnstad, S., Austdal, L.P.E., Roald, B., Glover, J.C., and Paulsen, R.E. (2015). Cracking the egg: Potential of the developing chicken as a model system for nonclinical safety studies of pharmaceuticals. Journal of Pharmacology and Experimental Therapeutics *355*, 386-396.
110. Krautwald-Junghanns, M.-E., Cramer, K., Fischer, B., Förster, A., Galli, R., Kremer, F., Mapesa, E.U., Meissner, S., Preisinger, R., Preusse, G., et al. (2017). Current approaches to avoid the culling of day-old male chicks in the layer industry, with special reference to spectroscopic methods. Poultry Science *97*, 749-757.
111. Bentham, J. (1781). An introduction to the principles of Morals and Legislation, Volume Persistent link: https://EconPapers.repec.org/RePEc:hay:hetboo:bentham1781 (McMaster University Archive for the History of Economic Thought, number bentham1781).
112. Singer, P. (2013). Praktische Ethik, Volume 3: revid. und erw. (15. März 2013), (Reclam Verlag, Stuttgart).
113. Huisinga, M., Flick, B., Kreling, N., Weltje, L., Ott, K., Jessl, L., Scheider, J., Gröters , S., and van Ravenzwaay, B. (2017). Histopathological evaluation of gonads from chicks exposed in ovo as an alternative method for endocrine endpoints in birds. In Congress of the ESVP, ESTP and ECVP. (Lyon, France).
114. Huisinga, M., Flick, B., Kreling, N., Weltje, L., Ott, K., Jessl, L., Scheider, J., Gröters , S., and van Ravenzwaay, B. (2019). A fast screening tool for the early indicator phase of sexual development in birds: histopathological evaluation of avian gonads from chicks exposed in ovo. In Society of Toxicologic Pathology. (Raleigh, USA).
115. Jessl, L., Oehlmann, J. (2019). No effects of the antiandrogens cyproterone acetate (CPA), flutamide and p,p´-DDE on early sexual differentiation but CPA-induced retardation of embryonic development in the domestic fowl (*Gallus gallus domesticus*). Manuscript, ready to be submitted.

Annex

A.1 Publikationen als Teil der vorliegenden Arbeit

A.1.1 Gene expression of chicken gonads is side-and sex-specific

Jessica Scheider, Fabian Afonso-Grunz, Klaus Hoffmeier, Ralf Horres, Florian Groher, Lukas Rycak, Jörg Oehlmann, Peter Winter

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Was hat die Promovendin bzw. was haben die Koautoren beigetragen?

(1) zu Entwicklung und Planung

Promovendin JS:	30%
Co-Autor JO:	30%
Co-Autor PW:	20%
Co-Autor RH:	15%
Co-Autor FAG:	5%

(2) zur Durchführung der einzelnen Untersuchungen und Experimente

Promovendin JS:	60% (Injektion, Bebrütung, Gonadenisolation)
Co-Autor FAG:	20% (Präparation für den SuperSAGE)
Co-Autor KH:	10% (Präparation für den SuperSAGE)
Co-Autor FG:	10% (Präparation für den SuperSAGE)

(3) zur Erstellung der Datensammlung und Abbildungen

Promovendin JS:	45% (Aufarbeitung der Rohdaten, Abbildungserstellung)
Co-Autor FAG:	40% (Aufarbeitung der Rohdaten)
Co-Autor LR:	15% (Bereitstellung der Rohdaten aus Sequenzierungen)

(4) zur Analyse und Interpretation der Daten

Promovendin JS:	60% (Interpretation und Analyse, Publikationsrecherche)
Co-Autor FAG:	40% (Datenaufarbeitung und -Analyse)

(5) zum Verfassen des Manuskripts

Promovendin JS:	50%
Co-Autor FAG:	40%
Co-Autor JO:	5%
Co-Autor PW:	5%

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Gene Expression of Chicken Gonads Is Sex- and Side-Specific

Jessica Scheider[a] Fabian Afonso-Grunz[b, c] Klaus Hoffmeier[c] Ralf Horres[b, c]
Florian Groher[c] Lukas Rycak[c] Jörg Oehlmann[a] Peter Winter[c]

Institutes for [a]Ecology, Evolution and Diversity and [b]Molecular BioSciences, Goethe University Frankfurt am Main, and [c]GenXPro GmbH, Frankfurt/M., Germany

Key Words
Change of function · Chicken embryo · deepSuperSAGE · qRT-PCR · Sexual development · Stereology · Transcriptome

Abstract
In chicken, the left and right female gonads undergo a completely different program during development. To learn more about the molecular factors underlying side-specific development and to identify potential sex- and side-specific genes in developing gonads, we separately performed next-generation sequencing-based deepSuperSAGE transcription profiling from left and right, female and male gonads of 19-day-old chicken embryos. A total of 836 transcript variants were significantly differentially expressed ($p < 10^{-5}$) between combined male and female gonads. Left-right comparison revealed 1,056 and 822 differentially ($p < 10^{-5}$) expressed transcript variants for male and female gonads, respectively, of which 72 are side-specific in both sexes. At least some of these may represent key players for lateral development in birds. Additionally, several genes with laterally differential expression in the ovaries seem to determine female gonads for growth or regression, whereas right-left differences in testes are mostly limited to the differentially expressed genes present in both sexes. With a few exceptions, side-specific genes are not located on the sex chromosomes. The large differences in lateral gene expression in the ovaries in almost all metabolic pathways suggest that the regressing right gonad might have undergone a change of function during evolution.

Many mechanisms of avian sex determination are still unknown. Although sex in birds and mammals is genetically determined, the mammalian and avian sex chromosomes are not homologous [Fridolfsson et al., 1998]. In contrast to mammals, female birds are heterogametic (ZW), while males are homogametic (ZZ). It is not clear whether it is the presence of the W chromosome in females, the double representation of the Z chromosome in males vis-à-vis females, or both of these characteristics that are crucial for the determination of sex in birds [Ellegren, 2000].

In every gonochoristic species, genetic pathways are differentially activated in males and females in order to initiate testis or ovary development [Merchant-Larios and Moreno-Mendoza, 2001; Ayers et al., 2013]. These sex-biased genes are often linked to reproduction, rendering many of them subject not only to natural selection

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Jessica Scheider
Goethe University Frankfurt am Main
Institute for Ecology, Evolution and Diversity
Max-von-Laue-Strasse 13, DE–60438 Frankfurt/M. (Germany)
E-Mail j.scheider@bio.uni-frankfurt.de

but as well to the pressures of sexual [Civetta and Singh, 1999; Swanson and Vacquier, 2002] and anthropogenic selection (e.g. hormone-mimicking chemicals in food and environment). Sex-biased gene expression is relatively common in metazoans [Ranz et al., 2003; Marinotti et al., 2006; Yang et al., 2006; Eads et al., 2007] and has important evolutionary [Meiklejohn et al., 2003; Zhang et al., 2004; Ellegren and Parsch, 2007; Mank et al., 2007], medical [Ivakine et al., 2005; Lu et al., 2007; Xu et al., 2008], and genomic implications [Connallon and Knowles, 2005, 2007; Hambuch and Parsch, 2005]. Strong right-left differences in females, however, are restricted to birds – except for birds of prey [Jacob and Bakst, 2007] – and characterized by a regressing right ovary with a medulla only, while the left one develops ongoing from day 7 of incubation to a functional ovary with cortex and medulla [Ukeshima and Fujimoto, 1991; Ukeshima, 1996; Smith, 2007; González-Morán, 2011]. Previous studies on avian gonad development mainly focused on early embryonic events based on histological descriptions [González-Morán, 2011] or on mRNA expression profiles of a distinct subset of genes [Hoshino et al., 2005; Hudson et al., 2005a, b; Rodríguez-Léon et al., 2008; Carré et al., 2011] as e.g. those involved in steroidogenesis, paracrine signaling, transcription, and homeostasis [Yamamoto et al., 2003; Diaz et al., 2011]. PITX2 and RALDH2 were shown to be important for ovarian dimorphism in the chicken embryo [Rodríguez-Léon et al., 2008; Smith et al., 2008a]. However, the molecular consequences of this differential expression are far from being completely understood. At present, the publication of the chicken genome [Wicker et al., 2005] along with the advent of next-generation sequencing (NGS)-based genome-wide gene expression profiling techniques provides the opportunity to monitor the differential gene expression of developing chicken gonads and to infer common and diverted mechanisms of gonadal differentiation from these data.

DeepSuperSAGE, a genome-wide, open-architecture transcriptome profiling technique, is the adaptation of SuperSAGE [Matsumura et al., 2003] to NGS [Matsumura et al., 2010, 2011; Zawada et al., 2011]. For the generation of comprehensive transcription profiles, so-called SuperTAGs, originating from the 3′ end of a given cDNA, are sequenced in their millions and annotated to their respective genes in an according database [Matsumura et al., 2003, 2005]. These SuperTAGs contain sufficient information to reliably characterize the cDNA and thus the transcript they are derived from [Velculescu et al., 1995; Saha et al., 2002]. Contrary to microarrays, deepSuperSAGE due to its open-architecture is able to quantify the expression of virtually all polyadenylated RNAs, including previously unknown and lowly expressed ones as well as natural antisense transcripts and polyadenylated noncoding RNAs without prior knowledge of the respective sequences.

There is only rudimentary research regarding the female right gonad, since most of the research was done focusing on the left gonads of both sexes. The present study provides a comprehensive description not only of the sex but especially of the side-specific differential sexual development of male and female chicken gonads on the level of gene expression at embryonic day 19 (E19). We wanted to see if there is any difference in male right-left gene expression in E19 and if there is any parallelism to the female gonads. Our genome-wide characterization of differences in the gene expression of mature gonads in an unprecedented depth represents an important step to a molecular characterization of side-specific gonadal development in birds and has implications beyond this taxon for other vertebrate classes, including mammals.

Materials and Methods

Gonad Tissue Isolation

Newly laid fertile chicken eggs (White Leghorn, deriving from the same genetic background) were obtained from a commercial local supplier (LSL Rhein-Main, Schaafheim, Germany) and bred in a ThermoStar 100 egg incubator (J. Hemel Brutgeräte, Verl, Germany). Incubation was performed at 37.6°C and 60% humidity and egg turning once every 2 h. On E19, 2 days before anticipated hatching, embryos were decapitated immediately after removal from the egg, dissected under a microscope, and sexed by gonad morphology.

Genetic Sexing

For genetic sex confirmation, blood samples from each embryo were collected in ethanol and stored at –20°C till isolation of the DNA with the DNeasy (Qiagen, Hilden, Germany) isolation kit. Genetic sexing was carried out by a standard PCR protocol using the primers 2550F (5′-GTT ACT GAT TCG TCT ACG AGA-3′) and 2718R (5′-ATT GAA ATG ATC CAG TGC TTG-3′) previously described by Fridolfsson and Ellegren [1999]. These primers are focused on the *CHD1* introns, placed on the Z (*CHD1Z*, 600 bp) and W chromosome (*CHD1W*, 450 bp). Thermal cycling comprised DNA polymerase activation at 95°C for 1 min followed by 30 cycles of denaturation at 95°C for 30 s, annealing at 52°C for 30 s, elongation at 72°C for 1 min, and a final extension step at 72°C for 3 min. All amplifications were performed on an advanced primus 96 thermocycler (Peqlab, Erlangen, Germany). The amplicons of this modified protocol were separated into 1 band (Z) in the case of male or 2 bands (Z + W) in the case of female animals on a 1.4% agarose gel.

Total RNA Isolation

Gonads were separated, cleaned from adhesive tissue, and immediately frozen individually in 200 µl RNA lysis buffer (Promega, Mannheim, Germany) for later RNA isolation. Total RNA was extracted using the SV Total RNA Isolation System Kit according to manual 048 (Promega). Deviating from the protocol, on-column DNaseI digestion of genomic DNA was elongated from original 15 min to 30 min. Additionally, DNaseI digestion was carried out with Baseline-Zero™ DNase (Epicentre; provided by Biozym Scientific GmbH, Hessisch Oldendorf, Germany) in solution to ensure that the samples were completely free of DNA. Total RNA concentration was estimated in a dilution series with the LabelGuard NanoPhotometer (Implen, München, Germany). RNA quality and quantity was further determined using a Caliper lab-on-a-chip system (Agilent, Hopkinton, Mass., USA). All isolated total RNA samples had an RNA Integrity Number (RIN) ranging from 8.5–10.0 (highest quality).

Library Preparation and Bioinformatics

DeepSuperSAGE libraries were constructed from total RNA of gonads from 10 pooled individuals by GenXPro GmbH essentially as described previously [Matsumura et al., 2010, 2011; Zawada et al., 2012] with slight modifications. Sequencing was performed on Illumina's Genome Analyzer IIx, and subsequent base calling was carried out by Illumina's GAPipeline v1.0. Distinct libraries were sorted out from the bulked sequencing data according to their respective indices, followed by elimination of PCR-derived tags identified by TrueQuant technology (GenXPro). The 26-bp SuperTAGs were extracted from the remaining sequences, counted, and subsequently combined to TAG groups of common origin (UniTAGs) according to Akmaev and Wang [2004]. For each library, the UniTAG read numbers were normalized to a million sequenced reads in total (tags per million; TPM) to establish comparability. The UniTAG numbers of the pooled datasets from left and right gonads were generated by in silico calculation of the arithmetic mean of the normalized read counts from the left and right gonad in the respective libraries. Logarithmic fold changes (base 2) were determined by pair-wise comparison of the normalized UniTAG numbers in the particular libraries, and statistical significance was assessed by χ^2 tests according to Man et al. [2000]. If a given UniTAG was only present in 1 of the 2 libraries, the respective TPM count was adjusted from 0 to 0.05 to allow for calculation of fold changes.

Annotation of SuperTAGs

The UniTAG reads were annotated in a multi-step procedure using the BLAST software [Altschul et al., 1997] to ensure an unambiguous assignment to their corresponding transcripts and to eliminate any remaining adaptor sequences. Reference datasets were extracted from the publicly accessible databases at the Harvard University (HU; ftp://occams.dfci.harvard.edu/pub/bio/tgi/data/Gallus_gallus) and the National Center for Biotechnology Information (NCBI; ftp://ftp.ncbi.nih.gov/refseq/release/vertebrate_other). A third reference comprising only sequences from *Gallus gallus* was generated based on the above-mentioned NCBI dataset, and finally, all 3 databases were trimmed to generate subsets (26-bp references) that only comprise sequences from the last possible anchoring site for a given SuperTAG. Beginning with a minimum required BLAST score of 52, UniTAG reads were successively aligned against these reference datasets in the following order of precedence: (1) 26-bp HU dataset, (2) 26-bp NCBI subset of *G. gallus*, (3) full-length HU, and (4) full-length NCBI subset. Reads which did not attain the specified BLAST score were re-annotated in the same fashion with a lowered required score of 48, and in a third round of 44. Still not annotated reads were finally also aligned against the complete NCBI dataset with a required BLAST score of 42 or above. In this final annotation step, the complete 26-bp NCBI dataset was used as the third reference, followed by the full-length HU and *G. gallus* NCBI reference and the complete full-length NCBI dataset as final reference. UniTAG reads, which could not be annotated with this multi-step procedure, were excluded from further analysis.

Validation of deepSuperSAGE Results with Selected Transcripts

For comparison of the tendencies in gene expression of both datasets (deepSuperSAGE vs. qRT-PCR), $\log_2$ fold changes were calculated with the TPM values of the respective UniTAGs and compared to the qPCR-based fold change (fig. 1). The quantitative real-time PCR assays (TaqMan/dual labeled probe qPCR assays) were performed on transcripts previously selected according to their differential expression profile in the sequencing data. Total RNA isolations of additional tissues were performed as described above. The primer assays for this study were designed and provided by GenXPro GmbH. Quantitative real-time PCR reactions were run as a one-step qPCR (Applied BioSystems StepOne RT-PCR systems) with dual labeled probes (FAM-BHQ) and ROX reference dye (Invitrogen, Darmstadt, Germany) as passive reference. An amount of 5 ng of total RNA served as template for each reaction of 15 µl. The applied One-Step RT-PCR Mastermix (Clontech-Takara QTaq-Mastermix) contained hot start Taq DNA polymerase, an optimized reaction buffer, 5 mM $MgCl_2$ (final concentration), 2.5–3.5 mM nucleotides (including 200 µM dUTP), and a reverse transcriptase combined with an RNase inhibitor. Specific primers were applied with a final concentration of 0.2–1.0 µM, and the dual labeled probes of 0.016–0.08 µM. The PCR regime consisted of the following steps: (1) reverse transcription: 48°C for 20 min; (2) activation of the hot start Taq DNA polymerase (inhibited by antibody during reverse transcription) at 95°C for 10 min, and (3) 45 cycles with denaturing at 95°C for 15 s, and an annealing/ extension step at 60°C for 1 min.

Amplification of the target genes was monitored by qPCR probe-released fluorescence (FAM dye) at each cycle. The threshold cycle (C_t), defined as the PCR cycle at which a statistically significant increase of reporter fluorescence is first detected (10× above background), was used as a measure for the starting copy numbers of the respective gene. Relative quantification of the amplified targets was performed via the comparative $\Delta\Delta C_t$ method [Pfaffl, 2001]. The amount of target, normalized to an endogenous reference (AURKAIP1) and relative to the passive reference, is given by $2^{-\Delta\Delta C_t}$.

Assignment of Transcription Profiles to Gene Ontology Categories

Though deepSuperSAGE and other modern transcriptome analysis technologies nowadays provide in-depth descriptions of gene expression, the assignment of a function to differentially expressed genes is not trivial in organisms that are not as comprehensively characterized as human and mice or rats. When there is no direct experimental evidence available, the most efficient way

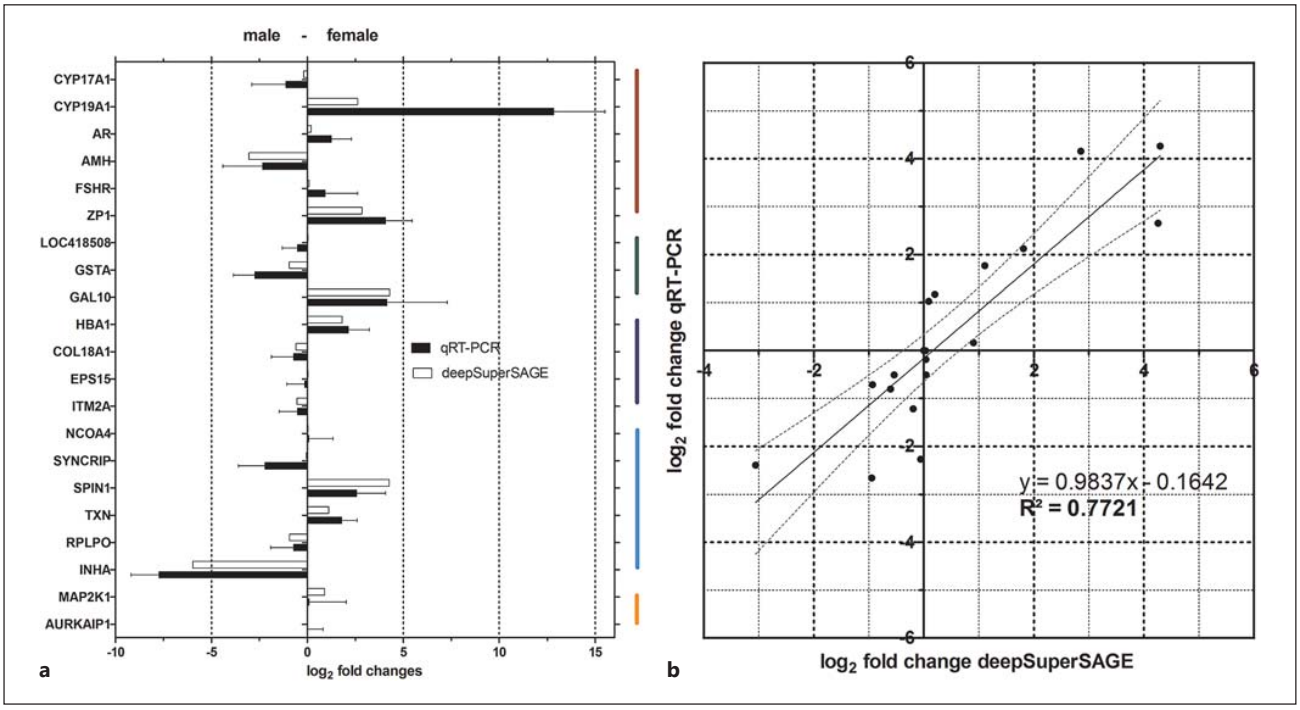


Fig. 1. Fold changes qRT-PCR/SuperSAGE. **a** Illustration of the $\log_2$ fold changes (male vs. female) from transcripts quantified by qRT-PCR and deepSuperSAGE. The qRT-PCR fold changes are derived from 5 male and 6 female individual gonads (right and left) with 3 replicates each. DeepSuperSAGE values were acquired with 10 pooled male and female gonads (right and left). Nomenclature was taken from NCBI. Color code identifies metabolic pathways; red = sexual differentiation, green = defense/immune reactivity, dark blue = blood, light blue = transcription factors/cofactors/ribosome/nuclei, orange = kinases. **b** Correlation between the $\log_2$ fold changes (male vs. female) derived from qRT-PCR and deepSuperSAGE (transformed with the equation $x^{(1/x)}$) calculated by linear regression ($R^2 = 0.77$). Individuals are the same as in **a**.

of function assigning is based on strict orthology, one of the central concepts of comparative genome analysis. By definition, orthologs are genes or proteins in 2 or more species that share significant similarity and that are thought to have diverged from a common ancestral gene that existed in their last common ancestor [Remm et al., 2001; Li et al., 2003; O'Brien et al., 2004; Chen et al., 2006; Hulsen et al., 2006]. Determination of orthology relations facilitates the knowledge transfer between species and can be used to improve both structural and functional annotation in organisms that are less well annotated. At present, there are several ortholog prediction methods and search tools available [O'Brien et al., 2005; Wright et al., 2005; Li et al., 2006; Hubbard et al., 2007]. However, the number of proteins from one species that is considered to be part of the same orthologous group in another species varies from one method to another due to differently employed algorithms and the diversity of species included in these methods [Hulsen et al., 2006]. Since most gene ontology (GO) annotations for newly sequenced species provided by the EBIGOA Project [Camon et al., 2004] are based on Electric Annotation (IEA) that is not assigned by a curator, these annotations do not necessarily include the gene products determined by deepSuperSAGE. Here, we used IEA-based GO (http://www.geneontology.org/) to categorize differentially expressed genes and to assign a potential function to them. The final data set containing information from all the libraries was subjected to GO enrichment analysis using the GenXPro in-house pipeline system.

Data Accessibility

The original SuperTAG sequences from this project will be available at Genome Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo).

Results

Annotation of SuperTAGs and qRT-PCR Validation of Determined Expression Profiles

For the description of gene expression profiles in the gonads, we created 4 deepSuperSAGE datasets with about 2 million 26-bp SuperTAGs each, based on the sequences from distinct pools of 10 individual right and left ovaries

and testes, respectively. Table 1 summarizes all tags and transcripts in the 4 libraries used for analysis.

From all annotated SuperTAGs, a total of 67,848 tags revealed high homology hits (BLAST score = 52) in 1 or more of the respective databases; 3,339 annotated tags were considered to be predicted by the respective databases and therefore excluded from further analysis.

The reliability and reproducibility of deepSuperSAGE data was tested via qRT-PCR analysis of a subset of 21 transcripts with similar and dissimilar expression between male and female gonads. In most cases, SuperTAG counts from the pooled gonads represented the mean of the $\Delta\Delta C_t$ values obtained for a given RNA from individual specimens. The overall similarity and the high correlation ($R^2 = 0.77$) between the tested individual qRT-PCR and pooled deepSuperSAGE expression ratios (fig. 1) demonstrates the exact quantification of transcript levels by deepSuperSAGE profiling.

Sex-Specific and Right-Left Expression Profile

The cumulated and normalized UniTAGs could be classified into 61,312 *G. gallus*-specific UniTAGs representing 48,079 UniTAGs with low abundance (0.13–10 tags per million), 6,075 UniTAGs of intermediate abundance (10–100 tags per million), and 1,216 highly abundant UniTAGs (>100 tags per million). These abundance categories contain on average 78, 10, and 2% of the total number of UniTAGs, respectively (upon request, data can be provided by the author). A total of 836 UniTAGs was found to be differentially expressed (M-F, $p < 10^{-5}$) between male and female gonads, and a considerable number of UniTAGs were exclusively detected in either the male or female gonads; 114 significantly differentially expressed UniTAGs ($p < 10^{-5}$) between the sexes showed more than a 2-fold difference in gene expression. Of these, 40 were up- and 74 down-regulated in testes compared to ovaries. The UniTags found laterally differently expressed are shown in table 2. The 20 most significantly regulated mRNAs between male and female gonads are summarized in table 3 which is an extract of table 2.

The list of transcripts up-regulated in male gonads mainly consists of ribosomal proteins and subunits, inhibin, CDH1-D, DMRT1, and SPARC as the most up-regulated mRNA in testes as compared to ovaries. *SPARC* (secreted protein acidic and rich in cysteine, originally described as osteonectin) is a highly conserved and abundant component of the extracellular matrix in bone tissues in vertebrate species [Termine et al., 1981; Bassuk et al., 1993]. *SPARC* binds to collagen type I–V in a calcium-dependent manner and is prominent in regions of tissue morphogenesis which correlates with its proposed function as a mediator of tissue remodeling [Sage et al., 1989; Wu et al., 1996; Delostrinos et al., 2006]. *CDH1-D* and its homologs are suggested to play a role in temporal and spatial regulation of the anaphase-promoting complex in and outside of the cell cycle in chicken embryos of stage E15–20 [Wan and Kirschner, 2001].

The female up-regulated transcripts comprise phosphatidylcholine 2-acylhydrolase precursor (PLA2G2E precursor), WPKCI, tetraspanin-8 (TSPAN8), gallinacin (GAL-9, GAL-10), and gastrotropin.

Further differentially expressed transcripts between sexes include galectin-2 (LGALS2), galectin-3TM1 (LGALS3-TM1), thrombomucin and its precursor, aromatase, GATA binding factor 2 and 5 (GATA2, GATA5), hRSPO1, tetraspanin-1 (TSPAN1), prolactin receptor precursor, truncated testis-specific box 1-B or box 1-less

Table 1. SuperTAG and *Gallus gallus*-specific UniTAG numbers of the sequenced libraries

	MR	ML	FR	FL
SuperTAGs	1,236,514	998,025	1,527,482	1,181,158
UniTAGs	43,339	42,253	49,691	42,852

MR = Male right; ML = male left; FR = female right; FL = female left gonads.

Table 2. Differentially expressed UniTAGs

	MR	ML	FR	FL
MR	**2,299**			
ML	↑139 ↓136	**1,991**		
FR	↑538 ↓462	↑328 ↓264	**4,786**	
FL	↑444 ↓372	↑240 ↓166	↑206 ↓163	**1,999**

Table of differentially expressed transcripts (FCh stronger than |1| and $p < 10^{-10}$). The numbers in bold represent UniTAGs exclusively expressed in the respective gonad. Pairwise comparison of the libraries takes the library on the ordinate to the base. Up-regulated UniTAGs are indicated by upward and down-regulated UniTAGs by downward arrows. Abbreviations are the same as in table 1.

Table 3. The 20 most differentially expressed transcripts

Description	Fch M-F		Fch M-FL		Fch ML-MR		Fch FL-FR	
Female up-regulated								
PLA2G2E	–9.30	*	–10.28	****	–4.90	n.s.	6.92	****
TSPAN8	–9.14	*	–10.06	***	3.57	n.s.	4.2	**
Gallinacin 10-pre	–4.32	****	–3.29	****	–1.21	*	–1.63	****
Ig-superfamily protein	–3.62	***	–2.08	****	0.83	*	–2.27	****
MGP-pre	–3.58	*	–3.74	****	0.23	n.s.	0.34	*
WPKCI	–3.43	*	–3.19	**	2.46	*	–0.43	*
Gastrotropin	–2.35	*	–3.33	****	0.00	n.s.	6.01	****
ELN-pre	–2.13	**	–1.86	****	–0.85	*	–0.49	*
Hemoglobin subunit beta	–1.84	**	–1.65	****	–0.45	*	–0.36	*
LOC100858011	–1.76	**	–1.77	****	0.04	n.s.	0.02	n.s.
TXN	–1.30	**	–0.87	****	–0.35	*	–0.75	***
Male up-regulated								
RPLP0	1.00	*	0.65	**	–1.04	**	0.81	*
PKM	1.04	*	0.77	*	–0.19	n.s.	0.58	*
LAMB1	1.04	*	1.08	*	–0.25	*	–0.08	n.s.
RPL37	1.05	*	0.72	*	–0.39	*	0.77	*
RPL17	1.08	**	0.84	**	–0.47	*	0.52	*
RPS17L	1.12	**	0.74	**	–1.34	****	0.91	**
Receptor expression-enhancing protein 5	1.17	*	1.39	*	–0.79	*	–0.42	*
INHA	3.77	*	3.97	*	–0.80	*	–0.37	n.s.
CDH1	5.43	*	4.97	*	0.27	n.s.	1.14	n.s.
SPARC	10.97	*	10.97	**	0.62	*	n.d.	n.d.

