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Discussion

Interpretation of sexual secondary characteristics (SSCs) in regulatory testing for endocrine activity in fish

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ABSTRACT

Secondary sexual characteristics (SSCs) are important features that have evolved in many fish species because of inter-individual competition for mates. SSCs are crucial not only for sexual selection, but also for other components of the reproductive process and parental care. Externally, they are especially clear in males (for instance, tubercles, fatpad, anal finnage, colouration) but are also externally present in the females (for instance, ovipositor). These characters are under hormonal control and as such there has been much interest in incorporating them as measures in fish test methods to assess the potential endocrine activity of chemicals. Here we describe the external SSCs in typical laboratory test species for endocrine testing - fathead minnow (*Pimephales promelas*), Japanese medaka (*Oryzias latipes*), zebrafish (*Danio rerio*) and the three-spined stickleback (*Gasterosteus aculeatus* L.). We also provide some examples and discuss the utility of SSC responses to the endocrine activity of chemicals in the field and the laboratory. This paper is not aimed to provide a comprehensive review of SSCs in fish but presents a view on the assessment of SSCs in regulatory testing. Due to the current regulatory importance of establishing an endocrine mode-of-action for chemicals, we also consider other, non-endocrine factors that may lead to SSC responses in fish. We conclude with recommendations for how the assessment of SSCs in fish could be usefully incorporated into the endocrine hazard and risk assessment of chemicals.

1. Introduction

Secondary sexual characteristics (SSCs, defined in the next section) are key features important for sexual selection processes during the reproduction of many animal species, including fish. The development of SSCs are under hormonal control, which has led to incorporation of these features into many of the newer regulatory fish test guidelines. In these testing methods, SSCs serve as potential indicators of hormone activity disturbance by chemicals. Consequently, there is a need to establish how SSCs can be interpreted in the evaluation of fish studies as valuable indicators for endocrine hazard and aquatic risk assessment.

2. Secondary sexual characteristics

Secondary sexual characteristics are features that appear at sexual maturity and distinguish the two sexes of a species under normal development. SSCs can be present in both males and females (Houde, 2001). SSCs are typically under hormonal control and are therefore potential markers for interactions of chemicals with the endocrine system (OECD, 2018). Purdom, (1993) distinguishes the sexes based on primary and secondary characters, where primary characters include the gonads and sex-specific hormone expression profiles. SSCs are divided into requisite and accessory features. Requisite characters include those for normal sexual expression such as efferent ducts or intromittent organs (e.g. gonopodium) whilst accessory characters such as size, colour and finnage function primarily in inter-individual competition and sexual selection. They may be permanent or temporary features. Although SSCs are typically more developed in males, females of many species also develop SSCs including weaponry and ornamentation in addition to aggressive behaviour (Houde, 2001;

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Andersen et al., 2002; Baldauf et al., 2010; Goymann and Wingfield, 2014).

As the development and manifestation of SSCs is, at least partly, under control of sex steroid hormones (Wootton and Smith, 2014) there has been great interest in using them to assess for potential endocrine effects of chemicals, which can affect their normal development and so SSCs are used as a diagnostic tool. One of the best described examples concerns the effects described near Canadian pulp mills. Howell et al., (1980) and Bortone and Cody, (1999) reported the development of a male SSC (a gonopodium) in females of the western mosquitofish *Gambusia affinis* downstream of a pulp mill effluent. Similar effects have been observed in mosquitofish exposed to New Zealand pulp effluent. Indeed, there are many accounts of the hypertrophy of male SSCs in several species exposed to paper/pulp mill effluents (Larsson and Förlin, 2002). *Vice versa*, long term exposure of fathead minnows (*Pimephales promelas*) from embryos to adults to effluents of a leached sulfite mill led to the development of female secondary characters in the majority of fish (Parrott et al., 2003). In bream (*Abramis brama*) from the River Elbe in Germany, Hecker et al., (2007), observed significantly reduced numbers of spawning tubercles in males. This effect was associated with altered steroidogenesis as evident from reduced 11-ketotestosterone (11-KT) levels. In addition, brain aromatase activity was reduced (approximately by 50% at some sites). However, the causative contaminant was unclear since some of the sampled stretches were probably subject to contamination from both sewage discharges and agricultural runoff. Masculinization in fish has also been observed in China, where Xie et al., (2010) have shown that some female mosquitofish (*G. affinis*) caught in the heavily sewage and industrial effluent-contaminated Hanxi River have masculinized (elongated) anal fins.

