7.0 DISCUSSION

The primary objectives of this study were two fold: 1) evaluate in an independent lab a short-term reproduction test developed by EPA (2001) for Tier 1 screening of endocrine disrupting chemicals (EDCs) with the fathead minnow, and 2) compare this test with two other assays, one a non-spawning 14-day adult fathead minnow screen (OECD 2001) and the other a shortened version of the assay described by EPA (2001), with respect to an ability to identify EDCs operating by different modes of action. The effectiveness of the ability of the three different short-term screening assays to detect substances that interact adversely with endocrine systems in fish (estrogen, androgen, and thyroid systems) were assessed after exposure to four chemical agents representing different endocrine disruptive modes of action, with the ultimate goal of selecting a preferred assay to be used in Tier 1 screening. In general, the Tier 1 screen should be relatively quick and simple to run, have the ability to detect multiple modes of action of EDCs, and be readily interpretable. The information gleaned from a Tier 1 assay will be used to determine whether the chemical under investigation could adversely affect the endocrine system of fish and, if necessary, direct the focus of further Tier 2 testing.

The four test chemicals used in this study have been previously tested by USEPA-MED using the 21-day protocol, providing a reference data set to allow comparison of results with the present study and indicate the transferability of the short-term fish screening assay. Before discussing transferability and performance of the various protocols, a comparison is made of control values for selected endpoints reported in this study with those of previous studies. This comparison is intended to provide some context for quantitative comparisons between current and past studies, in addition to comparison of the relative differences within a study among exposed and non-exposed fish.

7.1 Fecundity

The fecundity endpoint is a measure of the number of eggs produced by an individual female. Arguably, fecundity is the most useful indicator of the general reproductive condition of mature fish as it reflects successful integration of a variety of physiological processes. As a reference for daily fecundity averages in fathead minnows, a summary is presented in Table 7.1. The values in this table were taken from previous EPA-MED studies (Ankley et al. 2001, 2002, 2003) using the same 21-day protocol as that used in this study. Also included are representative fecundity data from other studies using pair breeding minnows or alternative group breeding configurations. Based on this comparison, the average daily fecundity of minnows maintained during the course of this study appear to be two to three times higher than those typically observed at the EPA-MED laboratory, but within the upper range reported in other studies. The increased fecundity in the present study appears directly related to the larger size of females (Table 7.1). There is a trend toward increased body size among females during the study, which is reflected in the progressively higher daily fecundity. This increased size is to be expected, as fecundity in batch spawning is a direct function of body size (Wootton 1990). Importantly, the daily variation in fecundity observed in this study (% CV) appears to be consistent with that reported in past studies.

Table 7.1. Average Daily Fecundity in Control Fathead Minnows (mean, mean ±SD)

Reference or 2-18 average	Fish Weight (g)	Average Fecundity eggs / female / day (±SD) Pair Breeding	Group Breeding
Benoit & Carlson 1977	1.5		34 - 52 [a]
Kramer et al. 1998	nr		9.6 – 15.7 [b]
Harries et al. 2000	nr	34 – 132[c]	
Jensen et al. 2001	nr	19 ±1	
Ankley et al. 2001	nr		20.5 ±1[d]
Ankley et al. 2001	nr		16.4 ±5.4[e]
Ankley et al. 2002	nr		20.5 ±5.7
Ankley et al. 2003	1.75		38 [f]
Ankley et al. unpublished	nr		57 [g]
This study:			
Methoxychlor controls	1.59		32.5 ±2.5
Trenbolone controls	1.72		43.7 ±9.5
Flutamide controls	1.82		55.7 ±9.2
Fadrozole controls	2.13		66.0 ±5.0

Note: All data are based on 21-day cumulative egg production except where noted below.
nr = not reported.
[a]Range of values from daily spawnings (average of 11-16 spawns) using a variety of minnow barns; includes non-adhering eggs laid (3 males / 6 females per tank).
[b]Daily average calculated from range reported per female after 13 spawning days (3 males / 3 females per tank).
[c]Range reported in the Harries et al. 2000 study from two separate experiments (including tank water and solvent control values).
[d]Control values reported with methoxychlor results.
[e]Control values reported with methyl-testosterone results (12 day data collection).
[f]Estimated from Figure 3 in the Ankley et al. 2003 paper.
[g]Estimated from unpublished control data collected during flutamide studies.