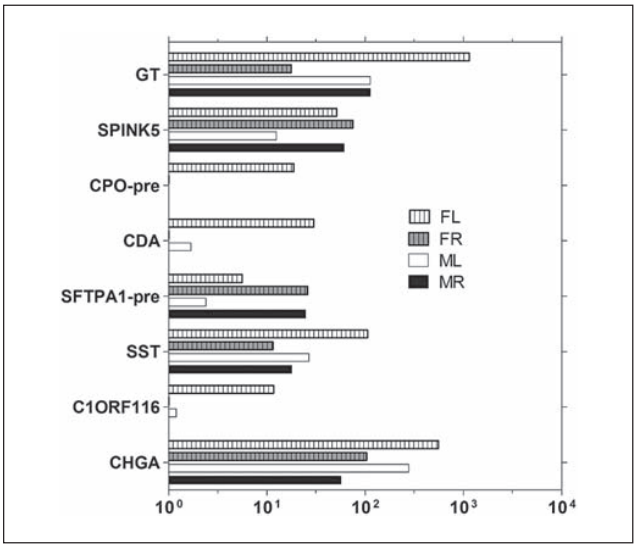
The annotated UniTAGs were screened for the most differentially expressed transcripts between sexes (male-female: M-F) and sorted accordingly. Nomenclature was taken from NCBI and Chicken Gene Nomenclature Consortium, if not tendered.

Fch = $\log_2$ fold change; n.s. = not significant; n.d. = not detected; -pre = precursor of the respective transcript; other abbreviations are the same as in table 1. * $p < 10^{-5}$; ** $p < 10^{-100}$; *** $p < 10^{-200}$; **** $p < 10^{-300}$.

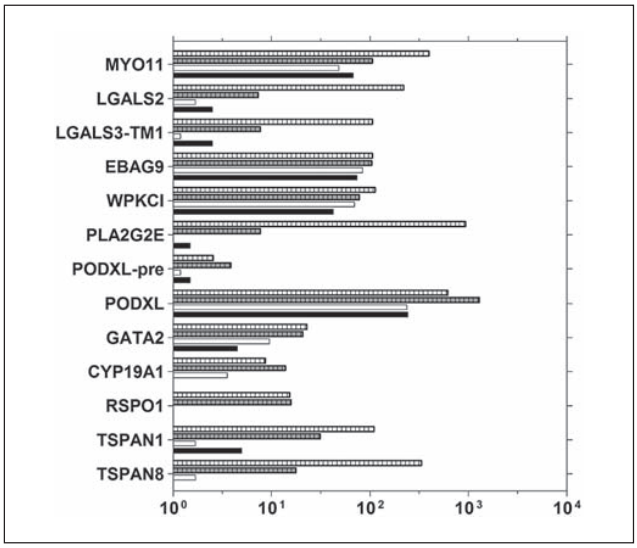
prolactin receptor, SOX9, AMH and its precursor (fig. 2b, c).

General right-left comparison revealed 1,056 and 822 right-left differentially (Fch > |1|; $p < 10^{-5}$) expressed UniTAGs for male and female, respectively. Interestingly, 72 of these UniTAGs are right-left specific in both sexes, with relatively low differences or significance between the sexes. The ovaries display a comprehensive side-specific expression of genes, while differential expression in right and left testes is limited to genes that are found to be generally right-left differentially expressed in both sexes (fig. 2a; table 2, 3). Of these, chromogranin A, specifically androgen-regulated isoform 1, androgen receptor-associated co, somatostatin precursor, followed by surfactant protein A precursor, and cytidine deaminase display the strongest fold changes, and might represent some of the key player genes involved not only in sexual differentiation but rather in lateral development in birds (fig. 2a).

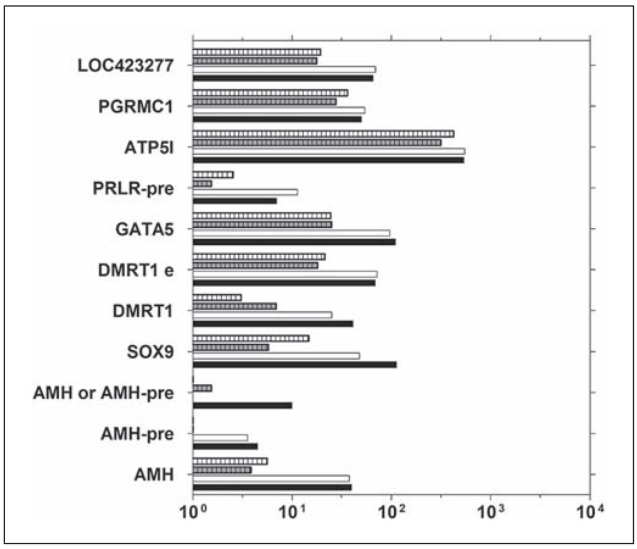
At day E19, differential gene expression in left and right ovaries clearly reflects the tendency of female gonads for growth or regressing in size. Growth-promoting expression patterns comprise the transcripts of e.g. TSPAN1, TSPAN8, GIIEp, galectin-2, gastrotropin, and SOX9. Cytidine deaminase and carboxypeptidase O are almost exclusively expressed in the growing left ovary. The most up-regulated mRNAs in the regressed right ovary (compared to female left ovary and male testes) are thrombomucin/thrombomucin precursor and aromatase. These genes are not located on the W or Z chromosome, but since the gonads are already well developed at day E19, this is not to be expected. These genes seem to be involved in the growth of the left or regression of the



a



b



c

Fig. 2. Expression profiles of selected genes in distinct chicken E19 gonads. For graphical reasons, TPMs were transformed to $\log_{10}$ values. If less than 1 UniTAG per million sequenced tags was present, the respective TPM count was adjusted to 0. The listed mRNAs are sorted for their most significant affiliation. Nomenclature was taken from NCBI and Chicken Gene Nomenclature Consortium if not indicated otherwise. -pre = Precursor of the respective transcript; MR = male right; ML = male left; FR = female right; FL = female left. **a** Right-left differentially expressed mRNAs. **b** Female up-regulated mRNAs. **c** Male up-regulated mRNAs.

right female gonad, rather than in the differentiation of new tissues (fig. 2; table 3).

Steroid Hormone Receptors

The subfamily 3 of nuclear receptors includes steroid receptors as e.g. estrogen, glucocorticoid, progesterone, and androgen receptors as well as the estrogen receptor-related receptors [Nuclear Receptors Nomenclature Committee, 1999; Germain et al., 2006]. Neither the estrogen, androgen, nor glucocorticoid receptors are differentially expressed between the sexes and between left and right gonads. Interestingly, the progesterone receptor is slightly up-regulated in the female right gonad, while there is no significant difference between male gonads.

The mRNAs encoding steroidogenic enzymes required to convert cholesterol to androgens are already present in the avian embryo before gonadal differentiation [Nomura et al., 1999]. Besides significantly differential expression of *P450arom* and W-chromosome-based *WPKCI* transcripts as key players in the differentiation of gonads, we found the chicken orthologs of estrogen-related transcripts minimally differentially expressed between male and females, as e.g. estrogen receptor binding

fragment associated gene 9 protein (EBAG9). The *EBAG9* gene is conserved in human, chimpanzee, dog, cow, mouse, rat, and zebrafish. For most of the human *EBAG9* counterparts, roles in ovarian or breast cancers have been described [Ikeda et al., 2000]. Their expression in chicken provides the opportunity to study their role in the development of sex-specific cancers in an easily accessible experimental system.

Functional Annotation of Gene Expression

The classification of transcripts from male and female gonads (M-F) into GO categories showed that 25,771 UniTAGs grouped to 'molecular function', 25,864 to 'cellular component', and 25,798 to 'biological process'. Differences in the abundance of tags in male and female gonads were considered significant at an enriched $p < 10^{-5}$. The most regulated GO:terms are listed in table 4. The GO classification shows that the strongest ($p < 10^{-15}$) differences occur in *larval development* (GO:0002164, up-regulated in left gonads of both sexes), *nematode larval development* (GO:0002119, up-regulated in left gonads of both sexes), and *structural constituent of ribosome* (GO:0003735, up-regulated in males). Additionally, the GO:terms *post-embryonic development* (GO:0009791) and *embryo development ending in birth or egg hatching* (GO:00009792), both up-regulated in left gonads, are slightly lower significantly regulated than the *development* GO:terms.

Additionally, the GO:term *growth* (GO:0040007) is highly affected: it is up-regulated in females, especially in the growing left gonad. The good reliability of the presented data is proven by the fact that the affected GO:terms comprise different developmental stages from pre-hatching to post-hatching. This shows the validity of the identified transcription profiles: the embryonic development is nearly finished and the individual is shortly before hatching – 2 days left at E19.

Discussion

Since mammalian meiotic sex chromosome silencing [Turner, 2007] makes it difficult to investigate sexually antagonistic genes, birds are the model of choice for studying sex- and right-left biased gene expressions. The presented dataset gives the opportunity to analyze not only differential gene expression in females and males but above all lateral differences within a gender.

The fact that structural gonadal differentiation at day E19 is already very advanced in comparison to other surveys, though the left and right ovaries are still of almost similar size compared to mature gonads, has to be taken into account for a correct interpretation of the presented data. The maturation of gonads at E19 mainly involves growth or regression in size and not the development of new tissues. The chicken Z sex chromosome comprises over 700 known protein-coding and at least 45 non-coding genes (www.ensembl.org/Gallus_gallus). Any of these genes could play a role in sex determination and/or downstream of gonadal sex differentiation. The undifferentiated expression of these sex-determining genes at the already advanced stage of gonadal differentiation at E19 indicates that these mechanisms are not active anymore and explains the discrepancies between sex-chromosome-specific gene expression described in earlier developmental stages [McNagny et al., 1997; Carré et al., 2011] and our findings.

Pluripotent Markers for Asymmetry Are Down-Regulated, Other Genes Have Taken the Wheel

Pluripotent markers like *PITX2, Nanog,* or *PouV*, found in other surveys especially in earlier stages [Ryan et al., 1998; Lavial et al., 2007; Intarapat and Stern, 2013] did not display an asymmetric expression in male or female gonads at E19. Additionally, neither *Sox2* nor *ERNI* were found to be differentially expressed in the left or right gonads. All of them had been associated with asymmetric expression patterns in the gonads of both sexes by other authors in Hamburger-Hamilton (HH) stages 33 and 35 [Hamburger and Hamilton; 1951]. Especially *PouV, ERNI,* and *Nanog* were reported to be less abundant in higher developmental stages [Lavial et al., 2007; Intarapat and Stern, 2013]. Our data confirm these findings for the above-mentioned genes for a speculation: the pluripotent genes lose importance in higher development stages, and other genes have taken the wheel, reacting to the status the pluripotent markers have predetermined.

In contrast to this, the germ cell marker *Cvh* as well as *TP53* are both slightly up-regulated in the male left gonad. *Cvh*-expressing cells are present in the central zone of the area pellucida in HH stage 10, *Cvh*-positive cells were found in the hypoblast layer and anteriorly in the germinal crescent due to morphogenetic movement [Tsunekawa et al., 2000]. *Cvh* plays an essential role in germline formation, and *Cvh*-positive primordial germ cells have been located in the left gonads of both sexes in HH stage 35 [Intarapat and Stern, 2013]. *TP53* belongs to a group of transport proteins carrying specific substances in the blood or across cell membranes. It is involved in cell cycle regulation as a trans-activator that negatively regulates cell division by controlling a set of genes required for this process.

Table 4. The 15 most significantly affected GO:terms between sexes (M-F) and right and left gonads within sexes (FL-FR and ML-MR)

	Identification (GO:term; GO level)	TF	reg	↑	↓	p
M-F	*Molecular function* (0003647;1)	25,771				
	structural constituent of ribosome (0003735;3)	977	258	56	2	***
	RNA binding (0003723;5)	2,257	481	46	2	*
	Cellular component (0005575;1)	25,864				
	mitochondrion (0005739;5, 6)	4,849	984	58	6	**
	ribosome (0005840;4, 6)	1,076	271	58	2	**
	cytosolic ribosome (0022626;5, 6)	738	200	52	2	**
	cytosol (0005829;5, 6)	4,215	856	71	14	**
	ribonucleoprotein complex (0030529;3, 5)	1,650	373	60	3	**
	cytosolic part (0044445;5, 6)	989	242	52	9	**
	Biological process (0008150;1)	25,798				
	nematode larval development (0002119;5, 6)	2,190	503	56	2	***
	larval development (0002164;4, 5)	2,207	506	56	2	***
	translation (0006412;6)	1,841	429	62	6	**
	post-embryonic development (0009791;3, 4)	2,511	547	57	4	**
	embryo development ending in birth or egg hatching (0009792;5)	4,189	847	67	7	**
	growth (0040007;2)	4,211	845	65	6	**
	reproduction (0000003;2)	4,669	926	75	8	**
FL-FR	*Molecular function* (0003647;1)	25,771				
	structural constituent of ribosome (0003735;3)	977	341	52	4	**
	structural molecule activity (0005198;2)	2,591	756	68	25	*
	Cellular component (005575;1)	25,864				
	ribosome (0005840;4, 6)	1,076	364	53	5	**
	cytosolic ribosome (0022626;5, 6)	738	264	46	5	**
	cytosol (0005829;5, 6)	4,215	1,202	77	23	**
	cytosolic part (0044445;5, 6)	989	326	48	12	**
	mitochondrion (0005739;5, 6)	4,849	1,347	77	20	*
	ribonucleoprotein complex (0030529;3, 5)	1,650	504	56	7	*
	Biological process (0008150;1)	25,798				
	translation (0006412;6)	1,841	583	57	12	**
	larval development (0002164;4, 5)	2,207	674	56	8	**
	nematode larval development (0002119;5, 6)	2,190	669	55	8	**
	growth (0040007;2)	4,211	1,185	74	16	*
	post-embryonic development (0009791;3, 4)	2,511	735	56	10	*
	embryo development ending in birth or egg hatching (0009792;5)	4,189	1,171	67	21	*
ML-MR	*Molecular function* (0003674;1)	25,771				
	RNA binding (0003723;5)	2,257	783	31	36	*
	structural molecule activity (0005198;2)	2,591	877	36	42	*
	Cellular component (0005575;1)	25,864				
	mitochondrion (0005739;5, 6)	4,849	1,603	44	66	**
	plastid (0009536;5,6)	898	340	13	16	*
	cytosolic part (0044445;5,6)	989	363	23	31	*
	cytosolic ribosome (0022626;5, 6)	738	279	21	26	*
	Biological process (0008150;1)	25,798				
	larval development (0002164;4, 5)	2,207	816	30	48	***
	nematode larval development (0002119;5, 6)	2,190	805	30	47	***
	post-embryonic development (0009791;3, 4)	2,511	900	34	49	**
	growth (0040007;2)	4,211	1,402	45	58	**
	translation (0006412;6)	1,841	657	36	40	**
	translational elongation (0006414;6, 7)	163	84	2	7	*
	embryo development ending in birth or egg hatching (0009792;5)	4,189	1,382	51	54	*
	reproduction (0000003;2)	4,669	1,525	52	58	*
	embryo development (0009790;4)	5,756	1,855	59	69	*

TF = Transcripts found in the respective GO:term; reg = transcripts differentially regulated $p < 10^{-10}$; ↑ = up-regulated transcripts; ↓ = down-regulated transcripts; p = enrichment p value; * $p < 10^{-5}$; ** $p < 10^{-10}$; *** $p < 10^{-15}$.

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Another mRNA implicated in left-right asymmetry is *BMP4* which is slightly up-regulated in the left gonad of both sexes and significantly up-regulated in the female compared to male gonads. BMP4 is a bone morphogenetic protein that is a potent inducer of bone formation. BMPs belong to the TGF-beta family and negatively regulate the structure and function of the limb apical ectodermal ridge [Pizette and Niswander, 1999]. It is conceivable that this asymmetric expression relates to a similar function in gonadal development [Hoshino et al., 2005].

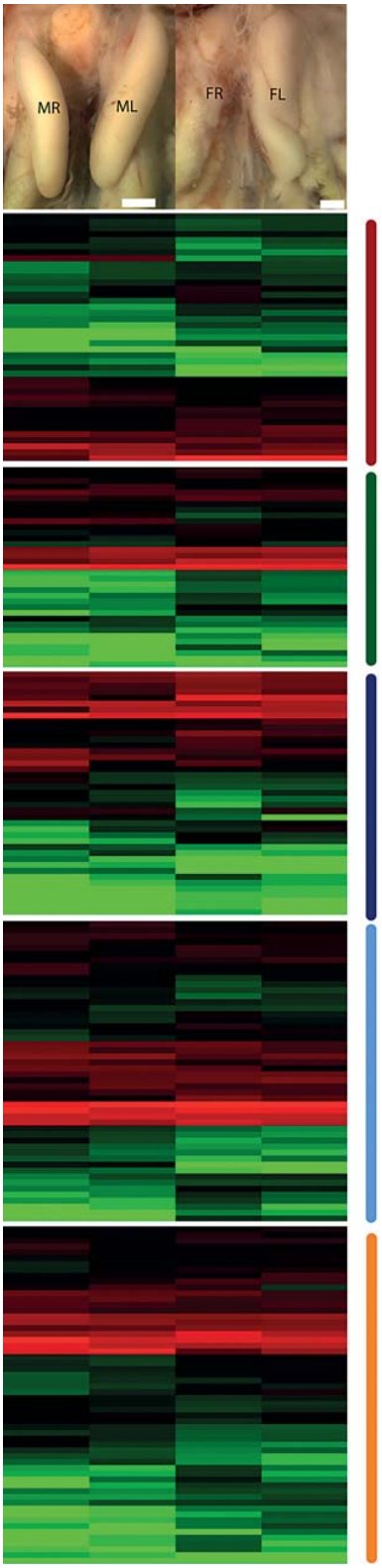
There are several other genes that are strongly up-regulated both in male and female left gonads (fig. 2a) and thus seem to be involved in later stages of side-specific development than the previously reported genes. Overexpression of genes in the right gonads is less prominent. The mRNAs encoding ovoinhibitor (serine peptidase inhibitor, Kazal type 5, SPINK5) and surfactant protein A, for instance, are only slightly up-regulated in male and female right gonads. These genes are not located on either of the sex chromosomes. Neither are the mRNAs almost exclusively expressed in the female left gonad as e.g. the tetraspanins *TSPAN 1* and *8*, *PLA2G2E, LGALS2*, and *LGALS3-TM1* as well as somatostatin and myosin-11 *(MYO11)*. TSPANs act as molecular facilitators and thus are involved in diverse processes. For example, proteins from the tetraspanin subfamily associate with integrin and support cell motility. Also, they have been ascribed a role in the development of the nervous system [Perron and Bixby, 1999]. *PLA2G2E* (GO:0004623) catalyzes the calcium-dependent hydrolysis of the 2 acyl groups in 3-sn-phosphoglycerides. *LGALS2* (GO:0030246) and *LGALS3-TM1* are interacting selectively and non-covalently with lactose and β-galactose, respectively. These genes are implicated in cell cycle regulation, and *LGALS3* is furthermore involved in the mammalian endometrical system with a substantial role in uteral embryo implantation [Yang et al., 2012]. *LGALS3* is also used as a tumor marker in mammals [Chiu et al., 2010; Righi et al., 2010]. Somatostatin acts as an inhibitor of growth hormone secretion [Florio et al., 1994]. Its functions include the modulation of neurotransmission, cell secretion, and cell proliferation [Patel, 1999]. None of the above mentioned genes – as many others differentially expressed between the sexes – is located on the W or Z chromosome which is to be expected in the advanced developmental stage of E19. Whether these differentially expressed genes have been (indirectly) activated by pluripotent markers or not warrants further research.

High Usual Suspects Ratio

We found a significantly higher expression of the W-linked *WPKCI* gene (also known as *HINTW* or *ASW*) in embryonic ovaries than in testes. It is already expressed at E4.5 in the developing female gonads and has been suggested to be involved in avian sex determination [Hori et al., 2000; O'Neill et al., 2000; Smith and Sinclair, 2004]. Early *WPKCI* expression (E5) has also been detected in the avian spinal cord, spinal ganglion, and myotomes [Hori et al., 2000; Scholz et al., 2006]. The conserved vertebrate embryo gene *DMRT1* is one of the mostly favored Z-linked candidate genes controlling gonadal sex differentiation [Nanda et al., 1999; Raymond et al., 1999; Smith et al., 2009; Chue and Smith, 2011], and our data confirms former observations [Shimada, 2002; Alam et al., 2008; Koba et al., 2008] that it is more expressed in testes versus ovaries. This is a further confirmation of the low effectiveness of dosage compensation of birds in higher developmental stages found in other surveys [Ellegren et al., 2007; Itoh et al., 2007]. The transcription profile generated via deepSuperSAGE allows for discrimination between *DMRT1* and its isoform e. Interestingly, expression levels in the female left and right gonad differ severely, although both are generally more abundant in testes (fig. 2c). *DMRT1* is about 1-fold up-regulated in the female right gonad compared to the left one, while its isoform e is more abundant in the female left gonad. The isoform e lacks the complete 3′ untranslated region from *DMRT1* which comprises several microRNA binding sites. The fact that *DMRT1* isoform e is far more abundant in all tissues but lacking these microRNA binding sites leads to the speculation of a post-transcriptional regulation of the *DMRT1* mRNA via microRNA-mediated degradation aside from alternative splicing mechanisms.

SOX9 is also found to be up-regulated in males, while *RSPO1* as well as aromatase are up-regulated in females, concretizing the findings of Ayers et al. [2013] as well as Chue and Smith [2011]. DMRT1 has been suggested to activate the testicular marker *SOX9* indirectly because of the time lag between *DMRT1* and *SOX9* expression (day 4.5 and day 6.5 of incubation, respectively), and the chicken homolog of hemogen *(cHEMGN)* was shown to link the expression of both [Nakata et al., 2013]. In line with this, we observed an exclusive expression of *cHEMGN* in male gonads.

The secretion of 17β-estradiol in chicken E8 embryos is higher in the left ovary than in the right [Pedernera et al., 1999], and the higher amount of estrogen produced in the left ovary is suggested to protect the left Müllerian duct from the effects of anti-Müllerian hormone (AMH) [Vil-



lalpando et al., 2000; Smith and Sinclair, 2001]. This is responsible for the regression of the right Müllerian duct in earlier stages [Oréal et al., 2002]. In our embryonic gonads, the AMH transcript is up-regulated in both male gonads compared to the females. Since males usually do not develop Müllerian ducts because of the elevated AMH level in early development stages, the up-regulation of *AMH* in male gonads at E19 seems to have another – or additional – function. *EBAG9* is the only mRNA related to estrogen that is slightly down-regulated in the male right gonad. The other estrogen receptors, in general, are not differentially expressed between the sexes. Additionally, we did not find any differential expression of *WNT4* or *β-catenin* in our dataset which are both thought to be especially involved in ovary development [Chue and Smith, 2011; Ayers et al., 2013]. The most likely reason for the discrepancy between our and previous findings is that the ovaries are nearly finished in their structural differentiation at E19 compared to days E4.5–12.5 [Smith et al., 2008b].

The Expression Profile of the Regressing Female Right Gonad and Possible Implications

Table 2 as well as the heat map in figure 3 both illustrate the differential expression of a selection of male and female as well as right-left differentially expressed genes. The male gonads in general do not display big differences in right-left comparisons. On the contrary, the regressing female right gonad exhibits strong differences in gene expression of transcripts involved in almost all metabolic pathways not only compared to left and right testes but also compared to the left ovary. Apart from genes involved in sexual development, also genes coding immune reactivity/defense/stress, blood building, as well as transcription factors and kinase transcripts show strong differences in male-female and, especially, right-left (female) gene expression.

Many strongly up-regulated genes in ovaries encode structural proteins (e.g. elastin, collagen, fibrilles), hemoglobin and – especially in the female right gonad – thrombomucin. This cell surface protein is distantly related to

Fig. 3. Heat map of the most differential male/female and right/left expressed genes. Photos of the gonads are displayed above the corresponding column, while selected, right-left differentially expressed genes from several metabolic pathways are shown in the heat map. Assignment of genes to metabolic pathways is based on the respective GO annotation, $p_{max} < 9E^{-3}$. The color code indicates the same metabolic pathways as in figure 1. Scale bar = 1mm. Abbreviations are the same as in table 1.

CD34 and besides haemoglobin one of the most used human hematopoietic stem cell markers defining thrombocytes and multipotent hematopoietic progenitors [McNagny et al., 1997]. Surprisingly, our results seem to broaden the findings of McNagny et al. [1997] that this gene is highly expressed in female gonads. McNagny and co-workers stained early embryonic stages (E3–E5) and found that thrombomucin is mainly expressed on the surface of intra- and extraembryonic hematopoietic cells. Additionally, immune globulins, cytidine deaminase followed by carboxypeptidase O and defensins (Gal-10), as well as fibrinogen are also up-regulated in the ovaries. Our findings lead to the speculation that, within the scope of evolution, the rudimental right ovary might have gained new function(s) like e.g. hematopoiesis, because mucins have been shown to play both positive and negative roles in adhesion processes and thrombomucin may prevent the differentiation of hematopoietic cells [McNagny et al., 1997].

Conclusions

By application of deepSuperSAGE as a potent technique for the identification of differential gene expression in chicken E19 gonads, we identified several genes that seem to be more important for sexual and lateral development than previously thought. Some of these might be specific for the highly developed gonads in E19 and consequently may exert important functions in developed gonads. The presented dataset is an outstanding possibility for research in the field of right-left development, especially in view of gynandromorph animals [Zhao et al., 2010; Chue and Smith, 2011], and is going to assist studies on gene expression, especially in the context of comparative genomics. Our results will facilitate the study of evolutionarily important traits at the molecular level, deepening our knowledge of avian genomics, avian proteomics, endocrinology, as well as developmental biology, including sex differentiation.

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References

Akmaev VR, Wang CJ: Correction of sequence-based artifacts in serial analysis of gene expression. Bioinformatics 20:1254–1263 (2004).

Alam MA, Kobayashi Y, Horiguchi R, Hirai T, Nakamura M: Molecular cloning and quantitative expression of sexually dimorphic markers *Dmrt1* and *Foxl2* during female-to-male sex change in *Epinephelus merra*. Gen Comp Endocrinol 157:75–85 (2008).

Altschul SF, Madden TL, Schäffer AA, Zhang J, Zhang Z, et al: Gapped blast and psi-blast: a new generation of protein database search programs. Nucleic Acids Res 25:3389–3402 (1997).

Ayers KL, Sinclair AH, Smith CA: The molecular genetics of ovarian differentiation in the avian model. Sex Dev 7:80–94 (2013).

Bassuk JA, Iruela-Arispe ML, Lane TF, Benson JM, Berg RA, Sage EH: Molecular analysis of chicken embryo SPARC (osteonectin). Eur J Biochem 218:117–127 (1993).

Camon E, Magrane M, Barrell D, Lee V, Dimmer E, et al: The gene ontology annotation (GOA) database: sharing knowledge in Uniprot with gene ontology. Nucleic Acids Res 32:D262–D266 (2004).

Carré GA, Couty I, Hennequet-Antier C, Govoroun MS: Gene expression profiling reveals new potential players of gonad differentiation in the chicken embryo. PLoS One 6:e23959 (2011).

Chen F, Mackey AJ, Stoeckert CJ, Roos DS: OrthoMCL-DB: querying a comprehensive multi-species collection of ortholog groups. Nucleic Acids Res 34:D363–D368 (2006).

Chiu CG, Strugnell SS, Griffith OL, Jones SJ, Gown AM, et al: Diagnostic utility of galectin-3 in thyroid cancer. Am J Pathol 176:2067–2081 (2010).

Chue J, Smith CA: Sex determination and sexual differentiation in the avian model. FEBS J 278:1027–1034 (2011).

Civetta A, Singh RS: Broad-sense sexual selection, sex gene pool evolution, and speciation. Genome 42:1033–1041 (1999).

Connallon T, Knowles LL: Intergenomic conflict revealed by patterns of sex-biased gene expression. Trends Genet 21:495–499 (2005).

Connallon T, Knowles LL: Recombination rate and protein evolution in yeast. BMC Evol Biol 7:235 (2007).

Delostrinos CF, Hudson AE, Feng WC, Kosman J, Bassuk JA: The c-terminal Ca^{2+}-binding domain of SPARC confers anti-spreading activity to human urothelial cells. J Cell Physiol 206:211–220 (2006).

Diaz FJ, Anthony K, Halfhill AN: Early avian follicular development is characterized by changes in transcripts involved in steroidogenesis, paracrine signaling and transcription. Mol Reprod Dev 78:212–223 (2011).

Eads BD, Colbourne JK, Bohuski E, Andrews J: Profiling sex-biased gene expression during parthenogenetic reproduction in *Daphnia pulex*. BMC Genomics 8:464 (2007).

Ellegren H: Evolution of the avian sex chromosomes and their role in sex determination. Trends Ecol Evol 15:188–192 (2000).