3. Secondary sexual characters in regulatory fish tests

SSCs are employed in several OECD fish test guidelines (OECD, 2009; 2011b, 2012b, 2015) and a fish test guidance document (OECD, 2011a). These tests fall into two categories, based on their position in the OECD's Conceptual Framework for Testing and Assessment of Endocrine Disrupters (as revised in 2012[1]). Broadly these tests describe *in vivo* assays for screening or generating definitive test data. Screening tests are used to identify if a substance has the potential to interact with the fish endocrine system. These screens typically fall in levels 3 and 4 of the framework and include the Fish Short Term Reproduction Assay (OECD, 2012b), A Short-Term Screening for Oestrogenic and Androgenic Activity, and Aromatase Inhibition (OECD, 2009) and the Fish Sexual Development Test (OECD, 2011b). The guidance document on the androgenised female stickleback screen (OECD, 2011a) is included at level 3 of the framework. Currently, there is only one definitive test at level 5 of the framework, the Medaka Extended One Generation Reproduction Test ('MEOGRT') (OECD, 2015). However, there are proposals to develop an analogous test design using zebrafish and a long history of performing life-cycle studies in fathead minnow (Crane et al., 2010). The purpose of tests at level 5 is to establish concentration-response relationships for adverse effects to be used in risk assessment and assist in determining whether any adversity is likely to be endocrine mediated or not.

Currently, SSCs are employed in the OECD test guidelines using fathead minnow (*Pimephales promelas*) or Japanese medaka (*Oryzias latipes*), and in a guidance document using female three-spined stickleback (*Gasterosteus aculeatus* L.). During the OECD test guideline development, SSCs for zebrafish (colouration and ovipositor) were considered, but due to their ambiguity and variability among zebrafish strains (Hutter et al., 2012) were not considered a robust endpoint in this species (OECD, 2012a). However, due to the multiplicity of endpoints providing mechanistic information (e.g. vitellogenin, gonad histopathology and potentially hormone titres) the species is still considered scientifically robust for the test purpose. The species-specific SSCs and associated guidance from the OECD Test Guidelines and Guidance Document are shown in Table 1. In all cases the main characters are male features that may be affected in males (reduced or over expressed) or induced in females. Below is a summary of the male SSCs in the different species.

3.1. Fathead minnow

The fathead minnow (*Pimephales promelas*) is one of the most widely used small fish models for research and regulatory toxicology testing (Ankley and Villeneuve, 2006). The relevant SSCs include body colour, colouration patterns (male banding), body shape, number and size of nuptial tubercles, size of dorsal fatpad and ovipositor. Fathead minnow exhibit SSCs once they come into breeding condition which is the case typically around 5–6 months of age (Jensen et al., 2001). Males are clearly larger than females and develop a dorsal fatpad and nuptial tubercles, while females develop a fleshy ovipositor. The pattern and number of nuptial tubercles is relatively consistent. Tubercles follow a bilaterally-symmetrical pattern in mature males and are absent in females (Jensen et al., 2001). Within the Test Guidelines (OECD, 2009; 2012b) tubercles are characterised following a procedure developed at the U.S. Environmental Protection Agency laboratory in Duluth, MN. Tubercles are counted and ranked for size (present, enlarged or pronounced) in 6 specific areas. This rating system generally results in overall tubercle scores of <50 in normal control males with typical tubercle counts of 18–20 (Jensen et al., 2001), which is supported by the published historical control databases mean male scores of 31.2 (15–53; $n = 77$ fish) (Watanabe et al., 2007) and 27 (16–35; $n = 11$ studies) (Coady et al., 2014). Test guidelines (OECD TG 229 etc.) also cite the size of the dorsal fatpad and the ovipositor as potentially important SSCs. However, the test guidelines provide no guidance for any form of quantitative assessment of these characters. Consequently, standard practise is to limit these assessments to visual observations i.e. presence or absence with commentary on any unusual appearance relative to control animals.