7.2 Histology and Gonadosomatic index (GSI)

The gonadosomatic index (GSI) is generally considered a good measure of gonad maturation and spawning readiness and is based on the broad assumption that proportionally larger gonads indicate greater development (West 1990). The GSI in female fathead minnows can also be quite variable depending on the time since last spawning (Jensen et al. 2001). Control values for GSI in females throughout this study ranged between 10% and 14%, and for males, between 1.1% and 1.4%. Although the male GSI values are consistent with previous studies, the female GSI values are slightly higher than those observed in past EPA-MED studies, which appears to vary between 8% and 12% (Jensen et al. 2001; Ankley et al. 2001). This difference may be related to the larger size of female fish used in the present study. The use of the GSI assumes a linear relationship between gonad weight and body weight, which may not be consistent across a broad range of body weights, as negative or positive allometric effects may skew the values (Weatherly 1990).

Histology is a qualitative to semi-quantitative tool used to describe alterations and localize specific changes in organ morphology. Histological methods have been recognized as the most accurate approach for staging reproductive development in fish (West 1990). However, a primary difficulty in applying histological analysis is that interpretation of changes in tissues may vary from one investigator to another. In the present study, quantitative aspects of the histological examination have been emphasized as much

as possible to reduce investigator bias. As a comparison to the analysis used in this study, the histological examination of the normal fathead minnow reproductive system described in Jensen et al. (2001) is perhaps the most relevant. With respect to ovarian development, the Jensen et al. (2001) study described approximately 35% to 55% of the oocytes as being in Stage I development (subdivided into 1a and 1b in the current study), and the remaining stages varying between 5% and 25% of the total number of oocytes. Notably, the oocytes in a late vitellogenic stage (equivalent to Stage 4 in the current study) varied systematically with time since last spawning, with approximately 35% of the oocytes at Stage 4 two days after spawning and 12% the same day after spawning. This pattern of oocyte growth is consistent with the findings from control fish sampled during the present study. Typically, oocytes in Stage 4 development comprised 20% of the sample, and were often highly variable in number, probably reflecting ovaries removed from females at different post-spawning intervals.

7.3 Vitellogenin and Plasma Steroid Levels

The biological role of VTG and regulation of its biosynthesis is well established in fish, including fathead minnows. Synthesis of VTG is under estrogen control and both male and female fish can be induced to synthesize VTG after estrogen exposure or after exposure to estrogen mimics. In sexually mature control minnows, it is expected that VTG levels are low to negligible in males and in the mg/mL concentration range in spawning females. Mean values of VTG in spawning females from previous EPA-MED studies appear to vary from 12 – 27 mg/mL (Table 7.2). Other studies have reported much lower values (for example Harries et al. 2001), which are likely due to differences in analytical methods, specifically the use of carp anti-VTG antibodies as opposed to fathead minnow anti-VTG used in past EPA-MED studies. In the present study, a commercially available kit for VTG detection was used, which used carp anti-VTG. The average values for VTG in control males using this kit were very low, as expected, and are similar or slightly higher than those observed in previous studies (Table 7.3). The VTG levels in control females ranged from 1 mg/mL to 6 mg/mL, or roughly 2 to 4 times lower than those from past EPA-MED studies. This difference is likely attributable to the use of carp versus minnow anti-VTG. It also appears inter-female variation in VTG levels are greater in the present study (e.g., %CV up to 95%, Table 7.2) than that found in previous EPA studies. As mentioned previously in the results section, a large part of this variation is due to some control females having exceptionally low VTG levels (<2% to 5% of the sample mean; see figures E21, E14M VTG-1; E21, E14FL VTG-1). For these fish, it is questionable whether they would be able to successfully spawn despite an external appearance of sexual maturity. This variation may represent an accepted inevitability with protocols using group spawners, in which it is difficult to identify individual females that are not laying eggs during pre-exposure observations.