Ellegren H, Parsch J: The evolution of sex-biased genes and sex-biased gene expression. Nat Rev Genet 8:689–698 (2007).
Ellegren H, Hultin-Rosenberg L, Brunstrom B, Dencker L, Kultima K, Scholz B: Faced with inequality: chicken do not have a general dosage compensation of sex-linked genes. BMC Biol 5:40 (2007).
Florio T, Rim C, Hershberger RE, Loda M, Stork PJ: The somatostatin receptor SSTR1 is coupled to phosphotyrosine phosphatase-activity in CHO-K1 cells. Mol Endocrinol 8:1289–1297 (1994).
Fridolfsson AK, Ellegren H: A simple and universal method for molecular sexing of non-ratite birds. J Avian Biol 30:116–121 (1999).
Fridolfsson AK, Cheng H, Copeland NG, Jenkins NA, Liu HC, et al: Evolution of the avian sex chromosomes from an ancestral pair of autosomes. Proc Natl Acad Sci USA 95:8147–8152 (1998).
Germain P, Staels B, Dacquet C, Spedding M, Laudet V: Overview of nomenclature of nuclear receptors. Pharmacol Rev 58:685–704 (2006).
González-Morán MG: Histological and stereological changes in growing and regressing chicken ovaries during development. Anat Rec 294:893–904 (2011).
Hambuch TM, Parsch J: Patterns of synonymous codon usage in *Drosophila melanogaster* genes with sex-biased expression. Genetics 170:1691–1700 (2005).
Hamburger V, Hamilton HL: A series of normal stages in the development of the chick embryo. J Morphol 88:49–92 (1951).
Hori T, Asakawa S, Itoh Y, Shimizu N, Mizuno S: Wpkci, encoding an altered form of PKCI, is conserved widely on the avian W chromosome and expressed in early female embryos: implication of its role in female sex determination. Mol Biol Cell 11:3645–3660 (2000).
Hoshino A, Koide M, Ono T, Yasugi S: Sex-specific and left-right asymmetric expression pattern of *Bmp7* in the gonad of normal and sex-reversed chicken embryos. Dev Growth Differ 47:65–74 (2005).
Hubbard TJ, Aken BL, Beal K, Ballester B, Caccamo M, et al: Ensembl 2007. Nucl Acids Res 35:D610–617 (2007).
Hudson QJ, Smith CA, Sinclair AH: Aromatase inhibition reduces expression of *FOXL2* in the embryonic chicken ovary. Dev Dyn 233: 1052–1055 (2005a).
Hudson QJ, Smith CA, Sinclair AH: Conserved expression of a novel gene during gonadal development. Dev Dyn 233:1083–1090 (2005b).
Hulsen T, Huynen MA, de Vlieg J, Groenen PM: Benchmarking ortholog identification methods using functional genomics data. Genome Biol 7:R31 (2006).
Ikeda K, Sato M, Tsutsumi O, Tsuchiya F, Tsuneizumi M, et al: Promoter analysis and chromosomal mapping of human *EBAG9* gene. Biochem Biophys Res Commun 273:654–660 (2000).
Intarapat S, Stern CD: Sexually dimorphic and sex-independent left-right asymmetries in chicken embryonic gonads. PLoS One 8:e69893 (2013).
Itoh Y, Melamed E, Yang X, Kampf K, Wang S, et al: Dosage compensation is less effective in birds than in mammals. J Biol 6:2–2 (2007).
Ivakine EA, Fox CJ, Paterson AD, Mortin-Toth SM, Canty A, et al: Sex-specific effect of insulin-dependent diabetes 4 on regulation of diabetes pathogenesis in the nonobese diabetic mouse. J Immunol 174:7129–7140 (2005).
Jacob M, Bakst MR: Anatomy of the female reproductive tract, in Jamieson BG (ed): Reproductive Biology and Phylogeny of Aves (Birds), vol 6A, pp 553–587 (USA: Science Publishers, Enfield 2007).
Koba N, Ohfuji T, Ha Y, Mizushima S, Tsukada A, et al: Profiles of mRNA expression of *Foxl2, P450arom, Dmrt 1, Amh, P450(c17), Sf1, Er alpha* and *Ar*, in relation to gonadal sex differentiation in duck embryo. J Poultry Sci 45: 132–138 (2008).
Lavial F, Acloque H, Bertocchini F, MacLeod DJ, Boast S, et al: The *Oct4* homologue *Pouv* and *Nanog* regulate pluripotency in chicken embryonic stem cells. Development 134:3549–3563 (2007).
Li H, Coghlan A, Ruan J, Coin LJ, Heriche JK, et al: Treefam: a curated database of phylogenetic trees of animal gene families. Nucleic Acids Res 34:D572–D580 (2006).
Li L, Stoeckert CJ, Roos DS: OrthoMCL: identification of ortholog groups for eukaryotic genomes. Genome Res 13:2178–2189 (2003).
Lu F, Bytautiene E, Tamayo E, Gamble P, Anderson GD, et al: Gender-specific effect of overexpression of *sFlt-1* in pregnant mice on fetal programming of blood pressure in the offspring later in life. Am J Obstet Gynecol 197: 418 (2007).
Man MZ, Wang X, Wang Y: Power_sage: comparing statistical tests for sage experiments. Bioinformatics 16:953–959 (2000).
Mank JE, Hultin-Rosenberg L, Axelsson E, Ellegren H: Rapid evolution of female-biased, but not male-biased, genes expressed in the avian brain. Mol Biol Evol 24:2698–2706 (2007).
Marinotti O, Calvo E, Nguyen QK, Dissanayake S, Ribeiro JM, James AA: Genome-wide analysis of gene expression in adult *Anopheles gambiae*. Insect Mo Biol 15:1–12 (2006).
Matsumura H, Reich S, Ito A, Saitoh H, Kamoun S, et al: Gene expression analysis of plant host-pathogen interactions by superSAGE. Proc Natl Acad Sci USA 100:15718–15723 (2003).
Matsumura H, Ito A, Saitoh H, Winter P, Kahl G, et al: SuperSAGE. Cell Microbiol 7:11–18 (2005).
Matsumura H, Yoshida K, Luo SJ, Kimura E, Fujibe T, et al: High-throughput superSAGE for digital gene expression analysis of multiple samples using next generation sequencing. PLoS One 5:e12010 (2010).
Matsumura H, Yoshida K, Luo S, Krüger DH, Kahl G, et al: High-throughput superSAGE. Methods Mol Biol 687:135–146 (2011).
McNagny KM, Pettersson I, Rossi F, Flamme I, Shevchenko A, et al: Thrombomucin, a novel cell surface protein that defines thrombocytes and multipotent hematopoietic progenitors. J Cell Biol 138:1395–1407 (1997).
Meiklejohn CD, Parsch J, Ranz JM, Hartl DL: Rapid evolution of male-biased gene expression in *Drosophila*. Proc Natl Acad Sci USA 100:9894–9899 (2003).
Merchant-Larios H, Moreno-Mendoza N: Onset of sex differentiation: dialog between genes and cells. Arch Med Res 32:553–558 (2001).
Nakata T, Ishiguro M, Aduma N, Izumi H, Kuroiwa A: Chicken hemogen homolog is involved in the chicken-specific sex-determining mechanism. Proc Natl Acad Sci USA 110: 3417–3422 (2013).
Nanda I, Shan ZH, Schartl M, Burt DW, Koehler M, et al: 300 million years of conserved synteny between chicken Z and human chromosome 9. Nat Genet 21:258–259 (1999).
Nomura O, Nakabayashi O, Nishimori K, Yasue H, Mizuno S: Expression of five steroidogenic genes including aromatase gene at early developmental stages of chicken male and female embryos. J Steroid Biochem Mol Biol 71: 103–109 (1999).
Nuclear Receptors Nomenclature Committee: A unified nomenclature system for the nuclear receptor superfamily. Cell 97:161–163 (1999).
O'Brien KP, Westerlund I, Sonnhammer EL: Orthodisease: a database of human disease orthologs. Hum Mutat 24:112–119 (2004).
O'Brien KP, Remm M, Sonnhammer EL: Inparanoid: a comprehensive database of eukaryotic orthologs. Nucleic Acids Res33:D476–D480 (2005).
O'Neill M, Binder M, Smith C, Andrews J, Reed K, et al: *ASW*: a gene with conserved avian W-linkage and female specific expression in chick embryonic gonad. Dev Genes Evol 210: 243–249 (2000).
Oréal E, Mazaud S, Picard JY, Magre S, Carre-Eusebe D: Different patterns of anti-mullerian hormone expression, as related to *DMRT1, SF-1, WT1, GATA-4, Wnt-4*, and *Lhx9* expression, in the chick differentiating gonads. Dev Dyn 225:221–232 (2002).
Patel YC: Somatostatin and its receptor family. Front Neuroendocrinol 20:157–198 (1999).
Pedernera E, Solis L, Peralta I, Velazquez PN: Proliferative and steroidogenic effects of follicle-stimulating hormone during chick embryo gonadal development. Gen Comp Endocrinol 116:213–220 (1999).
Perron JC, Bixby JL: Tetraspanins expressed in the embryonic chick nervous system. FEBS Lett 461:86–90 (1999).
Pfaffl MW: A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res 29:e45 (2001).

Pizette S, Niswander L: BMPs negatively regulate structure and function of the limb apical ectodermal ridge. Development 126:883–894 (1999).
Ranz JM, Castillo-Davis CI, Meiklejohn CD, Hartl DL: Sex-dependent gene expression and evolution of the *Drosophila* transcriptome. Science 300:1742–1745 (2003).
Raymond CS, Kettlewell JR, Hirsch B, Bardwell VJ, Zarkower D: Expression of *Dmrt1* in the genital ridge of mouse and chicken embryos suggests a role in vertebrate sexual development. Dev Biol 215:208–220 (1999).
Remm M, Storm CEV, Sonnhammer ELL: Automatic clustering of orthologs and in-paralogs from pairwise species comparisons. J Mol Biol 314:1041–1052 (2001).
Righi A, Jin L, Zhang S, Stilling G, Scheithauer BW, et al: Identification and consequences of galectin-3 expression in pituitary tumors. Mol Cell Endocrinol 326:8–14 (2010).
Rodríguez-Léon J, Esteban CR, Marti M, Santiago-Josefat B, Dubova I, et al: Pitx2 regulates gonad morphogenesis. Proc Natl Acad Sci USA 105:11242–11247 (2008).
Ryan AK, Blumberg B, Rodriguez-Esteban C, Yonei-Tamura S, Tamura K, et al: Pitx2 determines left-right asymmetry of internal organs in vertebrates. Nature 394:545–551 (1998).
Sage H, Vernon RB, Funk SE, Everitt EA, Angello J: SPARC, a secreted protein associated with cellular proliferation, inhibits cell spreading in vitro and exhibits Ca^{2+}-dependent binding to the extracellular-matrix. J Cell Biol 109: 341–356 (1989).
Saha S, Sparks AB, Rago C, Akmaev V, Wang CJ, et al: Using the transcriptome to annotate the genome. Nat Biotechnol 20:508–512 (2002).
Scholz B, Kultima K, Mattsson A, Axelsson J, Brunstrom B, et al: Sex-dependent gene expression in early brain development of chicken embryos. BMC Neurosci 7:12 (2006).
Shimada K: Sex determination and sex differentiation. Avian Poult Biol Rev 13:1–14 (2002).
Smith CA: Molecular genetics of avian, sex determination and gonadal differentiation, in Jamieson BG (ed): Reproductive Biology and Phylogeny of Birds, vol 6B, pp 479–506 (USA: Science Publishers, Enfield 2007).
Smith CA, Sinclair AH: Sex determination in the chicken embryo. J Exp Zool 290:691–699 (2001).
Smith CA, Sinclair AH: Sex determination: insights from the chicken. Bioessays 26:120–132 (2004).
Smith CA, Roeszler KN, Bowles J, Koopman P, Sinclair AH: Onset of meiosis in the chicken embryo; evidence of a role for retinoic acid. BMC Dev Biol 8:85 (2008a).
Smith CA, Shoemaker CM, Roeszler KN, Queen J, Crews D, Sinclair AH: Cloning and expression of R-spondin1 in different vertebrates suggests a conserved role in ovarian development. BMC Dev Biol 8:72 (2008b).
Smith CA, Roeszler KN, Ohnesorg T, Cummins DM, Farlie PG, et al: The avian Z-linked gene *Dmrt1* is required for male sex determination in the chicken. Nature 461:267–271 (2009).
Swanson WJ, Vacquier VD: The rapid evolution of reproductive proteins. Nat Rev Genet 3: 137–144 (2002).
Termine JD, Kleinman HK, Whitson SW, Conn KM, McGarvey ML, Martin GR: Osteonectin, a bone-specific protein linking mineral to collagen. Cell 26:99–105 (1981).
Tsunekawa N, Naito M, Sakai Y, Nishida T, Noce T: Isolation of chicken *vasa* homolog gene and tracing the origin of primordial germ cells. Development 127:2741–2750 (2000).
Turner JM: Meiotic sex chromosome inactivation. Development 134:1823–1831 (2007).
Ukeshima A: Germ cell death in the degenerating right ovary of the chick embryo. Zoolog Sci 13:559–563 (1996).
Ukeshima A, Fujimoto T: A fine morphological study of germ cells in asymmetrically developing right and left ovaries of the chick. Anat Rec 230:378–386 (1991).
Velculescu V, Zhang L, Vogelstein B, Kinzler K: Serial analysis of gene expression. Science 270:484–487 (1995).
Villalpando I, Sanchez-Bringas G, Sanchez-Vargas I, Pedernera E, Villafan-Monroy H: The p450 aromatase *(P450arom)* gene is asymmetrically expressed in a critical period for gonadal sexual differentiation in the chick. Gen Comp Endocrinol 117:325–334 (2000).
Wan Y, Kirschner MW: Identification of multiple CDH1 homologues in vertebrates conferring different substrate specificities. Proc Natl Acad Sci USA 98:13066–13071 (2001).
Wicker T, Robertson JS, Schulze SR, Feltus FA, Magrini V, et al: The repetitive landscape of the chicken genome. Genome Res 15:126–136 (2005).
Wright MW, Eyre TA, Lush MJ, Povey S, Bruford EA: HCOP: the HGNC comparison of orthology predictions search tool. Mamm Genome 16:827–828 (2005).
Wu JX, Pines M, Gay CV, Hurwitz S, Leach RM: Immunolocalization of osteonectin in avian tibial dyschondroplastic cartilage. Dev Dyn 207:69–74 (1996).
Xu HY, Hou XW, Wang NF, Hui B, Jin JF, et al: Gender-specific effect of estrogen receptor-1 gene polymorphisms in coronary artery disease and its angiographic severity in Chinese population. Clin Chim Acta 395:130–133 (2008).
Yamamoto I, Tsukada A, Saito N, Shimada K: Profiles of mRNA expression of genes related to sex differentiation of the gonads in the chicken embryo. Poultry Sci 82:1462–1467 (2003).
Yang H, Lei C, Zhang W: Expression of galectin-3 in mouse endometrium and its effect during embryo implantation. Reprod Biomed Online 24:116–122 (2012).
Yang X, Schadt EE, Wang S, Wang H, Arnold AP, et al: Tissue-specific expression and regulation of sexually dimorphic genes in mice. Genome Res 16:995–1004 (2006).
Zawada AM, Rogacev KS, Rotter B, Winter P, Marell R-R, Fliser D, Heine GH: Supersage evidence for CD14++CD16+ monocytes as a third monocyte subset. Blood (2011).
Zawada AM, Rogacev KS, Hummel B, Grün OS, Friedrich A, et al: SuperTAG methylation-specific digital karyotyping reveals uremia-induced epigenetic dysregulation of atherosclerosis-related genes. Circ Cardiovas Genet 5:611–620 (2012).
Zhang Z, Hambuch TM, Parsch J: Molecular evolution of sex-biased genes in *Drosophila*. Mol Biol Evol 21:2130–2139 (2004).
Zhao D, McBride D, Nandi S, McQueen HA, McGrew MJ, et al: Somatic sex identity is cell autonomous in the chicken. Nature 464:237–242 (2010).

A.1.2 The domestic fowl (*Gallus gallus domesticus*) embryo as an alternative for mammalian experiments - Validation of a test method for the detection of endocrine disrupting chemicals

Luzie Jessl, Jessica Scheider, Jörg Oehlmann

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Was hat die Promovendin bzw. was haben die Koautoren beigetragen?

(1) zu Entwicklung und Planung

Promovendin JS: 40%
Co-Autor LJ: 50%
Co-Autor JO: 10%

(2) zur Durchführung der einzelnen Untersuchungen und Experimente

Promovendin JS: 30% (Injektion, Bebrütung, Gonadenisolation, Histologie)
Co-Autor LJ: 70% (Injektion, Bebrütung, Gonadenisolation, Histologie)

(3) zur Erstellung der Datensammlung und Abbildungen

Promovendin JS: 30% (Aufarbeitung der Rohdaten, Abbildungserstellung)
Co-Autor LJ: 70% (Aufarbeitung der Rohdaten, Abbildungserstellung)

(4) zur Analyse und Interpretation der Daten

Promovendin JS: 20% (Interpretation und Analyse, Publikationsrecherche)
Co-Autor LJ: 80% (Interpretation und Analyse, Publikationsrecherche)

(5) zum Verfassen des Manuskripts

Promovendin JS: 20%
Co-Autor LJ: 60%
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The domestic fowl (*Gallus gallus domesticus*) embryo as an alternative for mammalian experiments – Validation of a test method for the detection of endocrine disrupting chemicals

Luzie Jessl*, Jessica Scheider, Jörg Oehlmann

Goethe University Frankfurt am Main, Institute for Ecology, Evolution and Diversity, Department Aquatic Ecotoxicology, Max-von-Laue-Str. 13, 60438 Frankfurt am Main, Germany

HIGHLIGHTS

- The first study to systematically examine the variability of individual parameters in control groups of chicken embryos.
- The low natural variability of the test system results in a good reproducibility in control and substance-treated groups.
- Reference values for developmental and gonadal endpoints, suggested as validity criteria, are provided.
- The chicken embryo is a suitable system for the detection of EDCs and a promising alternative to mammalian experiments.

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ABSTRACT

In recent decades the embryo of *Gallus g. domesticus* has been widely used as a model for the study of early sexual development and the potential impact of substances affecting development, including endocrine disrupting chemicals (EDCs). Since there is no standardized procedure available for experiments with the chicken embryo, the objective of our project is to expedite the protocol to assess the potential effects of EDCs on early sexual differentiation. The main aim of the present study was to systematically investigate the natural variability of individual developmental and histological key parameters in untreated and solvent-treated control groups, since this has been insufficiently addressed so far. A further aim was to provide robust values for all parameters investigated in control and substance experiments, using two known estrogenic compounds, bisphenol A (75/150/300 μg/g egg) and 17α-ethinylestradiol (20 ng/g egg). On embryonic day 1 eggs were injected with the estrogenic compounds. On embryonic day 19 histological gonadal data as well as morphological parameters were noted. In baseline experiments with control groups the selected endpoints showed reproducible results with low variabilities. Furthermore, gonadal endpoints responded sensitively to the treatment with the two model EDCs. Thus, these endpoints are recommended for the assessment of suspected EDCs in which the values provided for all parameters can serve as validity criteria in future experiments. The embryo of *G. domesticus* has shown to be a suitable alternative to currently accepted mammalian bioassays for the impact assessment of EDCs on reproductive tissues.

1. Introduction

Reproductive disorders in animals and humans caused by chemical substances that are suspected as endocrine disrupters have gained major interest for science and society. Especially the interference of these chemicals with sexual development and reproduction plays a major role. Endocrine disrupting chemicals (EDCs) are naturally or synthetically occurring compounds and may affect the natural balance of hormones or alter the endocrine control in animals. Various studies confirm the suspicion that EDCs may adversely affect wildlife and human health (Colborn et al., 1993; Giesy et al., 2003; Vandenberg et al., 2012; Mallozzi et al., 2016). In view of the large number of constantly used chemicals, e.g. in agriculture, industry or medicine, it is expected that EDCs

* Corresponding author.
E-mail addresses: jessl@bio.uni-frankfurt.de (L. Jessl), j.scheider@bio.uni-frankfurt.de (J. Scheider), oehlmann@bio.uni-frankfurt.de (J. Oehlmann).

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end up in the environment and may be incorporated by animals and humans and, thus, may impact their hormonal systems. Only a small proportion of these substances has been tested for potential effects on the hormonal system. The pending investigation of further substances will therefore produce a high demand for animal experiments in the coming years, since most of these substances have to be tested *in vivo*.

The Hershberger assay and uterotrophic assay are two internationally standardized tests for EDCs with androgenic or estrogenic activity, which are based on rodents (OECD, 2007, 2009). These tests use juvenile or adult rats or mice and, thus, not the most sensitive life stage for EDCs, the developing embryo (Lan and Katzenellenbogen, 1976; Cook et al., 1997; Grote et al., 2004). Moreover, the critical effect on sexual differentiation in higher vertebrates is currently studied by a standardized multigenerational test, using quail and other avian species (OECD, 1984). However, this test is time intensive and costly and a very high number of individuals is needed. As a result, nearly 2.7 million vertebrates are annually consumed for animal testing throughout Germany, almost 256,600 of them for toxicological studies (BMELV, 2015). This high number is hardly acceptable for animal welfare reasons, and this is why the search for a suitable animal replacement system is of great importance.

As early as 1959, Russel and Burch dealt with the subject of treating experimental animals in a more human way (Russel and Burch, 1959, reprinted 1992). Their principle of the 3Rs represents the widely accepted ethical standard for the use of animal experiments, which is already being implemented and applied in many laws and technical guidelines. The 3Rs stand for Replacement, Reduction and Refinement.

An interesting and promising approach to avoid animal testing is the development of a standardized procedure for the testing of potential EDCs in avian embryos. In recent years, the avian embryo has emerged as a model for the study of environmental pollutants, including EDCs (Fry and Toone, 1981; Fry, 1995; Berg et al., 1998, 1999, 2001a, 2001b, 2004; Berge et al., 2004; Biau et al., 2007; Brunstrom and Halldin, 2000). The present study is part of a project aiming to advance a replacement method for testing hormonally active compounds in birds, where fertilized eggs of the domestic fowl (*Gallus gallus domesticus*) are used (Berg et al., 1998, 1999). Chicken eggs provide significant advantages in the testing of chemicals, as they are available throughout the year and the injection of substances directly into the yolk allows specific and standardized dosages (Berg et al., 1999). As the hen affects the development of its offspring by transferred genetic materials as well as hormones (Carere and Balthazart, 2007), substances incorporated by the mother may consequently also influence the development of the offspring even originally or as metabolites in the allantoic fluid (Kamata et al., 2006). However, in contrast to developing mammals or aquatic species, the chicken egg is a closed system lacking any exchange with its environment except for the interchange of gases. Thus, one injection of a test compound results in chronic chemical exposure, because no exchange or loss of the substance is possible. A single injection may therefore be sufficient to influence the developing embryo (Davies et al., 1997; Gooding et al., 2003; McAllister and Kime, 2003; Zhang et al., 2007). The embryonic development is fully described (Keibel and Abraham, 1900; Hamburger and Hamilton, 1992; Starck and Ricklefs, 1997) and the individual developmental stages are clearly visible and easily accessible. Also the endocrine system of adult birds is largely similar to that of mammals (Lange et al., 2002) which allows a limited transfer of the resulting data to humans, as little differences still exist, e.g. the genetic and endocrine control of gonadal development is not identical to humans. It is known that the influence of xenohormones in birds during embryonic development can lead to irreversible malformations of the gonads or later to a disturbed gender-related behavior, whereas EDCs may exert less severe and often reversible effects in the less sensitive adult stage (Adkins-Regan, 1990; Ottinger and Abdelnabi, 1997). Therefore, the chicken embryo is a suitable model for the study of early sexual development and the potential impact of EDCs regarding the replacement for other vertebrate or even mammalian models.

However, the characterization of the normal development of the test organism without exposure to EDCs should be a first and fundamental step for the successful development of a test design based on chicken embryos. Based on previously published studies we decided for different common and gonad-based endpoints which have already been shown to be affected by EDCs (Scheib and Reyssbrion, 1979; Scheib and Baulieu, 1981; Scheib, 1983; Berg et al., 1998, 1999; Halldin et al., 2003). These endpoints are the surface area of left and right ovary or testis, the cortex thickness of left ovary or testis and the percentage of seminiferous tubules of left testis. In an extensive series of experiments, we investigated these endpoints and worked out a detailed morphological and histological description of embryonic gonads. Special focus was on the systematic examination of the variability of individual parameters in untreated and solvent-treated control to demonstrate the natural variability of the test system. Furthermore, this may allow the determination of reliable reference values for each endpoint which may serve as validity criteria in future experiments. Also, these values could be used for the comparison between controls and substance-treated groups. Moreover, the present study draws a comparison between untreated controls and solvent controls, which received dimethyl sulfoxide (DMSO) as a solvent. At last this should allow a statement about the conformity and validity of the data of both test groups and whether DMSO is a suitable solvent for the chicken egg test. With the knowledge about the normal development of the chicken embryos it will be possible to obtain a reliable comparison between control groups and test groups treated with different EDCs.

The second step in our investigations was to evaluate the effects of the chosen EDCs on gonadal differentiation as assessed by the previously chosen endpoints. After profound analysis of existing literature we determined two promising compounds and analyzed their effects on embryonic development with special focus on potential gross morphological and histological changes of the gonads. The selected estrogenic substances 17α-ethinylestradiol (EE_2), a synthetic hormone primarily used for contraception, and bisphenol A (BPA), a monomer used as basic material for polycarbonate plastics, have already been widely used in the study of EDC-related effects on different groups of organisms (Watts et al., 2001; Oehlmann et al., 2006; Pettersson et al., 2006; Birceanu et al., 2015) including the bird embryo (Berg et al., 1998, 1999, 2001a, 2001b, 2004; Biau et al., 2007; Oshima et al., 2012).

In the end we want to demonstrate that the test method based on chicken embryos is a suitable system for the detection of EDCs. If the chicken embryo proves to be similarly sensitive or even more sensitive to EDCs than already established tests, the successful development of a standardized test system based on chicken embryos is very promising. As an alternative to current assays, this test system could then contribute to a reduction in the number of consumed experimental animals.

2. Materials and methods

2.1. Dosing

All experiments were carried out with respect for the principles of laboratory animal care, in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC) and

the German Animal Welfare Act. Fertilized eggs of white Leghorn chicken (*Gallus gallus domesticus*) were obtained from a local breeder (LSL Rhein-Main, Dieburg, Germany). The eggs were incubated at 37.5 °C ± 0.5 °C and 60% ± 10% relative humidity and turned over eight times a day in a fully automated incubator (J. Hemel Brutgeräte, Verl, Germany). For the baseline experiments all trials involve the formation of two control groups, the negative control (NC), which includes unmanipulated eggs and the solvent control (SC) which received DMSO (CAS: 67-68-5; purity = 99.5%; Applichem, Darmstadt, Germany) as a carrier. In these experiments, either 15 µL or 60 µL of the solvent were used. In ovo-exposure experiments investigating the effects of EDCs additionally include an EE_2-treated group (20 ng/g egg weight; CAS: 57-63-6; purity: ≥ 98%; Sigma Aldrich Chemie GmbH, München, Germany) and three BPA-treated groups (75, 150, 300 µg/g egg weight; CAS: 80-05-7; purity: ≥ 99%; Sigma Aldrich Chemie GmbH, München, Germany). In previous studies, these substance concentrations have proven to strongly affect the reproductive organs of chicken embryos (Berg et al., 2001b, 2004). The corresponding doses were administered to the eggs dissolved in 15 µL of the solvent.

Solvent and substances were injected into the yolk via a small hole on the circle of the widest diameter of the egg using Hamilton microliter syringes and needles (ga22s/51mm/pst2). The injection was performed on day one of incubation. After injection the hole in the shell was sealed with agarose gel (3%, in phosphate buffered saline). During incubation, eggs were periodically checked by candling to identify and discard dead embryos or unfertilized eggs.

2.2. Dissection, tissue preparation and evaluation

The embryos were dissected on day 19 of incubation, two days before anticipated hatching. Unfertilized eggs were excluded from statistics. All embryos were examined for deformations of body or inner organs. Ovaries and testes were examined for deformations under a stereo microscope. Photos of all gonads (Diskus, Carl H. Hilgers, Königswinter, Germany) were taken for further analyses of the gonad surface area, in which the entire visible surface of each single gonad was determined with an image editing program (Fiji is just ImageJ, Open Source). Gonads were dissected and fixed in Bouin's solution for 24 h. The fixative was rinsed repeatedly with 80% ethanol. Ethanol was removed by saccharose solution (10, 20 and 30% in phosphate buffered saline) before gonads were embedded in Tissue-Tek® (Sakura Finetek Europe B·V., Alphen aan den Rijn, Netherlands). Gonads were sectioned (6 µm) by a freeze microtome (Microm HM 500 O, Thermo Fisher Scientific Germany, Bonn, Germany) at −23 °C. Tissue sections were stained with hematoxylin and eosin. Histological examination was performed using a light microscope (Olympus BX50, Olympus, Tokyo, Japan) and a camera (JVC Digital Camera, KY-F75U, Yokohama, Japan). In both sexes the thickness of the cortex and in male embryos additionally the percentage of the area of seminiferous tubules were measured with an image analysis system (Diskus, Carl H. Hilgers, Königswinter, Germany).