3.2. Japanese medaka

The medaka (*Oryzias latipes*) is a common test species in Japan and Asia (Shima and Mitani, 2004). Its key attributes of small size, short life cycle, sexual dimorphism and availability of genetic sex markers have led to its inclusion in test guidelines at all *in vivo* testing levels of the OECD Conceptual Framework (levels 3, 4 and 5). Males possess male-specific ossified processes called papillary processes. Their development is regulated by androgens, and the signalling pathway, initiating from the androgen receptor, has been elucidated by Ngamniyom et al., (2009). The processes are thought to rub the female to induce spawning (Kawajiri et al., 2015). Papillary processes normally appear only in adult males and are found on fin rays from the second to the seventh or eighth counting from the posterior end of the anal fin. Typically, the anal fins are either removed and fixed or photographed for the papillae to be subsequently enumerated. The method is the same for fish screening assays and the Medaka Extended One Generation

[1] https://www.oecd.org/env/ehs/testing/OECD%20Conceptual%20Framework%20for%20Testing%20and%20Assessment%20of%20Endocrine%20Disrupters%20for%20the%20public%20website.pdf.

Table 1
Species specific secondary sexual characters and associated guidance from the OECD Test Guidelines and Guidance Document.

Character	Species	Observation	Guidance	Test guideline
Nuptial tubercles	Fathead minnow	Number and size	Counting and rating guidance given	OECD 229 and 230 Maybe implemented into life-cycle studies
Dorsal fatpad		Size	No guidance	
Ovipositor		Presence	No guidance	
Vertical banding colouration		Presence	No guidance	
Papillary processes	Japanese medaka	Number of joint plates with papillae	Guidance given	OECD 229, 230, 234 and 240
Red male colouration	Three-spined stickleback	Presence	No guidance	OECD 234 and 148 (however SSCs are not included)

Reproduction Test (MEOGRT; OECD 240). The validation dataset for the MEOGRT shows a wide range of control male responses with mean male papillae number per fish ranging from 21 to 138 in adults and 15 to 95 in subadults (data from 8 substances groups presented by Flynn et al., 2017). There has also been a recent proposal for a new test guideline using juvenile medaka (ca. 35 days post fertilisation) exposed for 28-days followed by an assessment of papillary processes in genetic males as the key anti-androgenic measure (Nakari and Erkomaa, 2003; Horie et al., 2017).

3.3. Three-spined stickleback

The three-spined stickleback (*Gasterosteus aculeatus* L.) is not to date such a widely used laboratory test species in regulatory ecotoxicology. However, it has the potential as an attractive model species for endocrine testing as it possesses the spiggin protein (nest glue) (Hoar, 1963), a biomarker that has been shown to be useful for the identification of anti/androgenic substances (Katsiadaki et al., 2002). However, the assessment of external SSCs has not made it into the OECD test method using three-spined stickleback. In fact, the method was released as a guidance document (OECD Guidance Document 148 (OECD, 2011a)) rather than a test guideline. This reflects the lack of clarity in the test method's role within the OECD's Conceptual Framework and outstanding experimental work to confirm the method's specificity for anti/androgenic activity (OECD, 2011a). Similarly the Fish Sexual Development Test with stickleback does not describe the assessment of SSCs (OECD, 2011b).