Besides VTG, the measurement of plasma levels of E2, 11-KT, and T can be used as endpoints to assess endocrine status in male and female fish, provided there is some knowledge about normal population levels when the samples are taken. Plasma levels of E2 have limited utility in males, because levels of this steroid are usually low or non-detectable. The measurement of plasma E2 is most useful in sexually maturing females because of the gradual rise in this hormone during the period of vitellogenesis, and the cyclic nature of the levels during the reproductive cycle (Jensen et al. 2001). In male fishes, 11-KT is the sex steroid characteristic of sexual maturity, and reduced levels are considered synonymous with reproductive dysfunction. Very low levels of 11-KT (e.g., pg/mL range) are sometimes reported in adult female fish (Jensen et al., 2001; Simpson and Wright 1977), although the physiological significance, if any, is not understood. Testosterone levels are normally much lower than 11-KT in males at time of spawning (Jensen et al., 2001), although in females, levels of T can approach E2 levels, making this a useful endpoint to measure in assessing the endocrine status of sexually mature fish. An important concern with single measurements of plasma sex steroids in mature fish is the relevance of one measurement to reproductive function or dysfunction. As an aid in determining mode of action, steroid

Table 7.2. Average Plasma Markers in Control Female Fathead Minnows (mean or mean ±SD)

2-18 average	Vitellogenin mg / ml	Estrogen ng / ml	Testosterone ng / ml	11-Keto ng / ml	E2 / T Ratio
Jensen et al. 2001	12-17	5.97 ±1.1	3.08 ±0.34	0.36 ±0.11	1.94
Harries et al. 2000	0.1 – 0.35[a]	nd	nd	nd	
Makynen et al. 2000	nd	3.52 ±1.22	3.79 ±3.63	nr	0.93
Ankley et al. 2001[b]	13	9	3.25	nr	2.77
Ankley et al. 2001[bc]	25	4.5	3.5	nr	1.29
Ankley et al. 2002[b]	19.5	5.5	5.5	nr	1.0
Ankley et al. 2003[b]	27	4	4.25	nr	0.94
Ankley unpublished[d]	16	9	6	nr	1.50
This study:					
Methoxychlor	4.93 ±4.07	1.73 ±1.38	4.34 ±7.48	0.839 ±0.441	0.4
Trenbolone	1.29 ±1.16	1.36 ±1.38	1.33 ±1.33	nd	1.02
Flutamide	4.92 ±2.14	1.23 ±1.26	1.33 ±1.06	nd	0.92
Fadrozole	5.95 ±2.25	2.86 ±1.49	1.10 ±0.94	0.071 ±0.19	2.6
WA 2-18 study avg.					

nd = not determined; nr = not reported
[a]Determined using Carp anti-VTG ab.
[b]Values estimated from figures.
[c]Control values reported with Methyl-testosterone results (12 d data collection).
[d] Estimated from unpublished control data collected during Flutamide studies.

Table 7.3. Average Plasma Markers in Control Male Fathead Minnows (mean or mean ±SD)

2-18 average	Vitellogenin mg / ml	Estrogen ng / ml	Testosterone ng / ml	11-Keto ng / ml
Jensen et al. 2001	0.004 ±0.001	0.4 ±0.13	9.11 ±0.92	33.1 ±3.14
Harries et al. 2000	0.00003[a]	nd	nd	nd
Makynen et al. 2000	nd	0.8 ±0.16	5.32 ±4.82	7.09 ±9.11
Ankley et al. 2001[b]	<0.0001	0.1 ±0.13	7.5 ±0.92	29 ±3.14
Ankley et al. 2001[bc]	<0.0001	0.3	8	28
Ankley et al. 2002[b]	<0.0001		12.5	40.
Ankley et al. 2003[b]	nd	0.2	12	32
Ankley unpublished[d]	<0.02	0.15	25	45
This study:				
Methoxychlor	0.00011 ±0.00015	0.31 ±0.09	5.21 ±3.0	35.21 ±16.81
Trenbolone	0.0029 ±0.0036	0.32 ±0.27	1.41 ±0.60	12.93 ±18.17
Flutamide	0.0024 ±0.0046	0.19 ±0.09	2.22 ±1.17	16.88 ±8.48
Fadrozole	0.0023 ±0.0026	0.069 ±0.09	2.21 ±1.11	19.77 ±15.11
WA 2-18 study avg.				

nd = not determined
[a]Determined using Carp anti-VTG anti-bodies. Male VTG from solvent controls were <0.00007 mg/mL
[b]Values estimated from figures.
[c]Control values reported with Methyl-testosterone results (12 d data collection).
[d] Estimated from unpublished control data collected during Flutamide studies.

ratios, specifically E2 / T in females, may be more useful. The basis for this analysis is the hypothesis that sex steroid ratios, as opposed to their absolute values, are more important in determining sexual differentiation and, perhaps, sexual development (Jalabert et al. 2000). In fathead minnows, there does appear to be a correlation between plasma E2 and T levels in female fish, with E2 / T concentration ratios between 1 and 2 being typical (Jensen et al. 2001).