2.3. Determination of sexual genotype

DNA isolation for each individual was carried out with a tissue sample from the heart taken during dissection. Dead embryos, identified and removed before dissection, were also sampled. All embryos were typed for their sexual ZZ or ZW genotype according to Fridolfsson and Ellegren (1999), based on PCR with a single set of primers. Amplification was performed using qPCR and the primers 2550F "5′-GTT ACT GAT TCG TCT ACG AGA-3′" and 2718R "5′-ATT GAA ATG ATC CAG TGC TTG-3′". Following amplification, all qPCR products underwent a melting curve, which resulted in characteristic bands for each sex. Male embryos had a single 600-bp CHD1-Z specific fragment with a melting temperature of nearly 84 °C, while females had a 600bp-CHD1-Z specific fragment and an additional 450-bp CHD1-W female-specific fragment, with a melting temperature of nearly 82 °C.

2.4. Measurements and statistics

For the examination of the surface area of left and right gonads the data from 15 (baseline experiments with controls) or 4 (experiments with BPA and EE_2) individual experiments were analyzed. For the histological endpoints cortex thickness of left gonads as well as the percentage of seminiferous tubules in left testis, the data from 11 (baseline experiments) or 4 (experiments with BPA and EE_2) experiments were analyzed. For the baseline experiments in particular, the range of variation within the control groups, as well as any deviations among the control groups was in the focus of investigation. For experiments with BPA and EE_2 it was examined whether and how the endpoints specified in the baseline experiments respond to the treatment with EDCs.

The cortex thicknesses of male and female left gonads as well as the percentage of the area of seminiferous tubules in male left testes were measured. 10 (baseline experiments) or 5 (experiments with BPA and EE_2) sections for each embryo and endpoint were evaluated. The selected sections were exclusively taken from the gonads middle sectional plane. To determine the cortex thickness different representative areas around the gonad were chosen. From each section five measurements were performed to determine the cortex thickness. For males the area of all seminiferous tubules in a defined image section were measured to determine a representative percentage in the male left testis. Therefore random representative image sections were selected which showed only the medullary tissue but not the cortex region.

For each endpoint of the baseline experiments the mean value of each of the 11 (cortex thickness and seminiferous tubules) or 15 (gonad surface areas) individual experiments was calculated. From the mean values of the individual experiments, the arithmetic means of NC and SC were calculated. Statistical evaluation was carried out with GraphPadPrism® (version 5.01, GraphPad Software Inc., San Diego, USA). Data of all endpoints following normal distribution were verified by *t*-test. If data did not follow normal distribution, Man-Whitney-U-test was used for analysis. Quantal data were evaluated using Fisher's exact test. In the baseline experiments we first determined within the respective control groups whether the 11 (cortex thickness and seminiferous tubules) or 15 (gonad surface areas) individual experiments statistically differ from each other (one-way ANOVA with Newman-Keuls post test). It was also determined within the respective control groups whether the individual experiments statistically differ from their arithmetic mean (one-way ANOVA with Dunnett's post test). Finally, a direct comparison of the individual experiments of NC and SC was made to analyze whether they differ from each other statistically (one-way ANOVA with Newman-Keuls post test).

For the test series with BPA and EE_2, the results of 4 test runs were merged and analyzed. Statistical evaluation was carried out according to Green and Wheeler (2013). When the untreated negative control did not differ statistically from the solvent-treated control (unpaired *t*-test; $p > .05$), these two groups were merged as a common control. When both controls differed statistically from each other (unpaired *t*-test; $p < .05$) the solvent control was used as the reference-control. For the endpoints gonadal cortex thickness and area of seminiferous tubules as well as for the endpoint gonad surface area data were normalized to the control. Data were analyzed using Fisher's exact test, one-way ANOVA with Dunnett's

Table 1
Embryonic mortality in untreated (NC) and solvent-treated (SC) controls.

		NC	SC
Σ experiments		15	15
Mortality (%) [a] [b]	0	53.3 (8)	20.0 (3)
	>0-10	6.67 (1)	0.00 (0)
	>10-20	40.0 (6)	33.3 (5)
	>20-30	0.00 (0)	33.3 (5)
	>30-40	0.00 (0)	0.00 (0)
	>40-50	0.00 (0)	6.67 (1)
	>50-60	0.00 (0)	6.67 (1)
Σ fertilized eggs		256	258
Sex-specific mortality (%) [c]	Σ males	46.9 (120)	43.4 (112)
	males vital	44.9 (115)	36.8 (95)
	males dead	1.95 (5)	6.59 (17)
	Σ females	50.0 (128)	47.6 (123)
	females vital	48.0 (123)	42.2 (109)
	females dead	1.95 (5)	5.43 (14)
	sex not verified [d]	3.13 (8)	8.91 (23)

[a] Experiments were classified according to the percentage of mortality in the individual experiments (e.g. ">0–10" means a mortality rate between zero and ten percent).
[b] The number in parentheses represents the number of experiments with a defined mortality rate.
[c] The number in parentheses represents the number of affected embryos.
[d] The phenotypic and genetic sex of these embryos could not be determined, because they died in an early developmental stage in which no reproductive organs were visible and no sufficient tissue sample were available for genetic sexing.

multiple comparison test (normal distribution of data) or Kruskal-Wallis test with Dunn's multiple comparison test (no normal distribution of data) with GraphPad Prism 5.01 (GraphPad Software Inc., San Diego, USA).

3. Results

3.1. Baseline experiments in control groups

3.1.1. Embryonic mortality

The fertility rate in the individual experiments was at least 89% in both control groups. In the untreated control a maximum of 16% of embryos per experiment died. SC showed a higher variability of mortality up to 54%, whilst the majority of the experiments were in a range up to 25% mortality (Table 1). Mortality was significantly different between the two control groups ($p < .001$).

In both controls, the genetic sex matched 100% with the phenotypic sex, in males as well as in females. The sex ratio was balanced within both test groups, showing values around 1:1 for the total number of male and female embryos (Table 1). There was no indication for a sex-related effect of the solvent on mortality. In both control groups an equivalent proportion of embryos of both sexes died: 2% in the untreated control and around 6% in the solvent-treated control.

There was no correlation between the injection volume used for the SC and the mortality. Experiments performed with an injection volume of 60 μL per egg showed no statistically significant increase in mortality compared with experiments performed with an injection volume of 15 μL per egg.

3.1.2. Malformations

Observations of the different kinds of malformations were made on the treated embryos dying during the incubation period or stopped on embryonic day 19. In general, different types of malformations were noted in the same embryo. In the untreated control 3 of 256 embryos (1.17%) were malformed with a total number of 4 malformations. These malformations were exclusively found to be celosomia (75.0%) or affected the limbs (left wing; 25.0%). In the solvent-treated control 15 of 258 embryos (5.81%) were malformed with a total number of 22 malformations. These malformations mainly affected the eyes (left/right anophthalmia; 27.3%) often in context with malformations of the beak (torsion of beak or atrophy of upper beak; 18.2%), celosomia (22.7%) and less often malformations of the limbs (legs/feet; 13.6%), edema and exencephalia (9.10%, respectively). Statistical analysis showed that the number of malformed embryos and the total number of individual malformations in the DMSO-treated control increased significantly compared to untreated embryos ($p < .01$ and $p < .001$, respectively). There was no difference in the rate of malformations between the different volumes of DMSO.

3.1.3. Morphological observation of the gonads – gonad surface area

The left and right gonads of male and female chicken embryos are positioned on the ventromedial surface of the respective mesonephros with the dorsal aorta between the gonads. In female embryos, the difference in size between left and right ovary was clearly evident on embryonic day 19. The left ovary was significantly larger and more differentiated than the right one. On average, the right ovary attained around 20% of the surface area of the left ovary in both control groups (Table 2).

In male embryos, the difference in size between left and right gonad was less prominent compared to females. Both testes were fully differentiated and had about the same size, although the left testis was slightly larger. On average, the right testis attained about 90% of the surface area of the left one for both control groups (Table 2).

Regarding the parameters surface area of left and right testis and ovary, little statistical deviations were detected when comparing the mean values in the control groups of the 15 individual experiments against each other or against the arithmetic means of the respective control group. This shows a high statistical contingency, i.e. a high portion of untreated or solvent-treated controls in individual experiments with no statistical difference compared to the other controls of the respective group or compared with the arithmetic mean value of the respective control group. Within the untreated and solvent-treated control group, at least 93% of the experiments showed no statistically significant differences from each other or from the arithmetic mean of the respective control group ($p > .05$) (Table 3). Between untreated and solvent-treated control group, at least 94% of the experiments showed no statistically significant differences from each other ($p > .05$) (Fig. 1 A, B and

Table 2
Gonad surface area, cortex thickness and percentage of seminiferous tubules of untreated (NC) and solvent-treated (SC) chicken embryos on embryonic day 19.

Sex	Group	Gonad surface area			Cortex thickness [μm]	Seminiferous tubules [%]
		left [mm^2]	right [mm^2]	right/left [%]		
Male	NC	4.32 ± 0.58	4.00 ± 0.56 [b]	92.5 ± 7.81 [a]	9.32 ± 1.43	30.6 ± 2.75
	SC	4.17 ± 0.70	3.74 ± 0.71 [b]	89.8 ± 9.50 [a]	9.81 ± 1.59	30.1 ± 3.02
Female	NC	10.8 ± 1.37 [c]	2.24 ± 0.50 [b]	20.8 ± 4.60	158 ± 24.2	–
	SC	9.70 ± 1.53 [c]	2.04 ± 0.50 [b]	21.1 ± 4.14	163 ± 24.5	–

Statistical analysis by unpaired *t*-test. Identical superscripted letters indicate a significant difference (a: $p < 0.05$; b: $p < 0.01$; c: $p < 0.001$) between NC and SC.

Table 3
Statistical consistency as the percentage of experiments with no statistically significant difference of the mean values of individual experiments within untreated (NC) and solvent-treated (SC) control groups compared with each other (all vs. all) or against the arithmetic mean of the respective control group (individual vs. arithmetic mean) or between NC and SC.

Sex	Endpoint	Consistency of individual experiments (%)				
		within NC		within SC		between NC and SC
		all vs. all [a]	individual vs. arithmetic mean [b]	all vs all [a]	individual vs. arithmetic mean [b]	all vs. all [c]
Female	Surface area of left ovary	100%	100%	94%	93%	94%
	Surface area of right ovary	100%	100%	100%	100%	100%
	Cortex thickness	98%	100%	93%	91%	99%
Male	Surface area of left testis	100%	100%	99%	93%	100%
	Surface area of right testis	97%	93%	100%	100%	99%
	Cortex thickness	78%	82%	100%	100%	93%
	Seminiferous tubules	98%	91%	100%	100%	99%

[a] Statistical analysis by one-way ANOVA with Newman-Keuls post test. Comparison of all mean values of individual experiments within the respective control group.
[b] Statistical analysis by one-way ANOVA with Dunnett's post test. Comparison of all mean values of individual experiments with the arithmetic mean of the pooled control group.
[c] Statistical analysis by one-way ANOVA with Newman-Keuls post test. Comparison of all mean values of individual experiments between untreated and solvent-treated control group.

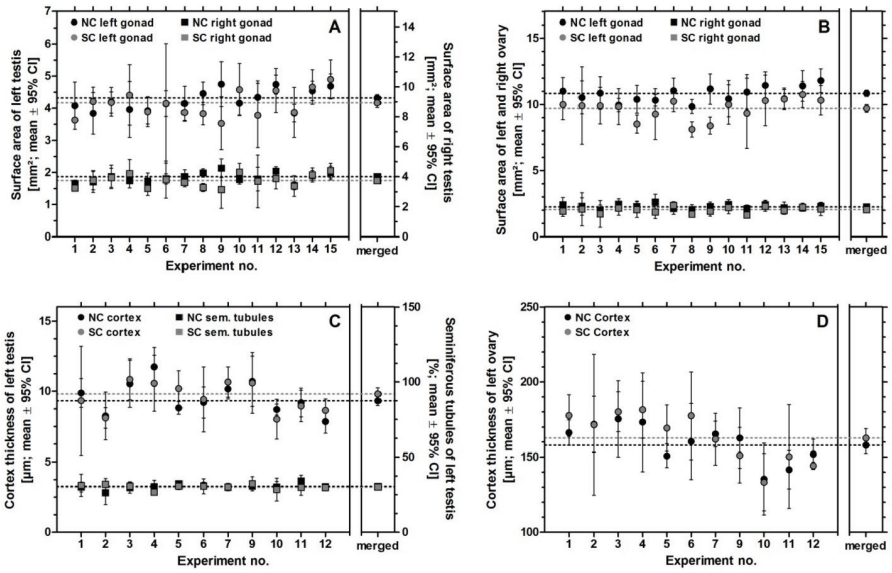

Fig. 1. Surface area of left and right testis (A) and ovary (B), cortex thickness and percentage of seminiferous tubules of left testis (C) and cortex thickness of left ovary (D) of embryos of the domestic fowl (*Gallus g. domesticus*) on embryonic day 19. Data points represent the mean ± 95% confidence interval (95% CI) of untreated negative control (NC) and solvent-treated control (SC) in each single experiment; dashed lines and data points "merged" represent the arithmetic mean of all NC and SC data from the 15 (A, B) or 11 (C, D) experiments. Experiment no. 8 (C, D) was not considered, since the gonadal tissues were used for other examinations.

Table 3).

Overall, the left and right gonads were slightly smaller in the solvent-treated control compared to the untreated control, for males as well as for females. In a predominant proportion of the experiments (left and right ovary: 100% and 87%, respectively; left and right testis: 53% and 67%, respectively) the means of the individual experiments of the untreated control were higher than those of the solvent-treated control. This effect was found to be more pronounced in female gonads.

Comparing the gonad surface area of male and female gonads in the solvent-treated group, experiments that were performed with an injection volume of 60 μL per egg showed a statistically significant difference compared with experiments that were performed with an injection volume of 15 μL per egg for the area of the left

ovary ($p < .001$) and the area of the right testicle ($p < .05$). The surface area of these gonads decreased with increasing volume of the solvent.

3.1.4. Histological observation of the gonads – left testis and ovary

On day 19 of incubation the female left ovary showed a well-differentiated medulla and cortex, while the right ovary had only medulla tissue with an outer thin layer of flattened cells, comparable to the cortex of male testicles. The medulla of the left ovary was loosely arranged and crossed by lacunar channels. The left and right male testes were nearly identically sized, mirror-inverted formed and characterized by a thin cortex layer of 2–3 cells and interstitial space and seminiferous tubules in the medulla, representing the location of spermatogenesis in postnatal developmental stages.

As already shown for the endpoint gonad surface area in section 3.1.3, also the parameters cortex thickness of left testis and ovary and percentage of seminiferous tubules in the left testis exhibited a high statistical consistency when comparing the mean values of the 11 individual experiments against each other or against the arithmetic means of the respective control groups. Within untreated and solvent-treated control group, at least 78% of the experiments showed no statistically significant differences from each other or from the arithmetic means of the respective control group ($p > .05$) (Table 3). Between untreated and solvent-treated control groups, 93–99% of the experiments showed no statistically significant differences from each other ($p > .05$) (Fig. 1C, D and Table 3).

Comparing the histological endpoints in gonads of male and female embryos, experiments that were performed with a solvent volume of 60 μL showed no statistically significant increase compared with experiments that were performed with 15 μL of the solvent.

3.2. Effects of in ovo exposure to BPA and EE_2

3.2.1. Embryonic mortality

In the experiments investigating the effects of BPA and EE_2 the fertility rate of the individual treatment groups was ≥87%. The untreated control showed the lowest mortality of nearly 6%, the solvent control a mortality rate of nearly 20% (Table 4). With a decreasing concentration of BPA a rising mortality was observed, which was between 17% and 30%. Only in the test group receiving 75 μg BPA/g egg weight, which showed the highest mortality rate with 30%, the deviation from the solvent control was statistically significant ($p < .01$). The mortality in the test group receiving EE_2 was 23.4%. There were no statistical differences between the individual experiments of the respective treatment groups. There was also no indication that one of the sexes was more affected by substances treatment than the other.

The genetic sex ratio was balanced within all test groups, showing a proportion of males between 37% and 59%. In both controls, as well as in treatment groups receiving different concentrations of BPA, the genetic sex matched 100% with the phenotypic sex. The group receiving 20 ng EE_2/g egg showed a significantly increased number of embryos, which were determined as phenotypic females but identified as genetic males. Almost 90% of the genetic males (17 of 19 vital males) were identified as intersex-males with ovotestes. The right testes of the affected males were noticeably smaller than those of control males while left testes visibly changed in shape and structure.

3.2.2. Malformations

Different kinds of malformations were observed for all treated embryos dying during the incubation period or stopped on embryonic day 19. While no malformations could be found in the control groups, in the EE_2-treated group 2 of 47 embryos (4.26%) showed malformations which were found to be celosomia or exencephalus (50.0%, respectively). Taking all groups receiving different concentrations of BPA together, 8 of 143 embryos (5.59%; 75 μg BPA/g egg: 4 of 50 embryos (8.00%); 150 μg BPA/g egg: 2 of 46 embryos (4.35%); 300 μg BPA/g egg: 2 of 47 embryos (4.26%)) showed malformations which were found to be celosomia (50.0%), exencephalus (12.5%) or in general deformations of body or head (37.5%). Compared to the control, only the test group receiving 75 μg BPA/g egg weight showed an increase in the incidence of malformations ($p < .01$).

3.2.3. Morphological observations of the gonads - gonad surface area

The gonad surface area (Fig. 2 A Table 5) was especially influenced by EE_2, which caused a significant reduction of female right ovaries by about 26% and of male right testes by about 55%. Female left gonad surface areas significantly increased when treated with 75 μg BPA/g egg. Left and right gonad surface areas of males and right gonad surface areas of females of all groups receiving BPA did not differ statistically from the control. In females, the right ovary attained around 20% of the surface area of the left ovary with marginal differences between controls and treatment groups receiving EE_2 or BPA as well as between the different treatment groups. In males, the right testis of controls and treatment groups receiving different concentrations of BPA attained around 90% of the surface area of the left. Since the gonad surface area of the right testis was significantly decreased when treated with EE_2, the ratio of right to left testis was significantly reduced to 42% ($p < .001$). The gonad surface area of right testes of EE_2-treated males was similar to right ovaries in control groups. Although the surface area of left testes was only marginally changed by EE_2 affected testes showed a female-like shape and a well visible female-typical thickened

Table 4
Embryonic mortality after in ovo exposure to EE_2 (20 ng/g egg) and bisphenol A (75, 150 or 300 μg/g egg).

	NC	SC	EE_2	BPA 75	BPA 150	BPA 300
Σ fertilized eggs	66	41	47	50	46	47
Σ males	45.5% (30)	48.8% (20)	48.9% (23)	54.0% (27)	37.0% (17)	48.9% (23)
males vital	42.4% (28)	43.9% (18)	40.4% (19)	44.0% (22)	34.8% (16)	38.3% (18)
males dead	3.03% (2)	4.88% (2)	8.51% (4)	10.0% (5)	2.17% (1)	10.6% (5)
Σ females	54.5% (36)	51.2% (21)	46.8% (22)	42.0% (21)	58.7% (27)	51.1% (24)
females vital	51.5% (34)	36.6% (15)	36.2% (17)	26.0% (13)	43.5% (20)	44.7% (21)
females dead	1.52% (1)	14.6% (6)	10.6% (5)	16.0% (8)	15.2% (7)	6.38% (3)
sex not verified [a]	1.52% (1)	0.00% (0)	4.26% (2)	4.00% (2)	4.35% (2)	0.00% (0)

[a] The phenotypic and genetic sex of these embryos could not be determined, because they died in an early developmental stage in which no reproductive organs were visible and no sufficient tissue sample were available for genetic sexing.

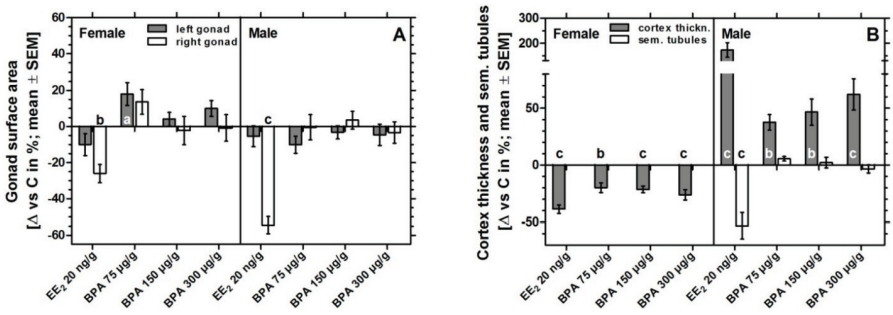

Fig. 2. Effects of in ovo exposure to bisphenol A (BPA) and 17α-ethinylestradiol (EE_2) on left and right gonad surface area (A) and cortex thickness and percentage of seminiferous tubules (B) of embryos of the domestic fowl (*Gallus g. domesticus*) on embryonic day 19. Statistical analysis by one-way ANOVA with Dunnett's multiple comparison test (B) or Kruskal-Wallis test with Dunn's post test (A, B). Lowercase indicate significant differences compared to control. Level of significance: a: $p < .05$; b: $p < .01$; c: $p < .001$.

Table 5
Gonad surface area, cortex thickness and percentage of seminiferous tubules of chicken embryos after in ovo exposure to EE_2 (20 ng/g egg) and bisphenol A (75, 150 or 300 µg/g egg).

Sex	Group	Gonad surface area			Cortex thickness [µm]	Seminiferous tubules [%]
		left [mm^2]	right [mm^2]	right/left [%]		
Male	NC	4.61 ± 0.85	4.03 ± 0.68	87.9 ± 6.62	11.9 ± 2.18	27.9 ± 3.42
	SC	4.48 ± 0.86	4.17 ± 0.81	91.7 ± 8.74	11.0 ± 2.40	26.8 ± 4.62
	C [#]	4.56 ± 0.85	4.09 ± 0.73	89.4 ± 7.62	11.6 ± 2.26	27.4 ± 3.92
	EE_2	4.31 ± 1.11	1.86 ± 0.83 [c]	42.0 ± 19.3 [c]	31.6 ± 11.1 [c]	12.8 ± 10.0 [c]
	BPA 75	4.10 ± 0.95	4.07 ± 1.32	94.0 ± 8.68	15.9 ± 3.07 [b]	28.9 ± 2.13
	BPA 150	4.41 ± 0.66	4.23 ± 0.80	95.9 ± 10.7	17.0 ± 4.44 [b]	28.0 ± 3.83
	BPA 300	4.35 ± 1.19	3.95 ± 1.09	89.5 ± 6.83	18.8 ± 5.03 [c]	26.5 ± 2.69
Female	NC	10.6 ± 1.38 [b]	2.36 ± 0.65	22.6 ± 6.16	158 ± 40.1	–
	SC	8.83 ± 1.80	2.02 ± 0.62	21.7 ± 3.71	154 ± 20.6	–
	C [#]	–	2.25 ± 0.65	22.3 ± 5.49	157 ± 35.7	–
	EE_2	7.94 ± 2.29	1.67 ± 0.47 [b]	22.3 ± 8.90	95.8 ± 26.1 [c]	–
	BPA 75	10.4 ± 2.14 [a]	2.56 ± 0.60	24.9 ± 4.36	125 ± 22.2 [b]	–
	BPA 150	9.19 ± 1.50	2.20 ± 0.79	23.6 ± 6.73	123 ± 18.1 [c]	–
	BPA 300	9.71 ± 1.73	2.23 ± 0.74	22.2 ± 5.59	116 ± 27.8 [c]	–

[#] If NC and SC were not statistically different (unpaired *t*-test, $p > .05$), they were pooled to a merged control C and tested against the treatment-groups. If NC and SC were statistically different (unpaired *t*-test, $p < .05$), C was not calculated and treatment-groups were tested against SC.
Statistical evaluation with One-way ANOVA and Dunnett's post test (female cortex thickness, male seminiferous tubules) or Kruskal-Wallis test with Dunn's post test (male cortex thickness, male and female gonad surface areas). Identical superscripted letters indicate asignificant difference (a: $p < 0.05$; b: $p < 0.01$; c: $p < 0.001$) compared to the pooled control C or the solvent control SC.

translucent cortex region when viewed under a stereomicroscope.

3.2.4. Histological observations of the gonads – left testis and ovary

All concentrations of BPA as well as the single concentration of EE_2 resulted in a significant reduction of the cortex thickness of female left ovaries ($p < .01$ and $p < .001$; Fig. 2 B, Table 5). EE_2 differed by nearly 39% and BPA concentrations by up to about 26% from the control. Estrogen-mediated effects in females were found to be concentration-independent as differences between EE_2 and BPA or between the different concentrations of BPA were marginal. In contrast to females, the cortex thickness of male left gonads was significantly increased by the administration of BPA and EE_2. EE_2-related effects were markedly stronger than those of BPA. Compared to the control the male cortex thickness was increased by up to 62% in BPA-treated groups and by 173% in the EE_2-treated group. The BPA-related increase of male cortex thickness was found to be concentration dependent. In the affected cortices of both treatment groups, BPA and EE_2, dividing cells were found which resembled the female oogonia. Since the study of these cells was very complex due to the relatively narrow cortex thickness of male embryos this observation was not quantified. The percentage of seminiferous tubules in male left gonads, however, was affected by EE_2 alone. Compared to the control, EE_2-treatment resulted in a significant drop by about 53%. BPA concentrations differed marginally ($p > .05$) by up to 5.5%.

4. Discussion

The aim of our project is the further development of a standardized test protocol for the assessment of the effects of EDCs in chicken embryos. Therefore, the focus of the present study was on the systematic investigation of the variability of individual parameters in untreated and solvent-treated control groups to provide therefore an important basis for a further validation of the test. In a large number of experiments ($n = 15$), carried out over a period of 3 years, the natural variability of the test system was determined. By merging the data of the 15 experiments we also compared for differences inside and between untreated and solvent-treated control groups. We determined normal mortality rates and gonadal parameters of 19-days-old embryos to establish validity criteria for future standardized test series. In a second step, we investigated the effects of two model estrogens, BPA and EE_2, on developmental and gonadal endpoints to provide robust values for all parameters investigated and to show good reproducibility of the method. This is to demonstrate that the test method based on the chicken embryo is a suitable system for the detection of EDCs.

4.1. Embryonic mortality

It has been shown that the mortality of embryos from unmanipulated control eggs in the 15 baseline experiments was up to a

maximum of 16%. Based on the studies of Romanoff and Romanoff (1972), expected mortality in untreated embryos is about 20%. Comparable low mortality rates were also confirmed in other studies (Wyatt and Howarth, 1976; DeWitt et al., 2005a, 2005b). In comparison, mortality of solvent-treated embryos was below 25% in a predominant proportion of the 15 experiments while in two experiments more than 30% of embryos died, suggesting that DMSO induces an increased mortality compared to the untreated control. Our data indicate that a mortality of 30% in the solvent group as well as in the untreated group should be considered as validity criterion for a future test design.

In studies on avian development different carriers have been used, such as organic solvents, vegetable oil, just water or an emulsion. Each carrier with its special characteristics has its advantages and disadvantages. DMSO is used in a variety of experiments because of its solubility properties and good miscibility with other solvents. In toxicological studies and pharmacological screenings it is used to enhance the solubility of hydrophobic chemicals (Castro et al., 1995) but it may increase membrane permeability and sustance uptake (Notman et al., 2006). Although a solvent such as DMSO is often inevitable care should be taken as an intrinsic toxicity of DMSO has been reported in chicken (Caujolle et al., 1967; Carew and Foss, 1972; Landauer and Salam, 1972; Morgan, 1974; Wyatt and Howarth, 1976) and various other organisms (Anderson et al., 2004; Chen et al., 2011; Galvao et al., 2014; Stevens et al., 2015). It has been shown that an increasing dose of the solvent is associated with an increase in mortality. Different studies indicate that higher injection volumes in general have harmful effects on developing chicken embryos, irrespective of the solvent used (Landauer and Salam, 1972; Morgan, 1974; Wyatt and Howarth, 1976; DeWitt et al., 2005b). However, it must be mentioned that also the day of application can affect embryonic mortality. Regarding the two volumes of DMSO (15 μL, 60 μL) used in the present study, there was no difference between the mortality in the individual developmental stages of the embryos. This suggests that even a higher volume of up to 60 μL DMSO administered into the egg yolk of one day old embryos does not cause an increase in mortality compared to the lower volume of 15 μL. However, based on our own data and the results of other studies mentioned above, the volume of the solvent used should not exceed 60 μL. It is rather useful to keep the volume as low as possible to minimize toxic effects of the solvent. Nevertheless, the volume of the solvent is generally dependent on the chemicals to be dissolved therein and the concentration of the chemical.