4. Interpretation OF SSCs

The primary purpose for incorporating SSCs in regulatory ecotoxicology studies has been their utility to assist in the diagnosis of potential endocrine activity. To date, this has focussed on the estrogen, androgen and steroidogenesis (EAS) pathways for which the tests and regulatory assessment methodologies are best developed. However, it should also be recognised that non-endocrine changes may induce or inhibit SSCs. Therefore, changes in SSCs cannot be considered exquisitely diagnostic of an interaction of a chemical with the endocrine system.

4.1. Evidence for endocrine mediated changes in SSCs

The sex-specific direction of responses in SSCs can be used to infer particular endocrine modes of action along the EAS pathways. As SSCs are primarily male characters, induction in females indicates a potential androgenic potential. Increases of SSCs in males could also indicate an androgenic activity or steroidogenic activity via accumulating endogenous androgens that fail to be converted to estrogens (Ankley et al., 2002). Whilst, reductions in males could indicate a potential anti-androgenic activity. Table 2 summarises representative examples of these responses for known potent endocrine active substances. More exhaustive reviews of SSC responses to known and suspected endocrine active substances can be found in (Dang et al., 2011; Ankley and Jensen, 2014). Again, this highlights that the clearest responses are along the androgen pathway. For other pathways there is greater variability and considerably less responsiveness in medaka (see Table 2). Potentially, this is a consequence of papillae growth being earlier in development whereas most studies have exposed adult fish in screening test designs. This is exemplified by Nakamura et al., (2014) who found exposure to two anti-androgens (flutamide and vinclozolin) only led to changes in papillary processes if medaka were exposed as juveniles. Fish exposed as adults, analogous to the existing short-term reproduction assay, did not exhibit significant changes. This has led to a recent proposal from Japan to the OECD to develop a separate juvenile medaka screening assay for substances suspected of having an anti-androgenic activity.

4.2. Evidence for non-endocrine mediated changes in SSCs

Although SSCs are diagnostic of endocrine activity under certain circumstances, consistent responses (amongst laboratories and species) for the SSCs have not been observed during validation studies for test guideline development. The peer review of the OECD test guidelines highlighted this issue across a range of less potent endocrine active substances tested (OECD, 2006). The potential for the non-specific (i.e. not diagnostic of endocrine) responses was demonstrated by the phase 2 validation 'testing negative substances (OECD, 2007). Here, fish short term reproduction studies with fathead minnow, medaka and zebrafish were performed with potassium permanganate and *n*-octanol as assumed non-endocrine active substances. Due to the lack of quantifiable SSCs in zebrafish only medaka and fathead minnow are relevant. In one potassium permanganate study with fathead minnow large decreases in male mean tubercle number and score were observed at high treatment levels where the fish were also clearly systemically stressed. In another laboratory, where fish were exposed to higher levels, statistically significant differences where observed in male tubercle score (OECD, 2007). No SSC responses were observed in medaka exposed to potassium permanganate or *n*-octanol or in fathead minnow exposed to *n*-octanol. These data confirm the contention that fish that are generally stressed may respond by reducing the expression of the non-essential SSCs due to the energy demand of elimination or detoxification mechanisms. In fact there is a large body of evidence demonstrating that systemic toxicity can perturb homeostasis and affect the general well-being in fish, which in turn can affect endocrine signalling (Wheeler and Coady, 2016; Marty et al., 2018).

Like other non-essential body functions, it is likely that the expression of SSCs may change in response to increased energy demands that inevitably result as organisms handle toxic stress

Table 2
Summary of secondary sexual character responses for known potent endocrine active substances. Representative examples only; not an exhaustive literature search. Detailed information for fathead minnow can be found in Ankley and Jensen, (2014).