The mean sex-steroid levels observed in control female and male fish from the current study are summarized in Table 7.2 and 7.3. The levels for E2 and T in females are lower than those previously observed in EPA-MED studies by a factor of roughly 2 to 3. However, the E2 / T ratios are similar to past studies and generally ranged between 1 and 2, as to be expected. The exception to this observation was control female fish sampled during the methoxychlor studies, which had unusually high T levels, lowering the ratio to less than 0.5 (Table 7.2). The T levels in male control fish sampled during this same time were also unusually high (Table 7.3), suggesting the cause may be analytical as opposed to biological (e.g., assay error rather than induction). This may be explained by the use of an ELISA-based method for analyzing steroids as opposed to the RIA-based method used in the EPA-MED studies. The sex-steroid levels in male fish were, in general, closer to values reported in previous EPA studies although T levels in this study are approximately 4 to 5 times lower than those previously observed.

7.3.1 Test Results: Transferability and Inter-Protocol Comparison

During the course of this study, an enormous quantity of raw data was collected and summarized in the statistical analysis presented in the previous results section. A number of measured parameters, such as the chemistry values, serve to establish that the environmental conditions and measured exposure levels were within acceptable limits. Other parameters such as body weight and condition factor provide some indication of the general health status of the fish. In the latter case, it was anticipated that condition factor would be unaffected by the chemical treatments, as sublethal doses were employed that have been previously established to be well-tolerated in EPA-MED studies. In general, the results of this study essentially confirm this expectation. Although in some treatments a significant difference in body was noted, this appeared to be a random occurrence. With regard to other biological endpoints, a number of the measurements concerning fertilization success, hatchability, and larval survivability / development indicated these were comparatively insensitive parameters, as consistently high values were recorded (>90%) regardless of chemical treatment. For the protocols incorporating reproduction, overall fecundity either expressed as total eggs or the daily average was the most useful indicator of reproductive performance.

In considering the merits of the different test protocols in this study, it is important to identify the endpoints that appear to be the most sensitive and pertinent to the evaluation of mode of action. Therefore, the following discussion focuses on fecundity, plasma VTG and sex steroids, GSI, and histology as the key endpoints for comparison. For each chemical treatment, a table is presented that summarizes the results as either not significantly different (---) from control values, or if a significant difference was observed, the direction of the change higher (↑) or lower (↓). For simplification, dose-response aspects of the treatments are ignored (e.g., differences between high and low treatments). Also, in some of the sex steroid analysis, it appeared that exceptionally high variability prevented a statistically significant conclusion, despite obviously large differences in the sample means. In these instances, a "~" symbol is shown on the tables to distinguish these conclusions from those that achieved statistical significance.

7.3.2 Methoxychlor (mode of action: weak estrogen)

Methoxychlor is a relatively non-persistent organochlorine insecticide that has been demonstrated to be a weak estrogen mimic in fish and both an estrogen and anti-androgen proprieties in mammals. It is

registered for insect control in post-harvest applications in a variety of crops, foliar treatment, soil, and seed treatment. It is most commonly used to control black flies, houseflies, and mosquitoes in association with human habitation (EPA 1992).

The results of the methoxychlor tests summarized below indicate a general lack of response in the measured endpoints. The notable exception was reduced fecundity at the high exposure during the 21-day spawning protocol. In contrast, fecundity was not statistically different during the 14-day protocol. However, problems with male mortality during the first week of the exposure reduced the sample size to two replicates at termination, and combined with high variation in fecundity, diminished the statistical power of this test. With regard to the biochemical endpoints, some differences were observed at the low- and mid-exposure levels but not at the high, making the biological significance of the findings questionable. Similarly, histological changes were observed at the lower treatments but not at the high-exposure rate in the non-spawning protocol. With the exception of fecundity, no significant effects on measured endpoints were observed at the high-exposure rate.