In ovo-treatment of chicken embryos with BPA and EE_2 led to a slight increase in mortality as expected. Nevertheless, mortality rates of controls and treatment groups were below 30%, which is in a reasonable range since low mortality rates or, conversely, high survival rates result in a sufficient number of vital embryos for follow-up examinations of gonadal tissues. In the 4 individual experiments the mortality rates of the untreated control were below 14% and the mortality rates of the solvent control below 27% which is in the same range as baseline experiments. According to the proposed validity criteria, as derived from the baseline experiments, all 4 experiments investigating the effects of EE_2 and BPA can be considered as valid.

The natural sex ratio of *Gallus g. domesticus* varies around 50%. The genetic sex ratio in the baseline experiments was balanced within both control groups, in vital embryos as well as in dead embryos. The genetic sex agreed 100% with the phenotypic sex for both control groups. There was no indication that one of the sexes was more affected by the solvent than the other.

In ovo-treatment to 20 ng EE_2/g egg, however, resulted in a large number of embryos that were phenotypic females but found to be genetic males. The size of the right testis of the affected males was noticeably decreased with the structural appearance of a right ovary. The left testis, although not changed in size, showed shape and structure of a left ovary. This indicates that already the visual evaluation of the gonads, together with the information about the genetic sex, can indicate possible influences of EDCs on sex differentiation in chicken embryos. From this it can be concluded that a more detailed examination of the gonads is potentially suitable to detect possible effects of EDCs.

4.2. Malformations

Baseline experiments with control groups show a malformation rate of 1.2% in unmanipulated embryos and 5.8% in solvent-treated embryos. Compared to the unmanipulated group the significant increase in the solvent-treated group indicates a slight teratogenic activity of DMSO. This finding is in line with the results of Dresser et al. (1992) who assessed the teratogenic activity of four solvents in the Frog Embryo Teratogenesis Assay with *Xenopus laevis* (FETAX) and reported DMSO to be the least toxic and teratogenic solvent examined. Caujolle et al. (1967) noted that spontaneous malformations may exist in the chicken embryo with an incidence of about 2% what coincides with the data shown here for the untreated control. However, Alsop (1919) and Byerly (1930) showed malformation rates around 6%. Although the number of malformed embryos in the solvent-treated control increased compared to the untreated control, the SC is in the same range as in the studies mentioned above.

Malformations in untreated embryos were exclusively found to be celosomia or malformations of the limbs. DMSO-treated embryos showed malformations which mainly affected the eyes (left/right anophthalmia) often in context with malformations of the beak (torsion of the beak or atrophy of the upper beak), celosomia and less often malformations of the limbs (legs/feet), edema or exencephalia. Byerly (1930) found different types of malformations in unmanipulated eggs of the white leghorn breed, e.g. terata of the eyes (mono-/microphthalmia) or the brain (exencephalia, hyperencephalia). The study of Caujolle et al. (1967) showed that the nature of spontaneous malformations in chicken embryos exhibits a common pattern: anophthalmia, crossed beak with or without anencephalia and celosomia but never malformations of the limbs. Furthermore, they found that a 50% solution of DMSO in 0.9% physiological saline caused malformations at doses approaching the LD_{50} (10.3 mg/embryo at E3 or 12.2 mg/embryo at E4). The most typical DMSO-induced malformations of embryos treated at E3 were left anophthalmia and left torsion of the beak with reduction of the upper beak and only to very small extent malformations of the limbs. DMSO-treatment at E4 produced almost 26% malformed embryos and generally caused lesions of the limbs and in lower percentages malformations of the beak and the eyes, anurous embryos and celosomia. In the baseline experiments of the present study the malformations found in the solvent-treated group support the findings of Caujolle et al. (1967) although our percentage of malformed embryos is significantly lower. There was no difference in the rate of malformation between the DMSO-treated groups of different volumes suggesting that also a dosage of 60 μL can be used without increasing the number of malformed embryos. A malformation rate of almost 6% appears to be acceptable, as there is a sufficient number of embryos remaining for subsequent histological examinations.

Analyzing the frequency of malformations in the 4 experiments investigating the effects of EDCs, malformations are found neither in the untreated control nor in the solvent-treated control while malformation rates in the BPA- and EE_2-treated groups are around 5%. The significant increase in the malformation rate of the substance-treated group receiving 75 μg BPA/g egg compared to the

control has to be assumed as concentration-independent as higher concentrations of the estrogen show marginal differences. However, all values are still in the range of the reported spontaneous malformation rate in chicken embryos of about 2% (Caujolle et al., 1967) to around 6% (Alsop, 1919; Byerly, 1930). Also, the malformation rates of around 5% in the estrogen-treated groups are in the same range as the malformation rates of the solvent control in our baseline experiments. In this context, the incidence of malformations in the substance-treated groups can be considered as inconspicuous. However, almost all of the BPA- or EE_2-treated groups show a marginally increased incidence of celosomia, compared to the control. In addition, exencephalia are exclusively found in substance-treated groups, while the formation of edema is completely absent there. Various malformations have already been described, among them, for example, terata of the eyes, the beak, the brain or the formation of celosomia (Byerly, 1930; Caujolle et al., 1967). These terata largely coincide with the malformations found in the solvent- and substance-treated groups. Although the pattern of malformations from solvent-treated control to substance-treated groups is slightly shifted, there is no statistical evidence that treatment with BPA or EE_2 specifically favors particular terata or generally results in increased malformation rates. It can be concluded that in ovo-exposure to both estrogenic substances does not increase the rate of malformations.

4.3. Morphological observations of the gonads - gonad surface area

In the baseline experiments the treatment with DMSO results in reduced gonad surface areas in both sexes. This effect is more distinct in female than in male gonads, as untreated control and solvent control differ significantly from each other. The size ratio of right to left gonad, however, remains unaffected for female embryos. The solvent seems to affect the growth of the female gonads in some way. For male embryos treated with DMSO there is only a slight tendency of smaller left and right gonad surface areas and the ratio of left to right gonad differs marginally between untreated control and solvent control. The reason may be a growth-inhibiting effect caused by the low basic toxicity of the solvent. An alternative explanation is a possible endocrine-mediated effect of the solvent. This is supported by the fact that the effect on the surface area of the gonads is sex-specific with female embryos being more affected while the change in male embryos is marginal. The hypothesis of a solvent-induced influence on the endocrine system of organisms is supported by various studies. In their review about the effects of different OECD-recommended carrier solvents, Hutchinson et al. (2006) describe an influence of DMSO on the reproduction of different fish species and an impact on biomarkers of endocrine disruption. The results of Pawlowski et al. (2004a, 2004b) show that DMSO did not affect different reproduction-related endpoints such as spawning and biomarker response of the fathead minnow, but a distinct reduction in the mean egg production. Further studies demonstrate an inhibition of various cytochrome P450 enzymes by DMSO (Chauret et al., 1998; Hickman et al., 1998; Busby et al., 1999; Easterbrook et al., 2001). It is concluded that since these enzymes are involved in the metabolism of endogenous substances such as steroid hormones interactions with solvents can result in a change in circulating hormone concentrations with subsequent effects on reproductive functions. This probable DMSO effect cannot be excluded for the chicken embryo used in our experiments.

Furthermore, data show that the larger the volume of the solvent used, the smaller the gonad surface areas, which again leads to the point that the solvent volume should be kept as low as possible. Similar studies measuring reproductive endpoints or endocrine disrupter biomarker responses also propose a maximum solvent concentration for the testing of aquatic organisms (Hutchinson et al., 2006).

As a consequence of this, in the testing of chemical substances, a comparison should always be drawn between untreated embryos and solvent-treated embryos in order not to over- or underestimate any possible influence of the solvent. The arithmetic means of the gonad surface area of male and female embryos found in the baseline experiments can serve as key values for future tests.

The phenotypic sex of birds can be determined by screening the sex organs. Along with the information about the genetic sex the endpoint gonad surface area indicates whether a test substance influences the differentiation of the sexes. In ovo-exposure of male and female chicken embryos to EE_2 causes a significant decrease in the surface area of right testis and ovary. This effect is more pronounced in males and although the left testis does not change significantly in size, it shows a completely different appearance as it develops into an ovotestis with a translucent cortex as is typical for the left ovary. On the contrary, BPA had marginal effects on the gonad surface area of male and female chicken embryos. Only the concentration of 75 μg BPA/g egg resulted in an increase of the female left gonad surface area, which was found to be concentration-independent. For chicken and quail it is known that substances with endocrine potential can induce morphological changes in the sex organs (Scheib, 1983; Berg et al., 1998, 1999, 2001a, 2001b; Matsushita et al., 2006; Razia et al., 2006). The knowledge about the change in the gonads in shape, size and structure leads to the conclusion that gonad-related endpoints are useful for the testing of potential endocrine active substances. Although BPA and EE_2 are both estrogens, their activity differs considerably (Metcalfe et al., 2001; Oehlmann et al., 2006). However, it was in our expectation that in the chicken egg test the observed effect profile is substance-specific as shown here. Nevertheless, we have successfully shown the suitability of the endpoint gonad surface area for the detection of endocrine potentials as already the low dosage of 20 ng EE_2/g egg causes a significant change in shape and size of male testes. As a next step it should be investigated whether further classes of EDCs such as androgens, anti-androgens and anti-estrogens are able to cause comparable effects in gonads of the chicken embryo regarding the focused endpoints. All in all the endpoint gonad surface area could help facilitate the screening of potential EDCs as possible effects can be detected easily. However, subsequent histopathological analysis of the gonads will then give a more detailed description of the effects of possible EDCs on sex organ development of chicken embryos.

4.4. Histological observation of the gonads – left testis and ovary

Gonad tissue-related endpoints appear to be useful in the investigation of endocrine compounds, because they specifically influence the differentiation of the sexes. Since this represents a system in which the chemical is in direct contact with the embryo throughout development, it is likely that any toxic or teratogenic effect will be readily observed. However, there is always the possibility that the chicken will not be a species susceptible to a particular compound, just as it has been shown that other commonly used species of animals do not respond to all chemicals in a similar manner. It is also possible for the chicken to be more sensitive to a chemical than other species. Finally, this technique may be applied also to the study of (over-)additive effects of chemicals. Through the detailed investigation of the untreated and solvent-treated controls, a reliable statement about substance-related deviations from these can be made.

In the baseline experiments there was a marginal tendency of an enhanced cortex thickness in the SC compared to the NC, for males as well as for females. Analogous to the endpoint gonad surface area, females tended to be more affected than males. The

percentage of seminiferous tubules was not affected by the solvent. For both sexes only a small percentage of the means of the individual experiments within a test group as well as between the individual experiments between NC and SC differed significantly from each other. It can be assumed that this deviation is within natural fluctuation and indicates a good reproducibility for the method used. Therefore, the arithmetic means of the endpoints cortex thickness and percentage of seminiferous tubules are proposed as key values for follow-up tests.

The role of natural hormones in gonadal differentiation of birds is still partly unknown. Their possible function may become clearer when the hormone level is artificially influenced, for example by in ovo application of EDCs that potentially interact with steroidogenic enzymes. It is known that synthetic hormones may induce irreversible malformations of the gonads in birds during embryonic development or can alter gender-related behavior later in life, whereas other, less potent EDCs may exert less severe and often reversible effects in the less sensitive adult stage (Adkins-Regan, 1990; Ottinger and Abdelnabi, 1997). Therefore, the chicken embryo appears as a suitable model for the study of early sexual development and the potential impact of EDCs.

Depending on their hormonal system, the response of male and female chicken embryos to certain substances or substance classes can be very different. In birds, the genetic male is homozygous (ZZ), while the genetic female is heterozygous (ZW). Initially, all embryos have the same basic sex, regardless of their genetic gender: they are designed as males. The differentiation in one of the sexes during embryonic development depends on the level of circulating steroid hormones. Without external influence the undifferentiated gonads of genetic males develop into testes. In genetic females the synthesis of P450 aromatase finally results in the production of estrogens (Kagami and Hanada, 1997) which is substantial for the formation of female sex organs.

The results of the experiments assessing the effects of in ovo exposure to BPA and EE_2 show that both estrogens cause a significant reduction of the female cortex thickness. In males both substances cause a significantly thickened cortex with oocyte-like cells and a female-like structure, which coincides with previously published studies (Berg et al., 1998, 1999, 2001a, 2001b). While BPA does not affect the percentage of seminiferous tubules in male gonads, testicular tissue of EE_2-treated embryos appears significantly altered with a visibly lower number and degree of differentiation of seminiferous tubules and female-typical structures as lacunae. Since there are marginal differences between untreated and solvent-treated control in the baseline experiments as well as in substance experiments, it can be concluded that the differentiation of cortex and seminiferous tubules is unaffected by the solvent in both sexes. Therefore, it can be assumed that the shown effects are substance-specific and do not originate from the solvent.

Comparing both estrogenic substances, EE_2-caused effects are much more pronounced than those of BPA indicating the higher estrogenic potency of EE_2. This coincides with the studies of Metcalfe et al. (2001) which rates EE_2 to be much more potent than BPA in the *yeast estrogen screen* and in experiments with Japanese medaka (*Oryzias latipes*). Various studies on domestic fowl (*Gallus g. domesticus*) and Japanese quail (*Coturnix japonica*) demonstrate the effective feminization of male embryos when treated with estrogens or estrogen-active EDCs (Romanoff, 1960; Scheib and Reyssbrion, 1979; Wolff, 1979; Samsel et al., 1982; Sotonyi and Csaba, 1986; Etches and Kagami, 1997; Berg et al., 1998, 1999, 2001a, 2001b; Shibuya et al., 2004). While the left testis is formed into an ovotestis or ovary, the differentiation of the right testis is largely inhibited, resembling a right ovary. In contrast, the treatment of female embryos with estrogens showed fewer effects on gonad differentiation. Since the key enzyme P450arom is not synthesized in male gonads (Ayers et al., 2013; Scheider et al., 2014), constitutional estrogen concentrations in testes are very low (Woods and Erton, 1978; Tanabe et al., 1979, 1983) and not sufficient to cause an effect. Though, for a short time during embryonic development the estrogen receptor is detectable in male gonads, which makes them basically vulnerable to estrogens (Gasc, 1980; Smith et al., 1997; Nakabayashi et al., 1998). The artificial presence of estrogen at this critical time point therefore causes the differentiation towards the phenotypically female sex. Therefore, male gonads are basically able to develop ovarian tissue, whereby the absence of estrogen is essential for the testes formation. Since the estrogen level in females is continuously high, the administration of additional estrogen such as EDCs does not affect gonadal differentiation as strongly as in males.

Our as well as other investigations (Berg et al., 1998, 1999, 2001a, 2001b) have shown that the gonad-related endpoints cortex thickness and percentage of seminiferous tubules reliably respond to the treatment with the model estrogens BPA and EE_2. Also we provide reference values for future experiments. As mentioned above the next step of our investigations will focus on whether these endpoints can display the effects of other classes of EDCs as androgens, anti-androgens and anti-estrogens.

5. Overall conclusions

The present study is part of a project aiming to improve a replacement method for testing hormonally active compounds in birds, where fertilized eggs of the domestic fowl (*Gallus gallus domesticus*) are used. In baseline experiments we focused on the investigation of untreated and solvent-treated control groups to study normal development of the embryos without exposure to EDCs. We examined developmental and gonadal endpoints and determined reliable reference values for each endpoint which can serve as validity criteria in future experiments. Both controls were easily reproducible with low variability within as well as between both control groups. Since solvent-related effects were low we recommend DMSO as solvent for subsequent experiments. In further experiments investigating the effects of two estrogenic EDCs, BPA and EE_2, we provided robust reference values for all endpoints which are suggested to serve as positive control values in future experiments. Overall, the chicken embryo has proven to be a suitable and reliable test system for the investigation of the effects on toxicology and reproductive tissues of various chemical substances. Based on these results the study of the effects of further substance classes as androgens, anti-androgens and anti-estrogens on sexual differentiation of the chicken embryo is very promising.

6. Declarations

Conflict of interest

None.

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References

Adkins-Regan, E., 1990. Hormonal Basis of Sexual Differentiation in Birds. Hormones, Brain and Behavior in Vertebrates, vol. 8. Karger, Basel, pp. 1–14.

Alsop, F.M., 1919. The effect of abnormal temperatures upon the developing nervous system in the chick embryos. Anat. Rec. 15 (6), 306–331.

Anderson, G.L., Cole, R.D., Williams, P.L., 2004. Assessing behavioral toxicity with Caenorhabditis elegans. Environ. Toxicol. Chem. 23 (5), 1235–1240.

Ayers, K.L., Sinclair, A.H., Smith, C.A., 2013. The molecular genetics of ovarian differentiation in the avian model. Sexual Development 7 (1–3), 80–94.

Berg, C., Halldin, K., Brunstrom, B., Brandt, I., 1998. Methods for studying xenoestrogenic effects in birds. Toxicol. Lett. 103, 671–676.

Berg, C., Halldin, K., Fridolfsson, A.K., Brandt, I., Brunstrom, B., 1999. The avian egg as a test system for endocrine disrupters: effects of diethylstilbestrol and ethynylestradiol on sex organ development. Sci. Total Environ. 233 (1–3), 57–66.

Berg, C., Holm, L., Brandt, I., Brunstrom, B., 2001a. Anatomical and histological changes in the oviducts of Japanese quail, *Coturnix japonica*, after embryonic exposure to ethynyloestradiol. Reproduction 121 (1), 155–165.

Berg, C., Halldin, K., Brunstrom, B., 2001b. Effects of bisphenol A and tetrabromobisphenol A on sex organ development in quail and chicken embryos. Environ. Toxicol. Chem. 20 (12), 2836–2840.

Berg, C., Blomqvist, A., Holm, L., Brandt, I., Brunstrom, B., Ridderstrale, Y., 2004. Embryonic exposure to oestrogen causes eggshell thinning and altered shell gland carbonic anhydrase expression in the domestic hen. Reproduction 128 (4), 455–461.

Berge, J.A., Brevik, E.M., Bjorge, A., Folsvik, N., Gabrielsen, G.W., Wolkers, H., 2004. Organotins in marine mammals and seabirds from Norwegian territory. J. Environ. Monit. 6 (2), 108–112.

Biau, S., Bayle, S., Barbara, P.D., Roig, B., 2007. The chick embryo: an animal model for detection of the effects of hormonal compounds. Anal. Bioanal. Chem. 387 (4), 1397–1403.

Birceanu, O., Servos, M.R., Vijayan, M.M., 2015. Bisphenol A accumulation in eggs disrupts the endocrine regulation of growth in rainbow trout larvae. Aquat. Toxicol. 161, 51–60.

BMELV, 2015. Tables of German Animal Experiments in 2013. In: http://www.bmel.de/SharedDocs/Downloads/Tier/Tierschutz/Versuchstierdaten2015.pdf?__blob=publicationFile.

Brunstrom, B., Halldin, K., 2000. Ecotoxicological risk assessment of environmental pollutants in the Arctic. Toxicol. Lett. 112–113, 111–118.

Busby, W.F., Ackermann, J.M., Crespi, C.L., 1999. Effect of methanol, ethanol, dimethyl sulfoxide, and acetonitrile on in vitro activities of cDNA-expressed human cytochromes P-450. Drug Metabol. Dispos. 27 (2), 246–249.

Byerly, T.C., 1930. The effects of breed on the growth of the chick embryo. J. Morphol. 50 (2), 341–359.

Carere, C., Balthazart, J., 2007. Sexual versus individual differentiation: the controversial role of avian maternal hormones. Trends Endocrinol. Metabol. 18 (2), 73–80.

Carew, L.B., Foss, D.C., 1972. Tolerance of chicks for dimethyl sulfoxide. Poultry Sci. 51 (1), 206–211.

Castro, C.A., Hogan, J.B., Benson, K.A., Shehata, C.W., Landauer, M.R., 1995. Behavioral-effects of vehicles - DMSO, ethanol, TWEEN-20, TWEEN-80, and EMULPHOR-620. Pharmacol. Biochem. Behav. 50 (4), 521–526.

Caujolle, F.M., Caujolle, D.H., Cros, S.B., Calvet, M.M.J., 1967. Limits of toxic and teratogenic tolerance of dimethyl sulfoxide. Ann. N. Y. Acad. Sci. 141 (A1), 110–126.

Chauret, N., Gauthier, A., Nicoll-Griffith, D.A., 1998. Effect of common organic solvents on in vitro cytochrome P450-mediated metabolic activities in human liver microsomes. Drug Metabol. Dispos. 26 (1), 1–4.

Chen, T.-H., Wang, Y.-H., Wu, Y.-H., 2011. Developmental exposures to ethanol or dimethylsulfoxide at low concentrations alter locomotor activity in larval zebrafish: implications for behavioral toxicity bioassays. Aquat. Toxicol. 102 (3–4), 162–166.

Colborn, T., Saal, F.S.V., Soto, A.M., 1993. Developmental effects of endocrine-disrupting chemicals in wildlife and humans. Environ. Health Perspect. 101 (5), 378–384.

Cook, J.C., Kaplan, A.M., Davis, L.G., Oconnor, J.C., 1997. Development of a tier I screening battery for detecting endocrine-active compounds (EACs). Regul. Toxicol. Pharmacol. 26 (1), 60–68.

Davies, I.M., Harding, M.J.C., Bailey, S.K., Shanks, A.M., LÄnge, R., 1997. Sublethal effects of tributyltin oxide on the dogwhelk Nucella lapillus. Mar. Ecol. Prog. Ser. 158, 191–204.

DeWitt, J.C., Meyer, E.B., Henshel, D.S., 2005a. Environmental toxicity studies using chickens as surrogates for wildlife: effects of injection day. Arch. Environ. Contam. Toxicol. 48 (2), 270–277.

DeWitt, J.C., Meyer, E.B., Henshel, D.S., 2005b. Environmental toxicity studies using chickens as surrogates for wildlife: effects of vehicle volume. Arch. Environ. Contam. Toxicol. 48 (2), 260–269.

Dresser, T.H., Rivera, E.R., Hoffmann, F.J., Finch, R.A., 1992. Teratogenic assessment of 4 solvents using the Frog embryo Teratogenesis assay - Xenopus (FETAX). J. Appl. Toxicol. 12 (1), 49–56.

Easterbrook, J., Lu, C., Sakai, Y., Li, A.P., 2001. Effects of organic solvents on the activities of cytochrome P450 isoforms, UDP-dependent glucuronyl transferase, and phenol sulfotransferase in human hepatocytes. Drug Metabol. Dispos. 29 (2), 141–144.

Etches, R., Kagami, H., 1997. Genotypic and phenotypic sex reversal. In: Harvey, S., Etches, R.J. (Eds.), Perspectives in Avian Endocrinology. Journal of Endocrinology Ltd, Bristol, pp. 57–67.

Fridolfsson, A.K., Ellegren, H., 1999. A simple and universal method for molecular sexing of non-ratite birds. J. Avian Biol. 30 (1), 116–121.

Fry, D.M., Toone, C.K., 1981. DDT-induced feminization of gull embryos. Science 213 (4510), 922–924.

Fry, D.M., 1995. Reproductive effects in birds exposed to pesticides and industrial-chemicals. Environ. Health Perspect. 103, 165–171.

Galvao, J., Davis, B., Tilley, M., Normando, E., Duchen, M.R., Cordeiro, M.F., 2014. Unexpected low-dose toxicity of the universal solvent DMSO. Faseb. J. 28 (3), 1317–1330.

Gasc, J.M., 1980. Estrogen target-cells in gonads of the chicken-embryo during sexual-differentiation. J. Embryol. Exp. Morphol. 55 (FEB), 331–342.

Giesy, J.P., Feyk, L.A., Jones, P.D., Kannan, K., Sanderson, T., 2003. Review of the effects of endocrine-disrupting chemicals in birds. Pure Appl. Chem. 75 (11–12), 2287–2303.

Gooding, M.P., Wilson, V.S., Folmar, L.C., Marcovich, D.T., LeBlanc, G.A., 2003. The biocide tributyltin reduces the accumulation of testosterone as fatty acid esters in the mud snail (*Ilyanassa obsoleta*). Environ. Health Perspect. 111 (4), 426–430.

Green, J., Wheeler, J.R., 2013. The use of carrier solvents in regulatory aquatic toxicology testing: practical, statistical and regulatory considerations. Aquat. Toxicol. 144, 242–249.

Grote, K., Stahlschmidt, B., Talsness, C.E., Gericke, C., Appel, K.E., Chahoud, I., 2004. Effects of organotin compounds on pubertal male rats. Toxicology 202 (3), 145–158.

Halldin, K., Holm, L., Ridderstrale, Y., Brunstrom, B., 2003. Reproductive impairment in Japanese quail (*Coturnix japonica*) after in ovo exposure to o,p '-DDT. Arch. Toxicol. 77 (2), 116–122.

Hamburger, V., Hamilton, H.L., 1992. A series of normal stages in the development of the chick-embryo (reprinted from Journal of Morphology, Vol. 88, 1951). Dev. Dynam. 195 (4), 231–272.

Hickman, D., Wang, J.P., Wang, Y., Unadkat, J.D., 1998. Evaluation of the selectivity of in vitro probes and suitability of organic solvents for the measurement of human cytochrome P450 monooxygenase activities. Drug Metabol. Dispos. 26 (3), 207–215.

Hutchinson, T.H., Shillabeer, N., Winter, M.J., Pickford, D.B., 2006. Acute and chronic effects of carrier solvents in aquatic organisms: a critical review. Aquat. Toxicol. 76 (1), 69–92.

Kagami, H., Hanada, H., 1997. Current knowledge of sexual differentiation in domestic fowl. World Poultry Sci. J. 53 (2), 111–123.

Kamata, R., Takahashi, S., Shimizu, A., Shiraishi, F., 2006. Avian transgenerational reproductive toxicity test with in ovo exposure. Arch. Toxicol. 80 (12), 846–856.

Keibel, F., Abraham, K., 1900. Normentafel zur Entwicklungsgeschichte des Huhnes. Fischer, Gallus domesticus. Jena.

Lan, N.C., Katzenellenbogen, B.S., 1976. Temporal relationships between hormone receptor-binding and biological responses in uterus - studies with short-acting and long-acting derivates of estriol. Endocrinology 98 (1), 220–227.

Landauer, W., Salam, N., 1972. Aspects of dimethyl sulfoxide as solvent for teratogens. Dev. Biol. 28 (1), 35–46.

Lange, I.G., Hartel, A., Meyer, H.H.D., 2002. Evolution of oestrogen functions in vertebrates. J. Steroid Biochem. Mol. Biol. 83 (1–5), 219–226.

Mallozzi, M., Bordi, G., Garo, C., Caserta, D., 2016. The effect of maternal exposure to endocrine disrupting chemicals on fetal and neonatal development: a review on the major concerns. Birth Defects Res. Part C Embryo Today - Rev. 108 (3), 224–242.

Matsushita, S., Yamashita, J., Iwasawa, T., Tomita, T., Ikeda, M., 2006. Effects of in ovo exposure to imazalil and atrazine on sexual differentiation in chick gonads. Poultry Sci. 85 (9), 1641–1647.

McAllister, B.G., Kime, D.E., 2003. Early life exposure to environmental levels of the aromatase inhibitor tributyltin causes masculinisation and irreversible sperm damage in zebrafish (*Danio rerio*). Aquat. Toxicol. 65 (3), 309–316.

Metcalfe, C.D., Metcalfe, T.L., Kiparissis, Y., Koenig, B.G., Khan, C., Hughes, R.J., Croley, T.R., March, R.E., Potter, T., 2001. Estrogenic potency of chemicals detected in sewage treatment plant effluents as determined by in vivo assays with Japanese medaka (*Oryzias latipes*). Environ. Toxicol. Chem. 20 (2), 297–308.

Morgan, W., 1974. Toxic effect of a radioprotectant (DMSO) on young chicken embryos. Poultry Sci. 53 (5), 1958.

Nakabayashi, O., Kikuchi, H., Kikuchi, T., Mizuno, S., 1998. Differential expression of genes for aromatase and estrogen receptor during the gonadal development in chicken embryos. J. Mol. Endocrinol. 20 (2), 193–202.

Notman, R., Noro, M., O'Malley, B., Anwar, J., 2006. Molecular basis for dimethylsulfoxide (DMSO) action on lipid membranes. J. Am. Chem. Soc. 128 (43), 13982–13983.

OECD, 1984. Test No. 206: Avian Reproduction Test. OECD Publishing.

OECD, 2007. Test No. 440: Uterotrophic Bioassay in Rodents. OECD Publishing.

OECD, 2009. Test No. 441: Hershberger Bioassay in Rats. OECD Publishing.

Oehlmann, J., Schulte-Oehlmann, U., Bachmann, J., Oetken, M., Lutz, I., Kloas, W., Ternes, T.A., 2006. Bisphenol A induces superfeminization in the ramshorn snail *Marisa cornuarietis* (Gastropoda : prosobranchia) at environmentally relevant concentrations. Environ. Health Perspect. 114, 127–133.