	Estrogen		Androgen		Steroidogenesis		
Agonist	17β-estradiol	↓♂ nuptial tubercles (Miles-Richardson et al., 1999)	17β-trenbolone	↑♀ tubercle score but not statistically significant (Seki et al., 2006)			Fathead minnow
			Gestodene	↑♀ tubercle count and dorsal fin spot ≥10 ng/L and ↑♀ % fatpad (Frankel et al., 2016)			
		No evidence ≤85 μg/L (Seki et al., 2006)	17β-trenbolone	↑♀ papillary processes ≥0.365 μg/L (Seki et al., 2006)			Medaka
Antagonist/ inhibitor			Flutamide	↓♂ nuptial tubercles (Panter et al., 2004)			
				↓♂ papillary processes (juveniles only) (Nakamura et al., 2014)			
Antagonist/ inhibitor			Flutamide	↓♂ nuptial tubercles (Panter et al., 2004)	Fadrozole	No evidence ≤57 μg/L (Ankley et al., 2002) ≤96 μg/L (Panter et al., 2004)	Fathead minnow
				↓♂ papillary processes (juveniles only) (Nakamura et al., 2014)			Medaka

(Barata et al., 2004). Indeed, this can be inferred from the correlation of such characters to body size. For example, Watanabe et al. (2007) showed that fathead minnow male fatpad weight is correlated to body weight. Similarly, Wheeler et al. (2019) in the context of control responses of regulatory Fish Short Term Reproduction Assays (i.e. OECD TG 229 and OSCPP 890.1350) show correlations between body weight and male tubercle score as well as other important endpoints that are not SSCs (fecundity, plasma vitellogenin and male Gonadal Somatic Index). Therefore, it is not out of scope that a test substance that generally reduces condition and body weight might secondarily impact on SSC expression. Where comparisons are made to unstressed controls secondary SSC responses might appear to be related to endocrine activity rather than be non-specific. Therefore, during evaluation, the relationship between body weight and SSC expression should be investigated.

Further, other non-chemical stressors are known to influence SSCs expression in fish. As sexual differentiation in several fish species is temperature-dependent (Ospina-Alvarez and Piferrer, 2008), it is to be expected that SSCs may respond to temperature changes. Indeed, changes in SSCs have been demonstrated in three-spined stickleback (Borg, 1982), fathead minnow (Brian et al., 2011), guppy (Breckels and Neff, 2013) and half-smooth tongue sole (Shao et al., 2014) in response to elevated temperature. Factors such as hypoxia can also induce male SCCs in medaka as shown by Cheung et al., (2014).

Although regulatory fish endocrine tests are well controlled, non-specific responses can be a real interpretative issue. For example, test concentration setting guidance should in principle minimise the probability of observing non-specific responses in SSCs. However, to date guidance has focussed on measures of lethality and as such may select test levels that still lead to significant sublethal systemic effects (Wheeler et al., 2013). Substances that have a high acute to chronic ratio may be prone to be tested at levels inducing chronic toxicity which could non-specifically influence SSC expression. Similarly, factors such as dissolved oxygen and temperature are controlled in test systems. However, certain toxicological modes of action (of the test substance), such as respiration inhibition, could induce similar effects to those observed in response to non-chemical factors. Consequently, SSCs should never be a 'standalone' endpoint as they need to be complemented by additional measures to support a conclusion on endocrine activity. All measures in such fish endocrine tests, not least SSCs should be interpreted appropriately within the study (Wheeler et al., 2019) and across studies (Gross et al., 2017) by using Weight-of-Evidence approaches.

5. Relevance for endocrine disruption and risk assessment

It is our contention that SSCs can be useful indicators for potential endocrine activity in fish. SSCs may also give indications as to what other effects in the overall toxicological response to a substance may lead to adverse consequences. As such, changes may also reflect part of the overall toxicological response which could culminate in reproductive effects consequent to endocrine **or** non-endocrine mechanisms. A framework to assist in the evaluation of the results from fish short term reproduction tests (Ankley and Jensen, 2014) is helpful in this regard. However, the currently available evidence suggests that SSCs should not be considered directly adverse in the context of the definition of endocrine disruption as they are not always consequent to an endocrine activity. As SSCs can potentially be an expression of non-endocrine indirect effects to reproductive processes. Therefore, they should not be interpreted in isolation to direct measures of adversity, such as growth, development and reproduction. This is important for the determination of robust points of departure (thresholds for adverse effects) for use in risk assessment. It is perhaps even more critical when SSCs are used in combination with other endocrine-related endpoints, to determine whether a substance is an endocrine disrupter or not. These determinations have regulatory implications which are severe in some regions (Wheeler et al., 2012).