The decreased fecundity at the high-exposure level is in agreement with a previous study by Ankley et al. (2001), which also observed a significant reduction in fecundity at an exposure rate of 5 μg/L. Changes in sex-steroid levels and VTG induction were also observed. In females, estradiol levels were decreased, whereas in males, decreased testosterone and 11-KT and increased VTG were documented (Ankley et al. 2001). These additional changes were not observed in the present study; however, high variability in these measurements made it difficult to assess statistical significance. Additionally, the confounding variable of the *mycobacterium* infestation discovered after the test may also be a contributing factor to the unusually high variability observed in the biochemical endpoints.

Table 7.4. Methoxychlor Summary

Endpoint	Exposure level (measured range in μg/L)						
	Low (0.8)			Mid (2.0)	High (3.22 - 4.1)		
	E-21	E-14	NS-14	NS-14	E-21	E-14	NS-14
Fecundity	---	---	nd	nd	↓	---	nd
VTG	---	F↓	---	---	---	---	---
Estradiol	---	M↓	---	---	---	---	---
Testosterone	---	---	F↑	F↑	---	---	---
11-keto-testost.	F↓	---	---	---	---	---	---
GSI Females	---	---	---	---	---	---	---
GSI Males	---	---	---	---	---	---	---
Histology-Females	---	---	---	---	---	---	---
Histology-Males	---	---	Y	Y	---	---	---

--- not significantly different from controls
Y a significant effect observed
M male
F female
↑ or ↓ significantly greater or less than controls.
nd not determined.

7.3.3 Trenbolone (anabolic steroid)

Trenbolone is a potent steroid that is often used in the cattle industry to promote growth and was selected to be used in this study as a model androgenic compound. In this study, trenbolone exposure caused pronounced changes in many of the test endpoints at the high, mid, and in some instances, low concentrations (Table 7.5). These results are as one would expect from a potent anabolic steroid and are summarized below.

In comparing the test results among the three different protocols, fecundity results between the 14- and 21-day spawning assay at the high and low concentrations were similar. Plasma biochemical measurements were also similar between the two spawning assays, with the only differences being female testosterone plasma levels. In these tests, the results of the high-exposure group showed a significant decrease in female testosterone levels in the 14-day assay but not in the 21-day spawning assay. The opposite was seen in the low treatments, in which a significant decrease in female testosterone plasma levels was observed in the 21-day assay but was not observed in the 14-day assay. Regarding the non-spawning assay, biochemical results were similar to both the 14- and 21-day spawning protocols at the high concentration. However, at the low concentrations, the 14-day non-spawning assay observed a significant decrease in female vitellogenin and testosterone, and male estradiol, testosterone, and 11-KT steroid plasma levels. This significant difference was not observed in the low-treatment groups of the 14-day and 21-day spawning protocols (the 21-day low treatment had reduction in female testosterone levels only). Regarding GSI, no significant differences were observed in the non-spawning assay, whereas GSI in females was significantly greater than that in controls in both treatment groups for both the 14- and 21-day spawning protocols. Female histology results were similar at high and low concentrations in both 14- and 21-day spawning protocols. At high concentrations, significant differences were observed in females for both spawning protocols. This significant difference in female histology was not observed at the high concentration in the 14-day non-spawning protocol. Male histology results were generally similar among the three protocols in that no significant differences were observed between treatments and controls. The only exception was the observed significant difference at the high concentration in the 14-day spawning protocol.

The results from this study are similar to results from previous studies using the 21-day spawning protocol (Ankley et al, 2003) with regard to a significant reduction in female fecundity and plasma steroid levels (testosterone, estradiol, vitellogenin concentrations). However, this study did not observe significant increases in estradiol and vitellogenin plasma concentrations in male fish, as was observed from EPA-MED studies.

Table 7.5. Trenbolone Summary

Endpoint	Exposure level (measured range in µg/L) Low (0.041-0.71) E-21	E-14	NS-14	Mid (0.45) NS-14	High (0.60-0.86) E-21	E-14	NS-14
Fecundity	---	---	nd	nd	↓	↓	nd
VTG	---	---	F↓	F↓	F↓	F↓	F↓
Estradiol	---	---	M↓	F↓	F↓	F↓	~F↓
Testosterone	F↓	---	~F/M↓	~F/M↓	---	F↓	F↓
11-keto-testost.	---	---	M↓	---	---	---	---
GSI Females	↑	↑	---	---	↑	↑	---
GSI Males	---	---	---	---	---	↑	---
Histology-Females	---	---	---	---	Y	Y	---
Histology-Males	---	---	---	---	---	Y	---

--- not significantly different from controls
Y a significant effect observed
M male
F female
↑ or ↓ significantly greater or less than controls.
nd not determined.