Oshima, A., Yamashita, R., Nakamura, K., Wada, M., Shibuya, K., 2012. In ovo exposure to nonylphenol and bisphenol A resulted in dose-independent

feminization of male gonads in Japanese quail (*Coturnix japonica*) embryos. Environ. Toxicol. Chem. 31 (5), 1091–1097.
Ottinger, M.A., Abdelnabi, M.A., 1997. Neuroendocrine systems and avian sexual differentiation. Am. Zool. 37 (6), 514–523.
Pawlowski, S., Sauer, A., Shears, J.A., Tyler, C.R., Braunbeck, T., 2004a. Androgenic and estrogenic effects of the synthetic androgen 17-alpha-methyltestosterone on sexual development and reproductive performance in the fathead minnow (*Pimephales promelas*) determined using the gonadal recrudescence assay. Aquat. Toxicol. 68 (3), 277–291.
Pawlowski, S., van Aerle, R., Tyler, C.R., Braunbeck, T., 2004b. Effects of 17-alpha-ethinylestradiol in a fathead minnow (*Pimephales promelas*) gonadal recrudescence assay. Ecotoxicol. Environ. Saf. 57 (3), 330–345.
Pettersson, I., Arukwe, A., Lundstedt-Enkel, K., Mortensen, A.S., Berg, C., 2006. Persistent sex-reversal and oviducal agenesis in adult *Xenopus (Silurana) tropicalis* frogs following larval exposure to the environmental pollutant ethynylestradiol. Aquat. Toxicol. 79 (4), 356–365.
Razia, S., Maegawa, Y., Tamotsu, S., Oishi, T., 2006. Histological changes in immune and endocrine organs of quail embryos: exposure to estrogen and nonylphenol. Ecotoxicol. Environ. Saf. 65 (3), 364–371.
Romanoff, A.L., 1960. The Avian Embryo: Structural and Functional Development. Macmillan, New York.
Romanoff, A.L., Romanoff, A.J., 1972. Pathogenesis of the Avian Embryo - an Analysis of Causes of Malformations and Prenatal Death. Wiley, New York.
Russel, W.M.S., Burch, R.L., 1959, reprinted 1992. The Principles of Humane Experimental Technique. Wheathamptonstead, England: Methuen.
Samsel, J., Zeis, A., Weniger, J.P., 1982. Feminization in the chick-embryo testis by diethylstilbestrol and antagonizing action of tamoxifen. Biochimie 64 (5), 369–376.
Scheib, D., Baulieu, E.E., 1981. Inhibiting effects of tamoxifen on the female differentiation of the gonads of quail embryos. Comptes Rendus Seances Acad. Sci. Ser. III - Sci. Vie 294 (7), 513–518.
Scheib, D., 1983. Effects and role of estrogens in avian gonadal differentiation. Differentiation 23, 87–92.
Scheib, D., Reyssbrion, M., 1979. Feminization of the quail by early diethylstilbestrol treatment - histoenzymological investigations on steroid dehydrogenases in the gonads. Archives d'Anatomie Microscopique et de Morphologie Experimentale 68 (2), 85–98.
Scheider, J., Afonso-Grunz, F., Hoffmeier, K., Horres, R., Groher, F., Rycak, L., Oehlmann, J., Winter, P., 2014. Gene expression of chicken gonads is sex- and side-specific. Sexual Development 8 (4), 178–191.
Shibuya, K., Mizutani, M., Wada, M., Sato, K., Nunoya, T., 2004. A new screening model using F1 (AWE x WE) Japanese quail embryo for evaluating sex reversal effects. J. Toxicol. Pathol. 17 (4), 245–252.
Smith, C.A., Andrews, J.E., Sinclair, A.H., 1997. Gonadal sex differentiation in chicken embryos: expression of estrogen receptor and aromatase genes. (vol 60, pg 295, 1997). J. Steroid Biochem. Mol. Biol. 62 (4), 361.
Sotonyi, P.T., Csaba, G., 1986. Effect of prenatal and or neonatal diethylstilbestrol (DES) or allylestrenol (AE) treatment on the postnatal-development of the chicken ovary. Acta Biol. Hung. 37 (3–4), 189–196.
Starck, M., Ricklefs, R., 1997. Avian Growth and Development: Evolution within the Altricial-precocial Spectrum, vol. 1. Oxford University Press, Oxford.
Stevens, A.-S., Pirotte, N., Plusquin, M., Willems, M., Neyens, T., Artois, T., Smeets, K., 2015. Toxicity profiles and solvent-toxicant interference in the planarian *Schmidtea mediterranea* after dimethylsulfoxide (DMSO) exposure. J. Appl. Toxicol. 35 (3), 319–326.
Tanabe, Y., Nakamura, T., Fujioka, K., Doi, O., 1979. Production and secretion of sex steroid-hormones by the testes, the ovary, and the adrenal-glands of embryonic and young chickens (*Gallus domesticus*). Gen. Comp. Endocrinol. 39 (1), 26–33.
Tanabe, Y., Yano, T., Nakamura, T., 1983. Steroid-hormone synthesis and secretion by testes, ovary, and adrenals of embryonic and post-embryonic ducks. Gen. Comp. Endocrinol. 49 (1), 144–153.
Vandenberg, L.N., Colborn, T., Hayes, T.B., Heindel, J.J., Jacobs, D.R., Lee, D.H., Shioda, T., Soto, A.M., Vom Saal, F.S., Welshons, W.V., Zoeller, R.T., Myers, J.P., 2012. Hormones and endocrine-disrupting chemicals: low-dose effects and nonmonotonic dose responses. Endocr. Rev. 33 (3), 378–455.
Watts, M.M., Pascoe, D., Carroll, K., 2001. Chronic exposure to 17-alpha-ethinylestradiol and bisphenol A-effects on development and reproduction in the freshwater invertebrate *Chironomus riparius* (Diptera : chironomidae). Aquat. Toxicol. 55 (1–2), 113–124.
Wolff, E., 1979. Old experiments and new trends in avian sex differentiation. Vitro Cell Dev. Biol.: J. Tech. Counc. ASCE 15 (1), 6–10.
Woods, J.E., Erton, L.H., 1978. Synthesis of estrogens in the gonads of the chick-embryo. Gen. Comp. Endocrinol. 36 (3), 360–370.
Wyatt, R.D., Howarth, B., 1976. Effect of dimethyl-sulfoxide on embryonic survival and subsequent chick performance. Poultry Sci. 55 (2), 579–582.
Zhang, J.L., Zuo, Z.H., Chen, Y.X., Zhao, Y., Hu, S., Wang, C.G., 2007. Effect of tributyltin on the development of ovary in female cuvier (*Sebastiscus marmoratus*). Aquat. Toxicol. 83 (3), 174–179.

A.1.3 Morphologic and transcriptomic effects of endocrine modulators on the gonadal differentiation of chicken embryos: the case of tributyltin (TBT)

Jessica Scheider, Fabian Afonso-Grunz, Luzie Jessl, Klaus Hoffmeier, Peter Winter, Jörg Oehlmann

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Jessica Scheider (JS), Fabian Afonso-Grunz FAG), Luzie Jessl (LJ), Klaus Hoffmeier (KH), Peter Winter (PW), Jörg Oehlmann (JO)

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Promovendin JS:	40%
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Co-Autor PW:	25%
Co-Autor JO:	25%

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Co-Autor FAG:	30% (Präparation für den SuperSAGE)
Co-Autor KH:	10% (Präparation für den SuperSAGE)

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Morphological and transcriptomic effects of endocrine modulators on the gonadal differentiation of chicken embryos: The case of tributyltin (TBT)

Jessica Scheider[a,*], Fabian Afonso-Grunz[b,c], Luzie Jessl[a,b], Klaus Hoffmeier[b], Peter Winter[b], Jörg Oehlmann[a]

[a] Goethe University Frankfurt am Main, Institute for Ecology, Evolution and Diversity, Department Aquatic Ecotoxicology, Max-von-Laue-Str. 13, 60438, Frankfurt/M., Germany
[b] GenXPro GmbH, Altenhöferallee 3, 60438, Frankfurt/M., Germany
[c] Goethe University Frankfurt am Main, Institute for Molecular BioSciences, Max-von-Laue-Str. 9, 60438, Frankfurt/M., Germany

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ABSTRACT

Morphological malformations induced by tributyltin (TBT) exposure during embryonic development have already been characterized in various taxonomic groups, but, nonetheless, the molecular processes underlying these changes remain obscure. The present study provides the first genome-wide screening for differentially expressed genes that are linked to morphological alterations of gonadal tissue from chicken embryos after exposure to TBT. We applied a single injection of TBT (between 0.5 and 30 pg as Sn/g egg) into incubated fertile eggs to simulate maternal transfer of the endocrine disruptive compound. Methyltestosterone (MT) served as a positive control (30 pg/g egg). After 19 days of incubation, structural features of the gonads as well as genome-wide gene expression profiles were assessed simultaneously. TBT induced significant morphological and histological malformations of gonadal tissue from female embryos that show a virilization of the ovaries. This phenotypical virilization was mirrored by altered expression profiles of sex-dependent genes. Among these are several transcription and growth factors (e.g. FGF12, CTCF, NFIB), whose altered expression might serve as a set of markers for early identification of endocrine active chemicals that affect embryonic development by transcriptome profiling without the need of elaborate histological analyses.

1. Introduction

Endocrine disrupting chemicals (EDCs) represent a potential risk for animals because of their ability to interfere with endogenous endocrine pathways through various molecular processes. Tributyltin (TBT) is a known endocrine disrupter and a highly toxic compound with a complex toxicity profile. This organotin compound affects *inter alia* calcium homeostasis, inhibits oxidative phosphorylation as well as ion transport processes, interferes with the cytochrome P450 dependent monooxygenase system by inhibition of aromatase as the key enzyme for the conversion of androgens to estrogens and is a potent agonist of the retinoid X receptor (RXR) (Alzieu, 2000; Dmetrichuk et al., 2008; Gooding and LeBlanc, 2001; Nishikawa et al., 2004; OECD, 2010; Oberdörster and McClellan-Green, 2000; Sekizawa et al., 2017). TBT has widely been used as an antifouling. Environmental levels of TBT have been found up to 197 ng TBT-Sn/L in sea water and up to 1,198 ng TBT-Sn/g dry weight in blue mussels (*Mytilus edulis*) (Chau et al., 1997; Rodríguez-González et al., 2006). Accelerated by UV radiation, increasing temperature, and biological activity by microorganisms, TBT may degrade through sequential dealkylation to dibutyltin (DBT), monobutyltin (MBT), and inorganic tin, becoming progressively less toxic in the process (Antizar-Ladislao, 2008; Gadd, 2000). Indeed, information on the mechanisms of TBT detoxification is still limited (Dubey and Roy, 2003). Since 2008, the use of TBT in antifouling paints is banned internationally (Antizar-Ladislao, 2008).

TBT affects a wide range of non-target organisms from various invertebrate phyla to different classes of vertebrates. It causes adverse effects by disruption of their endocrine system such as abnormal shell growth, imposex and intersex development (Alzieu, 2000; Oehlmann et al., 1998; Stroben et al., 1992). TBT may bioaccumulate in higher trophic levels (EFSA, 2004), and thus may pose a threat for predators, although data on TBT tissue concentration in birds is scarce and information on TBT concentration in bird eggs is not available. Mollusk and fish-eating coastal seabirds seem to be particularly concerned by

* Corresponding author.
E-mail address: J.Scheider@bio.uni-frankfurt.de (J. Scheider).

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accumulating TBT from their prey from a theoretical standpoint (Kannan and Falandysz, 1997; Kannan et al., 1998). In livers of the Japanese common cormorant (*Phalacrocorax carbo*) up to 9 ng Sn/g wet weight were found (Guruge et al., 1997) and in birds (grebes, tufted ducks, cormorants) in the Netherlands measured TBT concentrations ranged from 9 to 28 ng Sn/g dry weight (Stäb et al., 1996). Measured levels were considerably lower than TBT levels in fish and invertebrates from the same area serving as food for the birds. These results indicate a lower biomagnification potential for TBT in birds than expected which reflects their good capability to metabolize TBT via phase I pathways (cytochrome P450-dependent debutylation) (Veltman et al., 2006) and the seasonal moulting as an efficient elimination route of organotins. Feathers of cormorants have shown to contain up to 30% of the total body burden of butyltins probably due to feather proteins that have a high affinity for organotins (Guruge et al., 1997).

The adverse effects of TBT on the gonads have already been shown for both, sexually mature animals and developing embryos in a broad range of species. Virilization and even sex-reversal of female gastropods, as well as effects on sex differentiation in other invertebrate and vertebrate groups are clear examples of endocrine disruption by TBT in early and later life stages starting at 1–20 ng TBT-Sn/L in gastropods (Davies et al., 1997; Gooding et al., 2003; His and Robert, 1985; Matthiessen and Gibbs, 1998; OECD, 2010; Wang et al., 2010). At similar exposure levels, effects on sex ratio were also observed in a sexual development test with the zebrafish *Danio rerio* (McAllister and Kime, 2003). In mice, prenatal TBT-exposure resulted in lower body weight, prolonged diestrus or extended periods of estrus during adulthood (Si et al., 2012). In human placenta, Heidrich and co-workers (Heidrich et al., 2001) demonstrated an inhibition of aromatase activity in response to TBT with an IC_{50} of 0.7 µgTBT-Sn/L.

In general, ovarian follicles from lower vertebrates such as fish and amphibians exhibited a comparable sensitivity to butyltins like mammalian ovary tissue cultured *in vitro* (Ahn et al., 2007; Yamazaki et al., 2005). For many of the observed TBT effects on sexual differentiation in the various wildlife groups aromatase seems to be the most prominent target. The modulation of aromatase activity is likely to result in a hormonal imbalance with an altered estrogen/androgen level and finally in the formation of ovotestes during development (Ahn et al., 2007; Heidrich et al., 2001; Nakanishi et al., 2006). However, sensitivity of steroidogenic enzymes to exogenous chemicals differs between ovaries and testes. In mammals, male sexual differentiation is dependent on testosterone (Cunha et al., 2004; Marker et al., 2003; Ricke et al., 2004). In contrast, differentiation of reproduction organs and sexual behaviour in birds is regulated by estrogens. If estrogen synthesis is inhibited, genetically female birds may develop into phenotypic males (Bannister et al., 2011). Higher levels of estrogen, on the other hand, feminise male embryos: In Japanese quails (*Coturnix japonica*), Berg and colleagues showed that administration of synthetic estrogens results in development of an ovary-like tissue in the left testis, while malformations occur in the Müllerian duct of female quails (Berg et al., 1999). In line with this, the treatment of genetic females with male hormones like e.g. 17α-methyltestosterone (MT) results in a characteristic virilization that affects the gonads, accessory sex organs as well as behaviour in a wide range of organisms, such as molluscs, fish, turkeys, and rats (Alzieu, 2000; Feist et al., 1995; Memon et al., 2008; Singh, 2013; Yamasaki et al., 2003).

Like in mammals, the avian mother affects the development of its offspring not only by transferred genetic material, but also by transferring hormones (Carere and Balthazart, 2007). Substances incorporated by the mother may consequently also influence offspring development even originally or as metabolites in the allantoic fluid (Kamata et al., 2006). Contrasting to other test systems that employ mammals or aquatic species, however, the chicken egg represents a closed system that lacks any exchange with its environment except for the interchange of gases. A unique injection of a testing compound results in chronic chemical exposure, because no exchange or loss of the test compound is possible. A single test compound injection at a very early developmental phase may therefore be sufficient to significantly influence the developing embryo. Consequently, chicken eggs provide a different system for assessment of the effects of chronic chemical exposure compared to aquatic systems, where the test compound is introduced into the environment (Ahn et al., 2007; Bannister et al., 2011; Berg et al., 1999; Carere and Balthazart, 2007; Cunha et al., 2004; Davies et al., 1997; Feist et al., 1995; Gooding et al., 2003; Heidrich et al., 2001; His and Robert, 1985; Kamata et al., 2006; Marker et al., 2003; McAllister and Kime, 2003; Memon et al., 2008; Nakanishi et al., 2006; Ricke et al., 2004; Si et al., 2012; Singh, 2013; Yamasaki et al., 2003; Yamazaki et al., 2005; Zhang et al., 2007; Zhang et al., 2011; Zhang et al., 2013).

Here we present a comprehensive morphological and histological study of chicken embryonic gonads after exposure to the endocrine modulator TBT along with a genome-wide transcriptome analysis of sex-specific gene expression. We investigated whether effects of TBT on embryonic survival and development before hatching, with special emphasis on the structure of the gonads in the common domestic hen (*Gallus gallus domesticus*), are accompanied by characteristic shifts in gene expression of the gonads.

2. Materials and Methods

2.1. Substances

The tested substances comprised tributyltin chloride (TBT-Cl, CAS-no. 1461-22-9, 96% p.a., Sigma-Aldrich, Munich) and 17α-methyltestosterone (MT, CAS-no. 58-18-4, ≥ 97% HPLC, Sigma-Aldrich, Munich), which served as positive control. For histological analysis chicken embryos were exposed to four doses of TBT (0.5, 3, 10 and 30 pg TBT-Sn/g egg), while gene expression profiling was performed after exposure to 10 pg TBT-Sn/g egg. All TBT doses are provided as tributyltin as tin (TBT-Sn). These doses can be converted to tributyltin chlorid (TBT-Cl) by multiplying with factor 2.44 (1 pg TBT-Sn/g = 2.44 pg TBT-Cl/g). MT was injected at a dose of 30 pg MT/g egg. For both test substances sesame oil (Oleum Sesame Raffinatum, CAS-no. 8008-47-0, Sigma-Aldrich, Munich) was used as solvent with an injected volume of 8 µL per egg, irrespective of the dose. To simulate the maternal transfer, the solvent and tested substances were injected directly into the yolk, right under the embryonic tissue, with a Hamilton syringe. Injection holes were subsequently sealed with 3% agarose to allow the passage of air and humidity.

2.2. Ethics statement

All experiments were carried out with respect for the principles of laboratory animal care, in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC) and the German Animal Welfare Act. All guidelines have been followed, complying with the ARRIVE guidelines. These experiments are pre-tests for the development of a test system using younger chicken embryos within the scope of a new animal replacement (3R) system (Jessl et al., 2017; Russel and Burch, 1959; Scholz et al., 2013).

2.3. Exposure of embryos

Six exposure experiments were performed with a total of 301 newly laid fertile chicken eggs (*Gallus gallus domesticus*, White Leghorn), which were obtained from a commercial local supplier (LSL Rhein-Main, Schaafheim). The eggs were incubated in batches of up to 20 eggs per day, while remaining eggs were stored up to 5 days at 16 +- 1 °C to prevent the start of development. Eggs were bred in a ThermoStar 100 egg incubator (J. Hemel Brutgeräte, Verl, Germany) and spaced so that they were not in contact with one another. Incubation was performed at 37.6 +- 0.1 °C and 60 +- 5% humidity, and eggs were turned once

every 2 hours. After 24 hours of incubation (embryonic day (ED) 1), the eggs were candeled and randomized into 7 treatment groups: control (untreated/not injected), solvent control (only sesame oil injected), positive control (MT), and four TBT exposure groups (doses: 0.5, 3, 10 and 30 pg TBT-Sn/g egg), not fertilized eggs were excluded. Eggs were candled on ED 7, 14, and 17 and dead embryos observed during candling were removed from egg shells and examined for possible causes of death. Embryos were decapitated 2 days before anticipated hatching on embryonic day 19 (ED19), and dissected under a microscope. Phenotypical sexing and morphologic judgment was blindly performed according to the respective gonad morphology before further processing for histological and genetic analyses. If a female showed a smaller left and/or a bigger right gonad than normal, it was used as a first marker for virilization.

2.4. Gonad tissue isolation and processing for histological analysis

Gonads were isolated together with the underlying mesonephros to provide a landmark for stereoscopic adjustment after histological processing. Tissues were fixed using Bouin's solution (15 mL saturated aquatic solution of picric acid with 5 mL formalin 40% and 1 mL glacial acetic acid) followed by repeated EtOH 70% rinsing until picric acid was removed (picric acid colors EtOH yellowish, thus, all picric acid was removed if EtOH stayed colorless). 6 µm cryo-slices from the middle of the gonads were stained by Hematoxylin-Eosin followed by measuring of cortex thickness and counting of *Tubuli seminiferi* in female gonads under a microscope (Olympus BX 50) using image processing (Diskus 4.5, Hilgers, Königswinter, Germany). 10 slices per individual were used for anlaysis. If a female gonad showed a noticeable thinner cortex and/or more than 10 seminiferous cords (cut off ration to exclude false positives), it was considered as virilized in a blind treatment examination.

2.5. Genetic sexing

For genetic sex determination, blood samples from each embryo were collected in absolute EtOH. and stored at −20 °C until isolation of the DNA with the DNEasy isolation kit (Qiagen, Hilden, Germany). Genetic sexing was carried out by a PCR using the primers 2550F (5'-GTT ACT GAT TCG TCT ACG AGA-3') and 2718R (5'-ATT GAA ATG ATC CAG TGC TTG-3') (Fridolfsson and Ellegren, 1999). These primers target at CHD1 introns, located on the Z (CHD1Z, 600 bp) and W chromosome (CHD1W, 450 bp). Thermal cycling comprised DNA polymerase activation at 95 °C for 1 min followed by 30 cycles of denaturation at 95 °C for 30 s, annealing at 52 °C for 30 s, elongation at 72 °C for 1 min and a final extension step at 72 °C for 3 min. All amplifications were performed on an advanced primus 96 thermocycler (Peqlab). Amplicons derived from this modified protocol were separated on a 1.4% agarose gel, where they resolved into one band (Z) in the case of male or two bands (Z + W) in the case of female embryos.

2.6. Gonad tissue isolation for transcriptome analysis

As gonads could be used either for histology or genentic analyzing, 2-3 individuals per test series, sex and group were taken for genetic analysis. Because of the six test series, we used ten individuals altogether for transcriptome analysis. Their individual right and left gonads were excised, cleaned from adhesive tissue and immediately frozen in 200 µl RNA lysis buffer (Promega) for later RNA isolation. Total RNA of whole right and left gonads (ten individuals of each group: control, solvent control, TBT 10 pg TBT-Sn/g egg and MT 30 pg/g egg) was extracted using the SV Total RNA Isolation System Kit according to manual 048 (Promega). Deviating from the protocol, on-column DNaseI digestion of genomic DNA was elongated from original 15 min to 30 min. A second DNaseI digestion was carried out with Baseline-Zero™ DNase (Epicentre; provided by Biozym Scientific GmbH, Hessisch Oldendorf, Germany) in solution to ensure that the samples were completely free of DNA. Total RNA concentration was estimated in a dilution series with the LabelGuard NanoPhotometer (Implen, München, Germany). RNA quality and quantity was further determined using a Caliper lab-on-a-chip system (Agilent – CALIPER, Hopkinton MA, USA). All isolated total RNA samples had an RNA Integrity Number (RIN) ranging from 8.5 to 10.0 (highest quality).

2.7. Library preparation and bioinformatics

DeepSuperSAGE libraries were constructed and validated by qPCR as previously described (Scheider et al., 2014a). Sequencing was performed on Illumina's Genome Analyzer IIx, and subsequent base calling was carried out by Illumina's GAPipeline. The obtained raw data were further analyzed with GenXPro's SuperSAGE data processing pipeline. First, distinct libraries were sorted out from the bulked sequencing data according to their respective indices, followed by elimination of PCR-derived tags identified by TrueQuant technology (GenXPro). Next, 26 bp SuperTAGs were extracted from the remaining sequences, and subsequently mapped on the *Gallus gallus* genome Galgal4 (Version 72.4, published in November 2011 by the International Chicken Genome Consortium) using the short read mapper Novoalign v2.07.13 (Novocraft Technologies). Feature annotation for the mapped loci was performed on the basis of the standard Ensembl genebuild pipeline (please consult http://www.ensembl.org/info/docs/genebuild/2013_04_chicken_genebuild.pdf for a detailed description of the annotation), and tags were counted via the Python package HTSeq v0.5.4p2 (EMBL Heidelberg, https://pypi.python.org/pypi/HTSeq). The unambiguously mapped reads for each library were normalized to a million sequenced reads in total (tags per million; TPM). Read numbers for the pooled datasets from left and right gonads were obtained by *in silico* pooling of the raw sequencing data from the respective left and right gonad prior to the analysis via the GenXPro SuperSAGE data processing pipeline. Fold changes were determined by pair-wise comparison of the normalized tag numbers in two particular libraries, and the statistical significance was assessed by *chi* square tests according to Man and colleagues (Man et al., 2000). TPM counts of zero were adjusted to 0.05 to allow for calculation of fold changes, even if a given tag was only present in one of the libraries (see supplementary table T1).

2.8. Data accessibility

The pre-processed raw data are submitted to the Genome Expression Omnibus (Accession number: GSE108141).

3. Results and Discussion

3.1. Effect of sesame oil on gonadal differentiation and gene expression

In (eco-) toxicological studies the selection of the best suited solvent for a given substance is a critical decision. Ideally, the solvent should resolve the substance completely but should not elicit an effect on its own. Since TBT is lipophilic, we had to use an oily carrier. Because we did not want to use an emulsion of several compounds as previously described for chicken studies (Berg et al., 1998; Berg et al., 2001a; Berg et al., 2001b), we used sesame oil as solvent, which was previously applied in several vertebrate endocrine disruption studies (Chataigneau et al., 2004; Demissie et al., 2008; Fail et al., 1998; Liang et al., 2013). In other bird studies, sesame oil did not show any significant morphological effect (Eising et al., 2001; Muller et al., 2005; von Engelhardt et al., 2006). Our histological analysis revealed a slight but notable reduction in the cortex thickness of gonadal tissue from female chicken embryos treated with sesame oil (Fig. 1C), resulting in the occurrence of 21% virilized females in the solvent control compared to 0% in the untreated control (Table 1). This difference is not statistically significant (Fisher's exact test, $p = 0.061$). The treatment did not induce

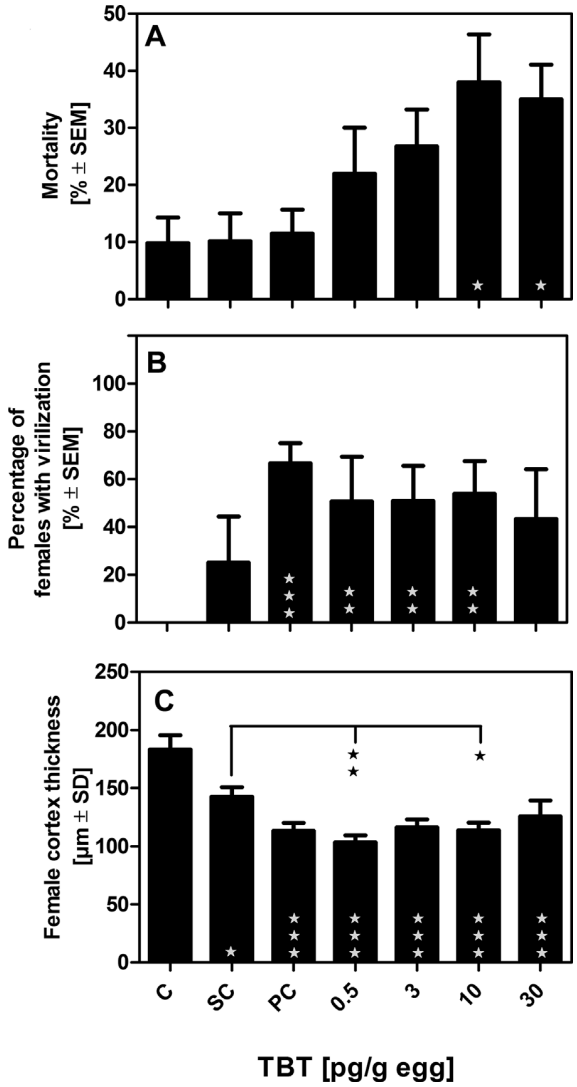

Fig. 1. Effect of TBT on chicken embryos. A: Mortality in response to TBT until embryonic day 19. B: Numbers of morphologically virilized genetic females based on structural anomalies in gonads. C: the corresponding cortex thickness of female animals. C = Control, SC = Solvent control; PC = Positive Control (MT). Statistics: 1-way ANOVA with Dunnett's Post test for A and C, Fisher's exact test for B * = $p < 0.05$, ** = $p < 0.01$; *** = $p < 0.001$.

any other changes in gonadal structure and was apparently non-toxic (Fig. 1A), which confirms the previous findings.

In line with the observed histological alterations, gene expression profiling of sesame oil-treated embryos revealed a strong impact of the solvent on the identified transcription profiles. In total, 417 and 606 genes were significantly differentially expressed in males and females, respectively (Fig. 4). Serum response factor (SRF) is a transcription factor that participates in regulation of embryonic development, and is found to be down-regulated by 50% in male embryos, while its abundance in female embryos corresponds to approximately a third of its abundance in completely untreated embryos. Homeobox protein Hox-A4 (HOXA4), another transcription factor that is involved in embryonic development, is significantly down-regulated in sesame oil-treated embryos (log_2 fold change of 2 in both sexes). The deregulated expression of both *SRF* and *HOXA4* may contribute to the observed reduction of cortex thickness in female embryos, and underlines the importance of a thorough characterization of the employed solvent to account for off-target effects in toxicological studies.

3.2. Effects of TBT on gonadal morphology and differentiation based on histological alterations

In total, gonadal morphology and differentiation on E19 was analyzed in 241 embryos in six test series. At the two highest TBT doses (10 and 30 pg TBT-Sn/g egg) mortality increased significantly by more than 30% relative to the untreated and the solvent control (approx. 10%, Fig. 1A). Embryos often died before embryonic day 7 at first candling.