The Adverse Outcome Pathway (AOP) concept (Ankley et al., 2010) is useful in this context as it describes the connections amongst responses at multiple levels of biological organisation. It is a particularly good fit to address questions concerning endocrine disruption, as the AOP concept aims to provide the components required to satisfy the definition of an endocrine disrupter (Wheeler and Weltje, 2015). It is informative to catalogue the endpoints, including SSCs, from the fish tests into the structured representations used by the AOP concept (see Table 3). In this way of presenting the whole toxicological response via the effects observable in appropriate endocrine fish tests, SSCs fit best as organ level responses. Therefore, SSCs are below the level typically considered important to indicate an adverse effect relevant to the protection goal of environmental assessments (Weltje et al., 2013) and Crane et al., 2019). This further supports the contention that SSCs should be interpreted within the framework of the AOP concept. The AOP can determine if a change in SSC, as a Key Event in the pathway, is sufficiently linked to an observable Adverse Outcome that is relevant to either the protection goal (risk assessment) or the definition of an endocrine disrupter (endocrine hazard assessment). Otherwise such a determination in isolation

Table 3
Toxicological endpoint and AOP classification table for fish regulatory test guideline endpoints.

Fish Test Guidelines	Adverse Outcome Pathway				
	Macro-molecular interactions	Cellular responses	Organ responses	Organism responses	Population responses
OECD TGs 229 and 230	Typically not applicable – measured in *vitro* test systems	• Vitellogenin • Hormone levels	• Secondary sexual characteristics • Histopathology e.g. gonads; liver; kidney • Gonadal-Somatic-Index	• Reproduction (fecundity, fertility)[a] • Behaviour	• Population size (abundance or biomass) • Population stability • Population recruitment
OECD TG 234		• Vitellogenin • Hormone levels	• Secondary sexual characteristics • Histopathology e.g. gonads; liver; kidney	• Growth • Behaviour • Sex ratio • Embryo time to hatch • Hatching success	
OECD TG 240 and variants of EPA OPPTS 850.1500		• Vitellogenin • Hormone levels	• Secondary sexual characteristics • Histopathology e.g. gonads; liver; kidney	• Reproduction (fecundity, fertility) • Time to maturity • Growth • Behaviour • Sex ratio • Embryo time to hatch • Hatching success	

[a] Optional in OECD TG 230.

disregards the weight of all the available information. In many cases, this information will include direct measures of adversity (e.g. fecundity) in the same study or in other cases robust signals (i.e. clusters of diagnostic effects in the absence of overtly systemic effects) from SSCs might be used to trigger additional testing to investigate if adverse effects become manifest. Thus, we advocate that SSCs are not directly adverse *per se* but can be used to infer, in combination with other effects, an endocrine mechanism using weight-of-evidence.

6. Conclusion

SSCs play important biological roles in many fish species and may be affected by a range of natural environmental factors, and by exposure to chemicals. SSCs are both male and female features under hormonal control that convey information concerning sexual maturity and reproductive fitness. Field observations have made the connection between SSC responsiveness and chemical exposure and natural physical factors. Their hormonal aetiology, external expression and ease of observation has embedded their assessment in test methods to assess potential endocrine activity in fish. We contend that SSCs should be considered, as other biomarker responses, indicative of potential endocrine activity but not adverse effects *per se* in themselves. Therefore, the 'sign-posts not traffic lights' analogy (Hutchinson et al., 2006) should apply to their utility to decide on the need for higher tier testing to establish adversity. Such evaluations will inevitably rely on within and across study weight-of-evidence evaluations that can balance the limitations of the employed test designs and consistency of effects across test systems.

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