Female body weights increased in E-21 high treatment.
Histology: E-21 test females had ↑ in atretic follicles and ↓ in corpus lutea at high treatment.
E-14 test females exhibited similar effects plus inhibition of stage 3-4 progression of oocytes (progression to late vitellogenesis).
In E-14 males, there was an ↑ in microscopic staging.

7.3.4 Flutamide (anti-androgen)

Flutamide is an anti-androgen and therapeutic agent that is used primarily to treat prostate cancer (Sogani et al. 1984). It is considered to be a pro-drug, and bioactivation to the 2-hydroxy metabolite is believed necessary for full biological activity (Simard et al. 1986). In this study, the effects of flutamide exposure on test endpoints were similar to methoxychlor in that relatively little or no effect was observed. A notable exception was in the fecundity data, which showed a decrease at the high-exposure level in both the 14- and 21-day spawning protocols. This result was consistent with the histological analysis, which indicated an increase in atretic follicles in these fish. With regard to sex-steroid levels, increased levels of testosterone in females and 11-KT in males are noteworthy. The former result indicates a decrease in the E2 / T ratio in females, which may be associated with decreased fecundity, although sex-steroid levels were unaffected in the 14-day spawning protocol. It should also be noted that no statistically significant differences in the endpoints were observed in the non-spawning protocol fish.

The relatively weak activity of flutamide in the fathead minnow protocols may be the result of limited bioactivation compared with mammals or inadequate exposure duration. The latter point is supported by the more pronounced effects observed on sex-steroid levels in the 21-day females compared to 14-day spawning females. In general, the results from the 21-day spawning protocol in this study are consistent with a previous unpublished study (Ankley 2002) using a similar exposure protocol. In the earlier study, fecundity was decreased at an exposure level of 640 µg/L. Similarly, testosterone levels in females were increased with an apparent decrease in E2 levels (statistical significance unknown). Thus, flutamide exposure appeared to decrease the E2 / T ratio in females. Other sex-steroid levels did not appear to be significantly altered, nor was GSI affected. There did, however, appear to be a slight induction of VTG in males at the 640-µg/L exposure level, which was not observed in the current study.

Table 7.6. Flutamide Summary

Endpoint	Exposure level (measured range in μg/L)						
	Low (43-47)			Mid (260)	High (504-519)		
	E-21	E-14	NS-14	NS-14	E-21	E-14	NS-14
Fecundity	---	---	nd	nd	F↓	F↓	nd
VTG	---	---	---	---	---	---	---
Estradiol	---	---	---	---	M↓	M↓	---
Testosterone	M↓	---	---	---	F↑	---	---
11-keto-testost.	---	---	---	---	M↑	---	---
GSI Females	---	---	---	---	---	---	---
GSI Males	---	---	---	---	---	---	---
Histology-Females	---	---	---	---	Y	Y	---
Histology-Males	---	---	---	---	---	---	---

--- not significantly different from controls
Y a significant effect observed
M male
F female
↑ or ↓ significantly greater or less than controls.
nd not determined.

Histology: E-21, 14 test females had ↑ in atretic follicles.

7.3.5 Fadrozole (aromatase inhibitor, indirect anti-estrogen)

Fadrozole is a competitive inhibitor of CYP19 (aromatase), a key enzyme in the biosynthetic pathway for estradiol synthesis. Estrogen synthesis inhibitors may also be considered indirect anti-estrogens. In this study, fadrozole exposure caused pronounced changes in several test endpoints after the high and in some instances, mid and low exposure levels. As expected with an aromatase inhibitor, estradiol levels in females were decreased in the high-exposure groups and in the low-exposure group from the 21-day spawning protocol. This decrease in estradiol was also associated with reduced fecundity and VTG in females. Testosterone levels were elevated in females, causing an overall large decrease in the E2 / T ratio. Histological examination of the ovaries was also consistent with the biochemical and fecundity results, with significantly reduced staging (preponderance of immature oocytes) observed in nearly all treatments and protocols. Fadrozole effects on male fish were less obvious, except for consistent increases in testosterone and 11-KT levels. The GSI in males from the 21-day protocol was elevated, which was associated with an increase in tubule diameter determined during histological examination.