In all TBT and the MT (positive control) exposed groups anatomical virilization of female gonads was observed. Virilization comprised the development of a longer right gonad and/or a rounder (less folded and less flat) left gonad (Figs. Fig. 1B, Figure 2B), resembling anatomical characteristics of a developing ovotestis (Koba et al., 2008). On the histological level, virilization effects were mainly characterized by a significant reduction of the cortex (Fig. 1C, Fig. 2D + E). Overall, around 50% of the female embryos were affected by virilization of the gonads in the three lower TBT exposure groups (0.5, 3 and 10 pg/g), while 30% were affected by the highest TBT dose (30 pg/g) and 68% in the positive control (30 pg MT/g) (Table 1). Both, the lacking significance of cortex reduction (regarding 3 and 30 pg/g) and the decrease in the percentage of virilization in the ovaries of females from the higher exposure groups does not necessarily reflect a reduced effect of higher concentrations of TBT, but might be linked either with the increasing dose-dependent mortality rate of exposed embryos (Fig. 1a), or related to the typical non-monotonic dose-response relationship of endocrine disrupters, where the effect is not increasing in a linear way but characterized for example by an u-shaped (Fig. 1C) or inverted u-shaped (Fig. 1B) response (Lagarde et al., 2015; Vandenberg et al., 2012) Contrary to ovaries, testes did not exhibit any anatomical or histological alterations in the TBT-treated groups and the positive control when compared to the untreated and solvent control (Fig. 2C + F).

3.3. Gene expression profiling

Of the sequenced 32.2 million reads 85% could be mapped to the chicken genome. In total 14,074 genes are represented by unambiguously mapped reads that did not return more than one hit (supplementary tab T1). As previously reported, gene expression in the gonads of untreated embryos strongly differs between sexes and between the left and right testes and ovaries (Scheider et al., 2014a).

Table 1
Total number of injected fertile eggs, surviving embryos until E 19, number of genetic males and females and number and proportion of virilized females in the different treatment groups. Virilized females exhibited a thinner cortex and/or at least 10 Tubuli seminiferi in histological sections of the gonad. Embryos that died before E19 are not listed.

	Σ fertile eggs	Σ surviving embryos	Σ genetic male	Σ genetic female	Σ virilized females (%)
Untreated control	51	47	27	20	0 (0)
Solvent Control	31	27	13	14	3 (21)
MT 30 ng/g egg	54	48	23	25	17 (68)
TBT-Sn 0.5 pg/g egg	40	33	14	19	10 (53)
TBT-Sn 3 pg/g egg	45	34	18	16	10 (63)
TBT-Sn 10 pg/g egg	44	28	10	18	8 (44)
TBT-Sn 30 pg/g egg	36	24	14	10	3 (30)

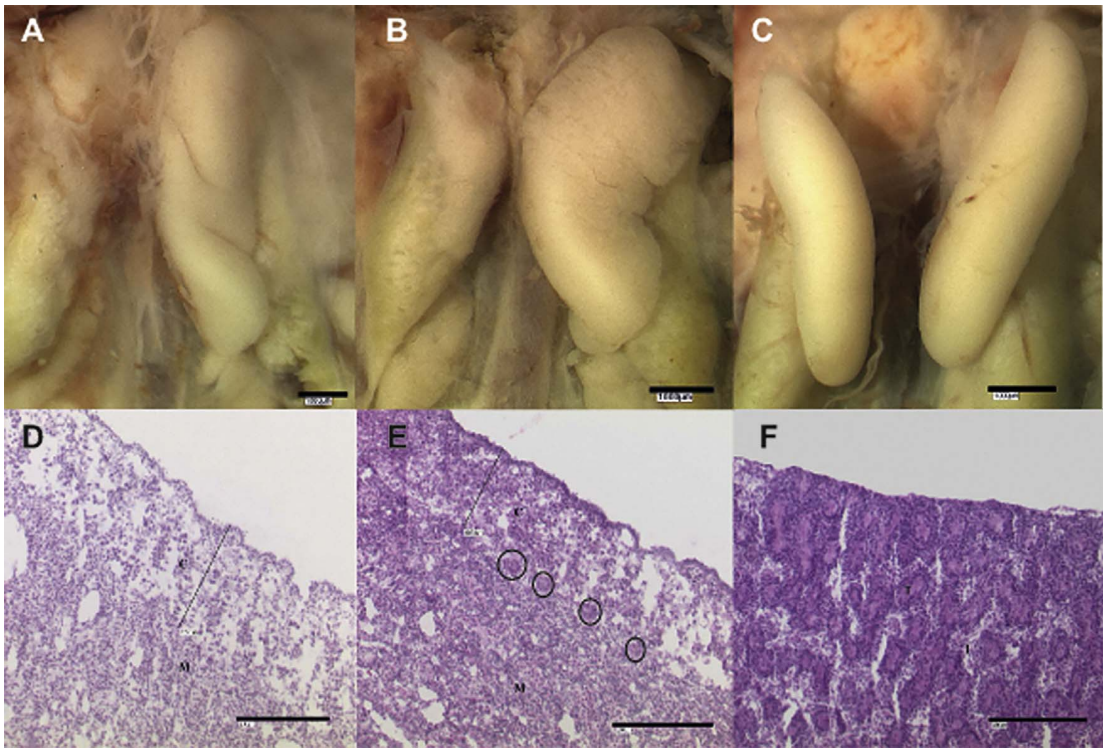

Fig. 2. Morphological (A-C; bar = 1 mm) and histological (D-F; bar = 200 µm) photographs of gonads in chicken embryos (E19) A + D: Control female with large and folded left and regressing right gonad, section through left gonad with cortex and medulla. B + E: Genetic female (10 pg TBT-Sn/g egg) with masculinized gonads: the left gonad gets rounder, more firm, and the right gonad gets taller. The section indicates Tubuli seminiferi in the outer medulla region (encircled) and a reduced cortex. C + F: Control male with two uniform testes and seminiferous cords and interstitial space.

Fig. 3 illustrates the most differentially expressed genes between the sexes in control as well as solvent, TBT or MT-treated chicken embryos. Apparently, the natural sex-specific gene expression is only partially continued in the response pattern caused by the exposure to the test chemicals. Most of the affected genes are activated or inactivated regardless of the sex, but some genes exhibit a sex-dependent differential expression under substance influence, partially differing between MT and TBT.

3.4. MT-induced changes in gonadal differentiation and gene expression

MT is the equivalent of endogenous testosterone, which is transformed to estrogen by the aromatase enzyme. In female embryos, the natural level of estrogen is higher than the testosterone level due to an increased activity of aromatase. The balance of both, estrogen and testosterone, is crucial for normal embryonic development of gonadal tissues in males and females. In our study, the cortex of female chicken gonads was still present, but thinner in MT-treated embryos (Fig. 1C). The change in cortex thickness in these embryos implies that the testosterone-estrogen relationship is shifted in favor of androgens such that the volume of the whole gonad is changed together with cortex thickness, while the gonad is not completely sex-reversed. Apparently, only complete left-side ovariectomy (Wallenburg, 1982) or *in ovo* treatment with the high-potential, non-steroidal aromatase inhibitor fadrozole (Abinawanto et al., 1998; Vaillant et al., 2001; Wartenberg et al., 1992) results in embryonic phenotypic female-to-male sex-reversal. The latter, however, has to be maintained by further treatment within juvenile development, otherwise this transformation is reversible in mature birds (Burke and Henry, 1999). Additionally, sex-reversed female chicken cannot be transformed into completely functional neo-males (Abinawanto et al., 1998; Vaillant et al., 2003; Yang et al., 2011). We therefore conclude that the *in ovo* application of 30 pg MT/g egg does generally not influence the development of the female gonad up to a neo-male, but nevertheless induces histomorphological changes of the tissue.

Even though MT affected development in several studies with

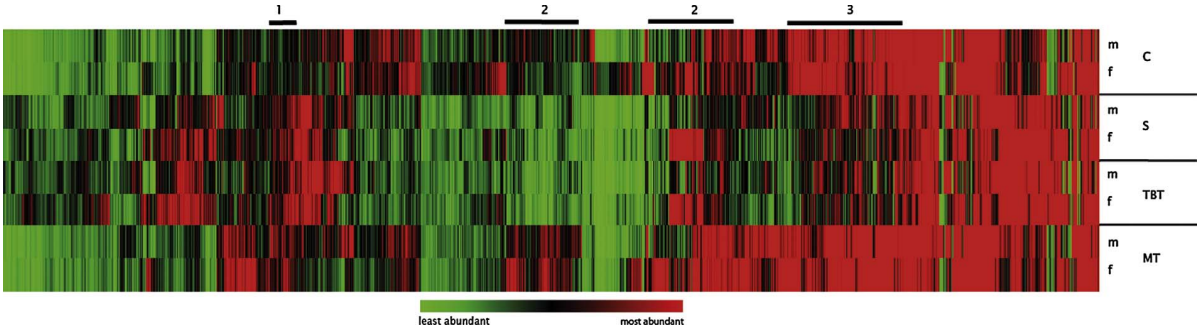

Fig. 3. Heat map of the most differential gene expression in the gonads of genetic males and females. $p_{max} < 9E^{-3}$, hierarchical clustering. 1 = S, TBT and MT differ from C, 2 = S, TBT and MT differ from C sex-dependently, 3 = S and TBT differ from MT and C.

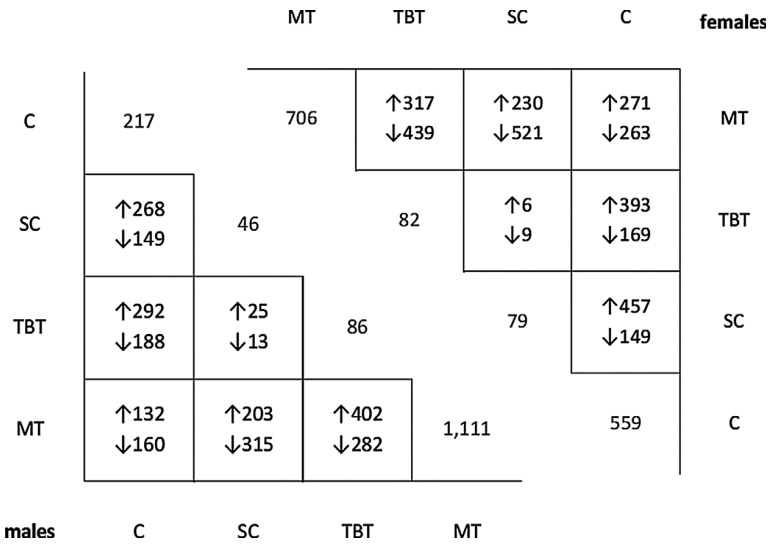

Fig. 4. Pair-wise comparison of differentially expressed transcripts in male (left) and female (right) gonads along with the number of exclusively expressed transcripts (gray numbers) for each group. Listed are unambiguously annotated reads with $\log_2$ fold changes stronger than |1.5| and p-values below 0.000001. Up- and downregulated transcripts are indicated by corresponding arrows for the library on the ordinate, respectively. Please consult Fig. 1 for further information.

different taxa (Cevasco et al., 2008; Faucounau et al., 1995; Feist et al., 1995; Giusti et al., 2013), MT and testosterone propionate did not have any morphological effects on the gonads of treated male and female quails (Shibuya et al., 2004; Utsumi and Yoshimura, 2009). In addition to the observed reduction of cortex thickness in our study, several MT- and TBT-treated females showed *Tubuli seminiferi* in the medulla, especially close to the cortex (Fig. 2E), and this did not occur in control and solvent-treated embryos. Our results therefore deviate from the studies in quails and confirm the findings of the androgen studies that reported an impact of MT during development. We assume that the technique of injection of the substance, the day of application of the compound as well as the microscopic analysis might be possible causes for this. Firstly, we injected the endocrine disrupter directly into the yolk, just below the embryo, while the injection mode in the quails studies (Shibuya et al., 2004; Utsumi and Yoshimura, 2009) was either into the egg white or into the air cell. Secondly, we injected on embryonic day 1 to imitate the maternal transfer and to fully assess the impact of our tested compounds during the whole embryonic development. Contrasting to this, Utsumi and colleagues (Utsumi and Yoshimura, 2009) applied the substance on embryonic day 13, followed by histological examination only three days later. The substances were therefore available for a shorter period compared to our study. Moreover, we administered the substance at a much earlier stage of development where changes in a few genes in the few cells that represent the gonads at E1 may have effects that have consequences in all subsequent cell divisions. Additionally, we processed an intensive histological investigation which was not considered in the other studies.

Gene expression profiling of gonadal tissue from male and female embryos subsequent to MT treatment indicates that the exposure to MT evokes transcriptional alterations that are distinct from those induced by the solvent. In comparison to the sesame oil-treated group more than 200 genes were significantly upregulated in both sexes, while 315 and 521 genes are downregulated in male and female chicken embryos, respectively (Fig. 4). Among the transcripts that are upregulated in both sexes upon MT treatment are the mRNAs that encode the two transcription factors V-Maf Avian Musculoaponeurotic Fibrosarcoma Oncogene Homolog F (MAFF) and transcription factor CP2 (TFCP2). Both are involved in embryonic development, and especially TFCP2 is known to regulate expression in chicken embryonic stem cells via activation of the cENS-1 promoter (Acloque et al., 2004; Lecoin et al., 2004). We additionally found a highly significant upregulation of the gene encoding (Sex Determining Region Y)-Box 9 (SOX9) in MT-treated males compared to the solvent control. SOX9, a transcription factor related to the mammalian testis-determining factor SRY, is expressed shortly after the Anti-Mullerian Hormone, which represents a key player for sexual differentiation (Oréal et al., 1998), and was identified as androgen-responsive gene in the trout testis (Rolland et al., 2013). Transforming Growth Factor Beta 3 (TGFB3) is involved in embryonic gonadal development (Memon et al., 2008), and the encoding mRNA is strongly downregulated in both sexes after MT treatment.

In our precedent study the mRNA encoding Gallinacin 10 (GAL10) was highly differential expressed between the sexes with an increased expression in the ovaries (Scheider et al., 2014b). Here, we report a dramatic, about 10-fold upregulation of *GAL10* transcription in male MT-treated embryos compared to the solvent control group while in female embryos it is not even doubled. The sex-dependent changes in *GAL10* expression therefore reflect the hormonal imbalance that leads to the observed histomorphological changes of gonad tissue in MT-treated chicken embryos. Taken together, the MT-induced differential expression seems to affect embryonic development by altered expression of important transcription factors such as MAFF, TFCP2, and SOX9, which in turn affect expression of secreted proteins such as GAL10.

3.5. Altered gonadal differentiation and gene expression in TBT-treated chicken embryos

TBT affects embryonic tissue in two different ways: the toxic and the hormonal component. Egg mortality increased significantly after treatment with the two highest TBT doses, which is clearly linked to increased toxicity. The second component of TBT is the hormonal reaction. As with MT, the cortex of female chicken gonads is generally thinner in TBT-treated embryos and likewise several females developed seminiferous cords in the medulla.

On the transcriptional level we find a significantly increased expression of *GAL10* in both sexes subsequent to TBT treatment compared to the solvent control whereof the male gonads reacted most prominently (Table 2). The upregulation in gonadal tissue from male embryos that were exposed to TBT corresponds to the 10-fold upregulation that is present subsequent to MT treatment. Interestingly, MT-treated female embryos do not show a strong differential expression of *GAL10*, while TBT treatment evokes a 3.3-fold upregulation. The fact that MT and TBT-treated males show an almost identical expression pattern of *GAL10*, which was previously identified as sex-specifically expressed in female chicken embryos, is an indicator of the hormonal reaction that is evoked by exposure to TBT. Two other members of the antimicrobial gallinacin family react to TBT treatment in a sex-dependent manner. The mRNAs encoding GAL1 and GAL6 are significantly downregulated after TBT treatment in females, while expression in male embryos is not

Table 2
Fold change of selected pluripotency and differentiation markers in response to MT and TBT in male and female gonads relative to the solvent control (S) in SuperSAGE data. Bonferroni correction * $p < 0.1$ ** $p < 0.5$ *** $p < 0.01$, ns = not significant, na = not available, red = upregulated, green = downregulated.

gene symbol	male				female			
	S-MT		S-TBT		S-MT		S-TBT	
CTCF	1.12	ns	1.37	ns	-1.14	ns	2.20	***
CTGF	1.05	ns	1.66	ns	-1.49	ns	2.54	***
CYP19A1	221.92	ns	na	na	-1.21	ns	-1.34	ns
FGF12	-3.13	***	-1.15	ns	-1.53	ns	2.57	***
GAL1	1.53	**	2.00	ns	1.16	ns	-2.29	***
GAL10	9.75	***	11.57	*	1.36	***	3.33	***
GAL6	1.03	ns	-1.55	ns	-2.01	***	-3.31	***
HSP70	-7.40	***	-1.57	ns	-6.54	***	-1.95	ns
MAFF	34.20	***	1.94	ns	2339.40	***	102.88	ns
NFIB	1.04	ns	9.87	***	-7.27	***	1.58	***
SOX9	3.97	***	-1.72	ns	272.62	ns	102.88	ns
TFCP2	14.29	***	-81.20	ns	6.49	ns	-63.54	ns
TGFB3	-12.03	***	1.33	ns	-10.02	***	4.20	ns

affected. Since *GAL6* is also significantly downregulated in MT-treated female embryos, downregulation of this gene is likely linked with the endocrine potential of TBT by provoking female-specific genes to be expressed in males. Downregulation of *GAL1*, on the other hand, might contribute to the stress response that is evoked by the toxic component of TBT.

The genes encoding connective tissue growth factor precursor (CTGF) and fibroblast growth factor 12 (FGF12) also display a sex-dependent differential expression subsequent to TBT treatment. The abundance of the mRNAs encoding both growth factors is significantly changed by the treatment and found to be more than doubled in female embryos. In contrast to the increased expression of *FGF12* in TBT-treated females, *FGF12* is also downregulated in gonadal tissue from MT-treated male embryos. Both growth factors are involved in embryogenesis and the increased expression might therefore contribute or result from the altered cortex structure that is observed in gonadal tissue from TBT-treated females at E19 (Pizette and Niswander, 1999). CCCTC-Binding Factor (CTCF) is an important transcriptional regulator that binds to tens of thousands of genomic sites in mammalian cells (Holwerda and de Laat, 2013), and the encoding mRNA is significantly upregulated in female gonad tissue compared to the solvent control. TGGCA-Binding Protein (NFIB) represents another important transcriptional regulator that is differentially expressed between the sexes subsequent to TBT treatment. The encoding mRNA is approximately 10-fold more abundant in male embryos in comparison to the sesame oil-treated group, but there is no differential expression between the corresponding female groups. However, we find an almost 7-fold downregulation of *NFIB* in gonadal tissue from MT-treated female embryos compared to the solvent control. TBT thus seems to evoke a contrary differential expression of *NFIB* in exposed female embryos as in MT-treated male embryos.

Interestingly, aromatase (CYP19A1) expression seems to be affected only by the solvent control. While its expression is increased about 4-fold subsequent to injection of the solvent, no significant differences are evoked by additional injection of TBT or MT. Since TBT is known to inhibit aromatase activity this implies that the induced expression of *CYP19A1* by the applied solvent forestalls a further differential expression or that this response has already passed by at embryonic day 19 (Bruggeman et al., 2002).

Other surveys that investigated the influence of organotin compounds on RNA expression generally focused on a small set of predefined genes. One of the few studies with a bigger set of genes was a 24 hour exposure of adult tunicates to 100 nM TBT where the expression of 13,400 protein coding genes was tested on a cDNA microarray (Azumi et al., 2004). Subsequent analysis revealed the differential expression of genes that are mainly involved in stress responses and detoxification. Due to the lack of a positive control the hormonal component of the reaction could not be determined in the data, and apparently the applied concentration of 100 nM of TBT already caused strong toxic effects. Among the stress-responsive genes in tunicates were members of the cytochrome P450 family as well as the 70 kd heat shock protein (HSP70). Although there are some indications for differentially expressed mRNAs that encode cytochrome P450 none of these genes exhibit any significant differential expression in comparison to the solvent control in our survey. *HSP70*, on the other hand, exhibits a strong differential expression in gonadal tissue from MT-treated embryos of both sexes, but also in tissue from the solvent control group. However, we did not detect a significant deregulation of *HSP70* expression in the TBT- groups.

4. Conclusions

Individuals are often most sensitive to endocrine disrupters in their early life stages (embryos, fetuses and juveniles). During these stages, endocrine disrupters interfere with developmental and organizational processes such as sexual differentiation (see e.g. (Ankley and Johnson, 2004; Holwerda and de Laat, 2013; Rolland et al., 2013)). In contrast to mammals, where steroid synthesis is initiated in the gonads after the termination of their differentiation, estrogen synthesis in birds begins in undifferentiated embryo gonads, which are thus more sensitive to hormonal manipulation (Trukhina et al., 2015). Exposure during this critical window of development may induce effects on sexual differentiation which are irreversible and thus persist in later life, whereas identical exposure levels during adulthood often result in temporary and reversible alterations of reproductive performance or of somatic parameters (Nichols et al., 2011). However, effects of early life stage exposures may only become apparent in the sexually mature organisms during the reproductive phase (Matthiessen and Johnson, 2007; Nichols et al., 2011). In view of their potentially serious consequences such delayed irreversible effects cause greatest concern (Si et al., 2012).

The present study provides the first survey linking the morphological alterations of gonadal tissue from chicken embryos that are induced by the known endocrine disrupter TBT with the underlying gene expression. We applied a single test compound injection at a very early developmental phase to simulate maternal transfer of the organotin compound. This treatment results in virilization of female gonads in a significant number of individuals and allowed us to identify differentially expressed transcription and growth factors that are associated with this phenotype in E19 embryos. Although the effect of the solvent cannot be neglected, MT that served as positive control allowed for discrimination of the toxic and hormonal effects induced by TBT. Surprisingly, we identified an aberrant expression of sex-dependent genes such as GAL10, but not of the mRNA encoding the TBT-inhibited aromatase. Since gonad development is well progressed at E19 aromatase inhibition might have already been compensated for. Our following studies will therefore aim to elucidate the transcriptional landscape after exposure to endocrine disruptive compounds in earlier embryonic stages of *Gallus gallus*. The final goal is to find parallel gene expression shifts in younger embryos to find a technique for an animal replacement study within the scope of a potential new 3R animal replacement method (Russel and Burch, 1959).

Conflict of interest statement

The authors have no conflict of interest to declare.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.toxlet.2017.11.019.

References

Abinawanto, Sai, C., Zhang, N., Matsuda, Y., Shimada, K., 1998. Identification of sperm-bearing female-specific chromosome in the sex-reversed chicken. J. Exp. Zool. 280 (1), 65–72 PubMed PMID: WOS000071437100008.

Acloque, H., Mey, A., Birot, A.M., Gruffat, H., Pain, B., Samarut, J., 2004. Transcription factor cCP2 controls gene expression in chicken embryonic stem cells. Nucl. Acids Res. 32 (7), 2259–2271 PubMed PMID: 15107494.

Ahn, R.S., Han, S.-J., Kim, S.C., Kwon, H.B., 2007. Effects of butyltin compounds on follicular steroidogenesis in the bullfrog (Rana catesbeiana). Environ. Toxicol. Pharmacol. 24 (2), 149–154. http://dx.doi.org/10.1016/j.etap.2007.04.005. PubMed PMID: WOS:000248980700011.

Alzieu, C., 2000. Impact of tributyltin on marine invertebrates. Ecotoxicology 9 (1–2), 71–76. http://dx.doi.org/10.1023/a:1008968229409. PubMed PMID: WOS:000088654100008.

Ankley, G.T., Johnson, R.D., 2004. Small fish models for identifying and assessing the effects of endocrine-disrupting chemicals. ILAR J. 45 (4), 469–483 PubMed PMID: WOS:000224480300010.

Antizar-Ladislao, B., 2008. Environmental levels, toxicity and human exposure to tributyltin (TBT)-contaminated marine environment. A review. Environ. Int. 34 (2), 292–308. http://dx.doi.org/10.1016/j.envint.2007.09.005.

Azumi, K., Fujie, M., Usami, T., Miki, Y., Satoh, N., 2004. A cDNA microarray technique applied for analysis of global gene expression profiles in tributyltin-exposed ascidians. Mar. Environ. Res. 58 (2–5), 543–546. http://dx.doi.org/10.1016/j.marenvres.2004.03.042. PubMed PMID: WOS000222199100063.

Bannister, S.C., Smith, C.A., Roeszler, K.N., Doran, T.J., Sinclair, A.H., Tizard, M.L.V., 2011. Manipulation of estrogen synthesis alters MIR202* expression in embryonic chicken gonads. Biol. Reprod.

Berg, C., Halldin, K., Brunstrom, B., Brandt, I., 1998. Methods for studying xenoestrogenic effects in birds. Toxicol. Lett. 103, 671–676 PubMed PMID: WOS:000078462400110.

Berg, C., Halldin, K., Fridolfsson, A.K., Brandt, I., Brunstrom, B., 1999. The avian egg as a test system for endocrine disrupters: Effects of diethylstilbestrol and ethynylestradiol on sex organ development. Sci. Total Environ. 233 (1-3), 57–66 PubMed PMID: WOS:000082518900006.

Berg, C., Halldin, K., Brunstrom, B., 2001a. Effects of bisphenol A and tetrabromobisphenol A on sex organ development in quail and chicken embryos. Environ. Toxicol. Chem. 20 (12), 2836–2840 PubMed PMID: WOS:000172420700024.

Berg, C., Holm, L., Brandt, I., Brunstrom, B., 2001b. Anatomical and histological changes in the oviducts of Japanese quail, Coturnix japonica, after embryonic exposure to ethynyloestradiol. Reproduction 121 (1), 155–165. http://dx.doi.org/10.1530/rep.0.1210155. PubMed PMID: WOS:000168328900016.

Bruggeman, V., Van As, P., Decuypere, E., 2002. Developmental endocrinology of the reproductive axis in the chicken embryo. Compar. Biochem. Physiol. A-Mol. Integrat. Physiol. 131 (4), 839–846 PubMed PMID: WOS:000174594600016.

Burke, W., Henry, M., 1999. Gonadal development and growth of chickens and turkeys hatched from eggs injected with an aromatase inhibitor. Poultry Sci. 78 (7), 1019–1033. http://dx.doi.org/10.1093/ps/78.7.1019.

Carere, C., Balthazart, J., 2007. Sexual versus individual differentiation: the controversial role of avian maternal hormones. Trends Endocrinol. Metab. 18 (2), 73–80. http://dx.doi.org/10.1016/j.tem.2007.01.003. PubMed PMID: WOS:000245067900006.

Cevasco, A., Urbatzka, R., Bottero, S., Massari, A., Pedemonte, F., Kloas, W., et al., 2008. Endocrine disrupting chemicals (EDC) with (anti)estrogenic and (anti)androgenic modes of action affecting reproductive biology of Xenopus laevis: II. Effects on gonad histomorphology. Compar. Biochem. Physiol. Part C: Toxicol. Pharmacol. 147 (2), 241–251. http://dx.doi.org/10.1016/j.cbpc.2007.10.001.

Chataigneau, T., Zerr, M., Chataigneau, M., Hudlett, F., Hirn, C., Pernot, F., et al., 2004. Chronic treatment with progesterone but not medroxyprogesterone acetate restores the endothelial control of vascular tone in the mesenteric artery of ovariectomized rats. Menopause – J. N. Am. Menopause Soc. 11 (3), 255–263. http://dx.doi.org/10.1097/01.gme.0000097847.95550.e3. PubMed PMID: WOS000221395400005.

Chau, Y.K., Maguire, R.J., Brown, M., Yang, F., Batchelor, S.P., Thompson, J.A.J., 1997. Occurrence of butyltin compounds in mussels in Canada. Appl. Organom. Chem. 11 (10–11), 903–912. http://dx.doi.org/10.1002/(SICI)1099-0739(199710/11)11:10/11<903:AID-AOC658>3.0.CO;2-C.

Cunha, G.R., Ricke, W., Thomson, A., Marker, P.C., Risbridger, G., Hayward, S.W., et al., 2004. Hormonal, cellular, and molecular regulation of normal and neoplastic prostatic development. J. Steroid Biochem. Mol. Biol. 92 (4), 221–236. http://dx.doi.org/10.1016/j.jsbmb.2004.10.017. PubMed PMID: WOS:000227022500002.

Davies, I.M., Harding, M.J.C., Bailey, S.K., Shanks, A.M., Lange, R., 1997. Sublethal effects of tributyltin oxide on the dogwhelk Nucella lapillus. Mar. Ecol. Prog. Series 158, 191–204. http://dx.doi.org/10.3354/meps158191. PubMed PMID: WOS:A1997YK60700017.

Demissie, M., Lazic, M., Foecking, E.M., Aird, F., Dunaif, A., Levine, J.E., 2008. Transient prenatal androgen exposure produces metabolic syndrome in adult female rats. Am. J. Physiol. Endocrinol. Metab. 295 (2), E264–E268. http://dx.doi.org/10.1152/ajpendo.90208.2008.

Dmetrichuk, J.M., Carlone, R.L., Jones, T.R.B., Vesprini, N.D., Spencer, G.E., 2008. Detection of endogenous retinoids in the molluscan CNS and characterization of the trophic and tropic actions of 9-cis retinoic acid on isolated neurons. J. Neurosci. 28 (48), 13014–13024. http://dx.doi.org/10.1523/jneurosci.3192-08.2008. PubMed PMID: WOS:000261191700043.

Dubey, S.K., Roy, U., 2003. Review Biodegradation of tributyltins (organotins) by marine bacteria. Appl. Organomet. Chem. 17 (1), 3–8. http://dx.doi.org/10.1002/aoc.394.

EFSA. Opinion of the Scientific Panel on Contaminants in the Food Chain on a request from the Commission to as sess the health risks to consumers associated with exposure to organotins in foodstuffs (Question N(EFSA-Q-2003-110) 2004.

Eising, C.M., Eikenaar, C., Schwabl, H., Groothuis, T.G.G., 2001. Maternal androgens in black-headed gull (Larus ridibundus) eggs: consequences for chick development. Proc. R. Soc. Lond. B: Biol. Sci. 268 (1469), 839–846. http://dx.doi.org/10.1098/rspb.2001.1594.

Fail, P.A., Hines, J.W., Zacharewski, T., Wu, Z.F., Borodinsky, L., 1998. Assessment of polystyrene extract for estrogenic activity in the rat uterotrophic model and an in vitro recombinant receptor reporter gene assay. Drug Chem. Toxicol. 21, 101–121. http://dx.doi.org/10.3109/01480549809007405. PubMed PMID: WOS:000078668100006.

Faucounau, N., Ichas, F., Stoll, R., Maraud, R., 1995. Action of testosterone on the estradiol-induced feminization of the male chick-embryo. Anat. Embryol. 191 (4), 377–379. http://dx.doi.org/10.1007/bf00534691. PubMed PMID: WOSA1995QM21700011.

Feist, G., Yeoh, C.G., Fitzpatrick, M.S., Schreck, C.B., 1995. The production of functional sex-reveresd male rainbow trout with 17-alpha-Methyltestosterone and 11-beta-Hydroxyandrostendione. Aquaculture 131 (1-2), 145–152. http://dx.doi.org/10.1016/0044-8486(94)00336-m. PubMed PMID: WOS:A1995QN19600013.

Fridolfsson, A.K., Ellegren, H., 1999. A simple and universal method for molecular sexing of non-ratite birds. J. Avian Biol. 30 (1), 116–121 PubMed PMID: WOS:000079569700017.

Gadd, G.M., 2000. Microbial interactions with tributyltin compounds: detoxification, accumulation, and environmental fate. Sci. Total Environ. 258 (1), 119–127. http://dx.doi.org/10.1016/S0048-9697(00)00512-X.

Giusti, A., Leprince, P., Mazzucchelli, G., Thome, J.P., Lagadic, L., Ducrot, V., et al., 2013. Proteomic analysis of the reproductive organs of the hermaphroditic gastropod Lymnaea stagnalis exposed to different endocrine disrupting chemicals. Plos One. 8 (11). http://dx.doi.org/10.1371/journal.pone.0081086. PubMed PMID: WOS:000327311900114.

Gooding, M.P., LeBlanc, G.A., 2001. Biotransformation and disposition of testosterone in the eastern mud snail Ilyanassa obsoleta. General Comp. Endocrinol. 122 (2), 172–180. http://dx.doi.org/10.1006/gcen.2001.7630. PubMed PMID: WOS:000168466300006.

Gooding, M.P., Wilson, V.S., Folmar, L.C., Marcovich, D.T., LeBlanc, G.A., 2003. The biocide tributyltin reduces the accumulation of testosterone as fatty acid esters in the mud snail (Ilyanassa obsoleta). Environmental Health Perspectives. 111 (4), 426–430. http://dx.doi.org/10.1289/ehp.5779. PubMed PMID: WOS:000182144300026.

Guruge, K.S., Iwata, H., Tanaka, H., Tanabe, S., 1997. Butyltin accumulation in the liver and kidney of seabirds. Mar. Environ. Res. 44 (2), 191–199. http://dx.doi.org/10.1016/S0141-1136(97)00001-9.

Heidrich, D.D., Steckelbroeck, S., Klingmuller, D., 2001. Inhibition of human cytochrome P450 aromatase activity by butyltins. Steroids 66 (10), 763–769. http://dx.doi.org/10.1016/S0039-128X(01)00108-8.

His, E., Robert, R., 1985. Developpement des véligères de Crassostrea gigas dans le Bassin d'Arcachon études sur les mortalites larvaires. Revue des Travaux de l'Institut des Pêches Maritimes 47 (1–2), 63–88 PubMed PMID: ZOOREC:ZOOR12200053784.

Holwerda, S.J., de Laat, W., 2013. CTCF: the protein, the binding partners, the binding sites and their chromatin loops. Philos. Trans. R. Soc. Lond., B, Biol. Sci. 368 (1620), 20120369 Epub 2013/05/08. 10.1098/rstb.2012.0369. PubMed PMID: 23650640; PubMed Central PMCID: PMC3682731.

Jessl, L., Scheider, J., Oehlmann, J., 2017. The domestic fowl (Gallus gallus domesticus) embryo as an alternative for animal experiments – baseline experiments to determine parameter variability in control groups. BMC Dev. Biol under revision.

Kamata, R., Takahashi, S., Shimizu, A., Shiraishi, F., 2006. Avian transgenerational reproductive toxicity test with in ovo exposure. Arch. Toxicol. 80 (12), 846–856 PubMed PMID: WOS:000242334300009.

Kannan, K., Falandysz, J., 1997. Butyltin residues in sediment, fish, fish-eating birds, harbour porpoise and human tissues from the Polish coast of the Baltic Sea. Mar. Pollut. Bull. 34 (3), 203–207. http://dx.doi.org/10.1016/S0025-326X(96)00146-4.

Kannan, K., Senthilkumar, K., Elliott, J.E., Feyk, L.A., Giesy, J.P., 1998. Occurrence of Butyltin Compounds in Tissues of Water Birds and Seaducks from the United States and Canada. Arch. Environ. Contam. Toxicol. 35 (1), 64–69. http://dx.doi.org/10.1007/s002449900350.

Koba, N., Mori, M., Ha, Y., Mizushima, S., Tsukada, A., Saito, N., et al., 2008. Effects of aromatase inhibitor (Fadrozole)-induced sex-reversal on gonadal differentiation and

mRNA expression of p450arom, AMH and ER alpha in embryos and growth in posthatching quail. J. Poult. Sci. 45 (2), 116–124 PubMed PMID: WOS:000255308400005.
Lagarde, F., Beausoleil, C., Belcher, S.M., Belzunces, L.P., Emond, C., Guerbet, M., et al., 2015. Non-monotonic dose-response relationships and endocrine disruptors: a qualitative method of assessment. Environ. Health 14 (1), 13. http://dx.doi.org/10.1186/1476-069x-14-13.
Lecoin, L., Sii-Felice, K., Pouponnot, C., Eychene, A., Felder-Schmittbuhl, M.P., 2004. Comparison of maf gene expression patterns during chick embryo development. Gene Express. Patterns 4 (1), 35–46 Epub 2003/12/18. PubMed PMID: 14678826.
Liang, T., Ouyang, J., Yi, L., Chu, Y., Liu, C., 2013. Behavioral changes of rats after short-term exposure to di-(2-ethyl hexyl) phthalate. J. South. Med. Univ. 33 (3), 401–405 PubMed PMID: MEDLINE:23529240.
Man, M.Z., Wang, X., Wang, Y., 2000. POWER_SAGE: Comparing statistical tests for SAGE experiments. Bioinformatics. 16 (11), 953–959. http://dx.doi.org/10.1093/bioinformatics/16.11.953.
Marker, P.C., Donjacour, A.A., Dahiya, R., Cunha, G.R., 2003. Hormonal, cellular, and molecular control of prostatic development. Dev. Biol. 253 (2), 165–174. http://dx.doi.org/10.1016/s0012-1606(02)00031-3. PubMed PMID: WOS:000180688400001.
Matthiessen, P., Gibbs, P.E., 1998. Critical appraisal of the evidence for tributyltin-mediated endocrine disruption in mollusks. Environ. Toxicol. Chem. 17 (1), 37–43. http://dx.doi.org/10.1897/1551-5028(1998)017<0037:caotef>2.3.co;2. PubMed PMID: WOS:000071120200006.
Matthiessen, P., Johnson, I., 2007. Implications of research on endocrine disruption for the environmental risk assessment, regulation and monitoring of chemicals in the European Union. Environ. Pollut. 146 (1), 9–18. http://dx.doi.org/10.1016/j.envpol.2006.05.036. PubMed PMID: WOS:000244595200003.
McAllister, B.G., Kime, D.E., 2003. Early life exposure to environmental levels of the aromatase inhibitor tributyltin causes masculinisation and irreversible sperm damage in zebrafish (Danio rerio). Aquat. Toxicol. 65 (3), 309–316. http://dx.doi.org/10.1016/s0166-445x(03)00154-1. PubMed PMID: WOS:000185544500007.
Memon, M.A., Anway, M.D., Covert, T.R., Uzumcu, M., Skinner, M.K., 2008. Transforming growth factor beta (TGFbeta1, TGFbeta2 and TGFbeta3) null-mutant phenotypes in embryonic gonadal development. Mol Cell Endocrinol. 294 (1-2), 70–80 Epub 2008/09/16. 10.1016/j.mce.2008.08.017. PubMed PMID: 18790002; PubMed Central PMCID: PMC2593935.
Muller, W., Groothuis, T.G.G., Eising, C.M., Dijkstra, C., 2005. An experimental study on the causes of sex-biased mortality in the black-headed gull – the possible role of testosterone. J. Anim. Ecol. 74 (4), 735–741. http://dx.doi.org/10.1111/j.1365-2656.2005.00964.x. PubMed PMID: WOS:000230601800018.
Nakanishi, T., J-i, Nishikawa, Tanaka, K., 2006. Molecular targets of organotin compounds in endocrine disruption: Do organotin compounds function as aromatase inhibitors in mammals? Environ. Sci. 13 (2), 89–100 PubMed PMID: BCI:BCI200600607848.
Nichols, J.W., Reen, M., Denver, R.J., Distefano, J.J., Edwards, J.S., Hoke, R.A., et al., 2011. Predicting chemical impacts on vertebrate endocrine systems. Environ. Toxicol. Chem. 30 (1), 39–51.
Nishikawa, J., Mamiya, S., Kanayama, T., Nishikawa, T., Shiraishi, F., Horiguchi, T., 2004. Involvement of the retinoid X receptor in the development of imposex caused by organotins in gastropods. Environ. Sci. Technol. 38 (23), 6271–6276. http://dx.doi.org/10.1021/es049593u. PubMed PMID: WOS:000225532900022.
OECD. Detailed review paper (DRP) on mollusc life-cycle toxicity testing. OECD Series on Testing and Assessment No. 121. ENV/JM/MONO(2010)9. 2010.
Oberdörster, E., McClellan-Green, P., 2000. The neuropeptide APGWamide induces imposex in the mud snail, Ilyanassa obsoleta. Peptides 21 (9), 1323–1330. http://dx.doi.org/10.1016/s0196-9781(00)00274-6. PubMed PMID: WOS:000165202000003.
Oehlmann, J., Bauer, B., Minchin, D., Schulte-Oehlmann, U., Fioroni, P., Markert, B., 1998. Imposex in Nucella lapillus and intersex in Littorina littorea: Interspecific comparison of two TBT-induced effects and their geographical uniformity. Hydrobiologia 378, 199–213. http://dx.doi.org/10.1023/a:1003218411850. PubMed PMID: WOS:000078311300023.
Oréal, E., Pieau, C., Mattei, M.G., Josso, N., Picard, J.Y., Carre-Eusebe, D., et al., 1998. Early expression of AMH in chicken embryonic gonads precedes testicular SOX9 expression. Dev. Dyn. 212 (4), 522–532 PubMed PMID: MEDLINE:9707325.
Pizette, S., Niswander, L., 1999. BMPs negatively regulate structure and function of the limb apical ectodermal ridge. Development 126 (5), 883–894.
Ricke, W.A., Ricke, E.A., Simko, J., Cunha, G.R., 2004. Antiandrogens act as chemopreventatives in prostate cancer progression. Cancer Epidemiol. Biomarkers Prevent. 13 (11), 1839S PubMed PMID: WOS:000225073100043.
Rodríguez-González, P., Encinar, J.R., Alonso, J.I.G., Sanz-Medel, A., 2006. Contamination of the Coastal Waters of Gijón (North West Spain) by Butyltin Compounds. Water, Air, Soil Pollut. 174 (1), 127. http://dx.doi.org/10.1007/s11270-005-9057-y.
Rolland, A.D., Lardenois, A., Goupil, A.S., Lareyre, J.J., Houlgatte, R., Chalmel, F., et al., 2013. Profiling of androgen response in rainbow trout pubertal testis: relevance to male gonad development and spermatogenesis. Plos One. 8 (1), e53302 Epub 2013/01/10. 10.1371/journal.pone.0053302. PubMed PMID: 23301058; PubMed Central PMCID: PMC3536770.
Russel W, Burch R. The Principles of Humane Experimental Technique. Reprinted by UFAW, 1992: 8 Hamilton Close, South Mimms, Potters Bar, Herts EN6 3QD England ed. Methuen, editor. London 1959. ()
Scheider, J., Afonso-Grunz, F., Hoffmeier, K., Horres, R., Groher, F., Rycak, L., et al., 2014a. Gene expression of chicken gonads is sex- and side-specific. Sexual development: genetics, molecular biology, evolution, endocrinology, embryology, and pathology of sex determination and differentiation. Sex. Dev. 8 (4), 178–191. http://dx.doi.org/10.1159/000362259. PubMed PMID: MEDLINE:24820130.
Scheider, J., Afonso-Grunz, F., Hoffmeier, K., Horres, R., Groher, F., Rycak, L., et al., 2014b. Gene expression of chicken gonads is sex- and side-specific. Sex Dev. 8 (4), 178–191 Epub 2014/05/14. 10.1159/000362259. PubMed PMID: 24820130.
Scholz, S., Sela, E., Blaha, L., Braunbeck, T., Galay-Burgos, M., García-Franco, M., et al., 2013. A European perspective on alternatives to animal testing for environmental hazard identification and risk assessment. Regulat. Toxicol. Pharmacol. 67 (3), 506–530. http://dx.doi.org/10.1016/j.yrtph.2013.10.003.
Sekizawa Jun, Suter II Glenn, Linda B. 2017 Case study: Tributyltin and triphenyltin compounds: WHO/IPCS/IRA/01/12; 2001. Available from: http://www.who.int/ipcs/publications/new_issues/ira/en/.
Shibuya, K., Mizutani, M., Wada, M., Sato, K., Nunoya, T., 2004. A new screening model using F1(AWE × WE) japanese quail embryo for evaluating sex reversal effects. J. Toxicol. Pathol. 17, 245–252.
Si, J., Han, X., Zhang, F., Xin, Q., An, L., Li, G., et al., 2012. Perinatal exposure to low doses of tributyltin chloride advances puberty and affects patterns of estrous cyclicity in female mice. Environ. Toxicol. 27 (11), 662–670. http://dx.doi.org/10.1002/tox.21756.
Singh, A.K., 2013. Introduction of modern endocrine techniques for the production of monosex population of fishes. Gen. Compar. Endocrinol. 181 (0), 146–155. http://dx.doi.org/10.1016/j.ygcen.2012.08.027.
Stäb, J.A., Traas, T.P., Stroomberg, G., van Kesteren, J., Leonards, P., van Hattum, B., et al., 1996. Determination of organotin compounds in the foodweb of a shallow freshwater lake in The Netherlands. Arch. Environ. Contam. Toxicol. 31 (3), 319–328. http://dx.doi.org/10.1007/bf00212670.
Stroben, E., Oehlmann, J., Fioroni, P., 1992. Hinia reticulata and Nucella lapillus – comparison of 2 gastropod tributyltin bioindicators. Mar. Biol. 114 (2), 289–296. http://dx.doi.org/10.1007/bf00349532. PubMed PMID: WOS:A1992JV69000016.
Trukhina, A.V., Lukina, N.A., Nekrasova, A.A., Smirnov, A.F., 2015. Sex inversion and epigenetic regulation in vertebrates. Russ. J. Genet. 51 (3), 231–237. http://dx.doi.org/10.1134/s1022795415020155. PubMed PMID: WOS:000351529900002.
Utsumi, T., Yoshimura, Y., 2009. Sensitive embryonic endpoints with in ovo treatment for detecting androgenic and anti-androgenic effects of chemicals in Japanese quail (Coturnix japonica). Poult. Sci. 88 (5), 1052–1059. http://dx.doi.org/10.3382/ps.2008-00326. PubMed PMID: WOS:000265405900023.
Vaillant, S., Magre, S., Dorizzi, M., Pieau, C., Richard-Mercier, N., 2001. Expression of AMH, SF1, and SOX9 in gonads of genetic female chickens during sex reversal induced by an aromatase inhibitor. Dev. Dyn. 222 (2), 228–237 PubMed PMID: WOS:000171382200009.
Vaillant, S., Guemene, D., Dorizzi, M., Pieau, C., Richard-Mercier, N., Brillard, J.P., 2003. Degree of sex reversal as related to plasma steroid levels in genetic female chickens (Gallus domesticus) treated with fadrozole. Mol. Reprod. Dev. 65 (4), 420–428 PubMed PMID: WOS:000184122500009.
Vandenberg, L.N., Colborn, T., Hayes, T.B., Heindel, J.J., Jacobs, J.D.R., Lee, D.-H., et al., 2012. Hormones and Endocrine-Disrupting Chemicals: Low-Dose Effects and Nonmonotonic Dose Responses. Endocrine Rev. 33 (3), 378–455. http://dx.doi.org/10.1210/er.2011-1050.
Veltman, K., Huijbregts, M.A.J., van den Heuvel-Greve, M.J., Vethaak, A.D., Hendriks, A.J., 2006. Organotin accumulation in an estuarine food chain: Comparing field measurements with model estimations. Mar. Environ. Res. 61 (5), 511–530. http://dx.doi.org/10.1016/j.marenvres.2006.02.005.
Wallenburg, J., 1982. Macroscopy, light and electron microscopy studies on the genesis and function of the gonads after experimental sex-reversal following left-side ovariectomy of hen chicks (Gallus domesticus). Gegenbaurs Morphologisches Jahrbuch 128 (4), 463–529 PubMed PMID: MEDLINE:6891351.
Wang, X., Fang, C., Hong, H., Wang, W.-X., 2010. Gender differences in TBT accumulation and transformation in Thais clavigera after aqueous and dietary exposure. Aquat. Toxicol. 99 (3), 413–422. http://dx.doi.org/10.1016/j.aquatox.2010.06.001. PubMed PMID: WOS:000281493400012.
Wartenberg, H., Lenz, E., Schweikert, H.U., 1992. Sexual differentiation and the germ cell in sex reversed gonads after aromatase inhibition in the chicken embryo. Andrologia. 24 (1), 1–6. http://dx.doi.org/10.1111/j.1439-0272.1992. tb02599.x.
Yamasaki, K., Takeyoshi, M., Sawaki, M., Imatanaka, N., Shinoda, K., Takatsuki, M., 2003. Immature rat uterotrophic assay of 18 chemicals and Hershberger assay of 30 chemicals. Toxicology. 183 (1–3), 93–115. http://dx.doi.org/10.1016/S0300-483X(02)00445-6.
Yamazaki, T., Shimodaira, M., Kuwahara, H., Wakatsuki, H., Horiuchi, H., Matsuda, H., et al., 2005. Tributyltin disturbs bovine adrenal steroidogenesis by two modes of action. Steroids 70 (14), 913–921. http://dx.doi.org/10.1016/j.steroids.2005.06.005. PubMed PMID: BCI:BCI200600205951.
Yang, X., Zheng, J., Qu, L., Chen, S., Li, J., Xu, G., et al., 2011. Methylation status of cMHM and expression of sex-specific genes in adult sex-reversed female chickens. Sex. Dev. 5 (3), 147–154. http://dx.doi.org/10.1159/000327712. PubMed PMID: WOS:000291350600006.
Zhang, J., Zuo, Z., Chen, Y., Zhao, Y., Hu, S., Wang, C., 2007. Effect of tributyltin on the development of ovary in female cuvier (Sebastiscus marmoratus). Aquat. Toxicol. 83 (3), 174–179. http://dx.doi.org/10.1016/j.aquatox.2007.03.018. PubMed PMID: WOS:000248277300002.
Zhang, J., Zuo, Z., Wang, Y., Yu, A., Chen, Y., Wang, C., 2011. Tributyltin chloride results in dorsal curvature in embryo development of Sebastiscus marmoratus via apoptosis pathway. Chemosphere 82 (3), 437–442. http://dx.doi.org/10.1016/j.chemosphere.2010.09.057.
Zhang, J., Zuo, Z., Xiong, J., Sun, P., Chen, Y., Wang, C., 2013. Tributyltin exposure causes lipotoxicity responses in the ovaries of rockfish, Sebastiscus marmoratus. Chemosphere 90 (3), 1234–1299. http://dx.doi.org/10.1016/j.chemosphere.2012.10.078. PubMed PMID: WOS:000312978700053.
von Engelhardt, N., Carere, C., Dijkstra, C., Groothuis, T.G.G., 2006. Sex-specific effects of yolk testosterone on survival, begging and growth of zebra finches. Proceedings of the Royal Society B-Biological Sciences 273 (1582), 65–70. http://dx.doi.org/10.1098/rspb.2005.3274. PubMed PMID: WOS:000234648100010.

A.4 Publikationen und Tagungsbeiträge

ORIGINALARBEITEN

- JESSL L, LENZ R, MASSING FG, **SCHEIDER J**, OEHLMANN J (2018): Effects of estrogens and antiestrogens on gonadal sex differentiation and embryonic development in the domestic fowl (*Gallus gallus domesticus*). PeerJ 6, e5094. doi: 10.7717/peerj.5094.
- JESSL L, **SCHEIDER J**, OEHLMANN J (2018): The domestic fowl (*Gallus gallus domesticus*) embryo as an alternative for animal experiments - validation of a novel method for the detection of endocrine disrupting chemicals. Chemosphere **196:** 502-513.
- **SCHEIDER J**, AFONSO-GRUNZ F, JESSL L, HOFFMEIER K, WINTER P, OEHLMANN J (2018): Morphological and transcriptomic effects of endocrine modulators on the gonadal differentiation of chicken embryos: the case of tributyltin (TBT). Toxicology Letters **284:** 143-151.
- MÜLLER S, RYCAK L, AFONSO-GRUNZ F, WINTER P, ZAWADA AM, DAMRATH E, **SCHEIDER J**, SCHMÄH, J, KOCH I, KAHL G, ROTTER B (2014): APADB: A database for alternative polyadenylation and microRNA regulation events. Database, DOI: 10.1093/database/bau076.
- **SCHEIDER J**, AFONSO-GRUNZ F, HOFFMEIER K, HORRES R, GROHER F, RYCAK L, OEHLMANN J & WINTER P (2014): Gene expression of chicken gonads is sex- and side-specific. Sexual Development **8**(4): 178-91 (DOI: 10.1159/000362259).
- DUIS K, **SCHEIDER J**, WARNECKE D, VAN DER VEEN A, COORS A, KNACKER T, SCHÄFERS C (2014): Substances of very high concern under REACh – an evaluation of uncertainties in the environmental risk assessment of endocrine active substances. Final report, UBA-project FKZ 3710 63 416.
- **SCHEIDER J**, WINTER P, OEHLMANN J (2012): A novel animal replacement system for the detection of endocrine disruptive capabilities in sexual development. Naunyn-Schmiedebergs Arch. Pharmacol. **385** (Suppl. 1): 109.
- SANTOS MC, WAGNER M, WU B, **SCHEIDER J**, OEHLMANN J, CADORE S, BECKER JS (2009): Biomonitoring of metal contamination in a marine prosobranch snail (*Nassarius reticulatus*) by imaging laser ablation inductively coupled plasma mass spectrometry (LA-ICP-MS). Talanta **80**: 428-433.

- **SCHEIDER J** & OEHLMANN J (2009): The chicken embryo as a model organism for detection of potential endocrine disruptors. Comp. Biochem. Physiol. A **154**, 19.
- **SCHEIDER J**, WINK M, WILTSCHKO W, STUBBE M (2009): Zur Phylogenie und Taxonomie der Schwarzmilan- Subspezies. – In: STUBBE M, MAMMEN U (eds.): Populationsökologie von Greifvogel- und Eulenarten **6**: 233-241.
- **SCHEIDER J**, WINK M, STUBBE M, HILLE S, WILTSCHKO W (2004): Phylogeographic relationships of the Black kite *Milvus migrans.* In: Chancellor RD & Meyburg BU (eds.): Raptors worldwide, 467-472.

POSTER UND VORTRÄGE

- HUISINGA M, FLICK B, KRELING N, WELTJE L, OTT K, JESSL L, **SCHEIDER J**, GRÖTERS S, VAN RAVENZWAAY B: A fast screening tool for the early indicator phase of sexual development in birds: Histopathological evaluation of avian gonads from chicks exposed *in ovo*. Society of Toxicologic Pathology, Raleigh, USA (06/19), Poster.
- HUISINGA M, FLICK B, KRELING N, WELTJE L, OTT K, JESSL L, **SCHEIDER J**, GRÖTERS S, VAN RAVENZWAAY B: Histopathological evaluation of gonads from chicks exposed *in ovo* as an alternative method for endocrine endpoints in birds. Joint European Congress of the ESVP, ESTP and ECVP, Lyon, Frankreich (08/17), Poster.
- DUIS K, **SCHEIDER J**, WARNECKE D, VAN DER VEEN A, COORS A, SCHÄFERS C: Uncertainties in the assessment of environmental effects of chemicals with endocrine activity – a literature study. SETAC Europe Basel, Schweiz (05/14), Poster.
- **SCHEIDER J**, WINTER P & OEHLMANN J: A novel animal replacement system for the detection of endocrine disruptive capabilities in sexual development. SETAC World Berlin (05/12), Vortrag.
- MODIRNOURI E, **SCHEIDER J**, WINTER P, & OEHLMANN J: Vermännlichung? Verweiblichung? Einfluss von Bisphenol A auf die Genexpression beim Modellsystem Hühnerembryo. SETAC GLB Landau (09/11), Poster.
- **SCHEIDER J,** WINTER P & OEHLMANN J: Entwicklung und Evaluierung eines sensitiven und kostengünstigen Tierersatzsystems für die Abschätzung des Hormon-toxischen

Potenzials von Chemikalien als Disruptoren der embryonalen Gonadenentwicklung. Berlin, BMBF (05/11), Vortrag.

- **SCHEIDER J,** HOFFMEIER K, WINTER P & OEHLMANN J: Der Einfluss von (TBT) auf die Geschlechtsentwicklung von Hühnern: Eine Analyse via SuperTAG Digital Gene Expression (STDGE) Profiling. 4. Gemeinsame Tagung von SETAC GLB und GDCh Dessau (09/10), Vortrag.
- WINTER P, **SCHEIDER J** & OEHLMANN J: Entwicklung einer Ersatzmethode am Hühnerei zur Testung endokriner Disruptoren. 4. Gemeinsame Tagung von SETAC GLB und GDCh Dessau (09/10), Poster.
- MODENBACH A, **SCHEIDER J**, OEHLMANN J & WINTER P: Auswirkungen von Bisphenol A auf die embryonale Geschlechtsentwicklung von Hühnerembryonen. 4. Gemeinsame Tagung von SETAC GLB und GDCh Dessau (09/10), Vortrag.
- **SCHEIDER J**, ROTTER B, HOFFMEIER K, OEHLMANN J & WINTER P: Testes and Ovary-specific SuperTAG Digital Gene Expression (STDGE) Profiles in Chicken Embryos. Plant and Animal Genome Conference (PAG) San Diego (01/10), Poster.
- **SCHEIDER J**, HOFFMEIER K, RYZAK L, ROTTER B, WINTER P & OEHLMANN J: The Impact of Tributyltin (TBT) on Chicken Sexual Development: Analysis via SuperTAG Digital Gene Expression (STDGE) Profiling. SETAC-Europe Sevilla (05/10), Poster.
- **SCHEIDER J** & OEHLMANN J: The Chicken Embryo as a Model Organism for Detection of Potential Endocrine Disruptors. 5. Workshop on Endocrine Disrupters Kopenhagen (05/09), Poster.
- **SCHEIDER J** & OEHLMANN J: Wirkung von TBT auf die Geschlechtsausprägung von Hühnern. 3. Gemeinsame Tagung von SETAC GLB und GDCh Frankfurt (10/08), Poster.
- **SCHEIDER J,** WINK M, STUBBE M, HILLE S & WILTSCHKO W: Phylogeographic relationships of the Black kite Milvus migrans. World Working Group of Birds of Prey and Owls in Budapest (05/03), Poster.