In comparing the test results from the different protocols, the biochemical, fecundity, and histological results were similar between the 14- and 21-day spawning protocols, although estradiol levels were decreased at the low-exposure level only in the 21-day protocol. With regard to the non-spawning protocol, it is notable that no effects were seen at the low treatment, which is in contrast to the results from the protocols that include spawning.

The results from this study are in close agreement with a previous study using the 21-day spawning protocol (Ankley et al. 2002). In that study, fadrozole exposure altered the biochemical endpoints in females in a manner nearly identical to that observed in this study, including decreases in estradiol at 10, 50, and 2 μg/L, and decreases in VTG at 10 and 50 μg/L fadrozole exposures. Fecundity was reduced at 2 μg/L and spawning ceased within 2 days at the higher treatment levels (Ankley et al. 2002). Histological analysis of the ovaries was similar to the current study, with essentially the absence of

oocytes beyond Stage 3 development (Ankley et al. 2002). In males, the GSI, testosterone, and 11-KT levels were increased at the high-treatment rates, which is also consistent with results from the present study.

Table 7.7. Fadrozole Summary

Endpoint	Exposure level (measured range in µg/L)						
	Low (5.1-5.5)			Mid (31.7)	High (55.7-60.0)		
	E-21	E-14	NS-14	NS-14	E-21	E-14	NS-14
Fecundity	---	---	nd	nd	↓	↓	nd
VTG	F↓	F↓	---	F↓	F↓	F↓	F↓
Estradiol	F↓	---	---	---	nd	F↓	F↓
Testosterone	F,M↑	F,M↑	---	---	F,M↑	F,M↑	~M↑
11-keto-testost.	M↑	M↑	---	---	M↑	M↑	~M↑
GSI Females	---	↑	---	---	↑	↑	↓
GSI Males	---	---	---	---	↑	---	---
Histology-Females	Y	Y	---	Y	Y	Y	Y
Histology-Males	---	---	---	---	Y	---	---

--- not significantly different from controls
Y a significant effect observed
M male
F female
↑ or ↓ significantly greater or less than controls.
nd not determined.

Female body weights greater in high treatment E-21,14.
Histology: E-21,14; NS-14 test females exhibited less advanced staging (↑ pre-vitellogenic oocytes) at the mid and high doses and both increased numbers of atretic follicles. E-21 males had ↑ tubule diameter.

7.4 Conclusion

The results suggest the four endocrine-disruptive chemicals tested in this study can be divided into two groups: weak-acting with little measured effect (methoxychlor and flutamide) or strong-acting, causing pronounced effects (trenbolone and fadrozole). It is interesting to note that the weak-acting chemicals require bioactivation for biological effect. Undoubtedly, both inter-species (e.g., mammals versus fish) and inter-individual differences contribute to the extent of metabolite formation, and for pharmaceutical agents such as flutamide, it is unclear whether the active metabolite binds to fish androgen receptors (Wells and Van Der Kraak 2000). Nonetheless, this distinction can be useful in determining which protocol is superior as a screening protocol, since it likely both strong and weakly active compounds will be encountered during large-scale screening efforts.

7.5 Protocol Selection

The short-term reproduction assay (21-day protocol) was demonstrated to be the most comprehensive and sensitive protocol for use as a fish endocrine-disruptor screening assay. However, the 14-day spawning protocol shows good promise as a cost-effective alternative. The reasons for this can be summarized as follows:

- Consistency in overall response to the 21-day protocol

- Insufficient need for quantitative pre-exposure fecundity measurements
- Reduced costs compared with the 21-day protocol
- Lack of response at the low-exposure level and frequent negative responses at the high-exposure level in the non-spawning protocol. This observation suggests a higher risk of false-negative results, particularly with weak-acting endocrine disruptors.

Throughout the study, the 14-day protocol gave results that were generally consistent with the 21-day protocol, including fecundity. In nearly all chemical treatments and exposure levels, the conclusions drawn from the fecundity measurements were identical for both the 14-day and 21-day treatments, with the notable exception of methoxychlor. For the most part, results from sex-steroid analysis were similar, with a slight tendency for more obvious results in the 21-day protocol with flutamide and fadrozole. With regard to the pre-exposure evaluation period, quantitative fecundity estimates did not improve the power of the statistical analysis, which is the primary limitation in the use of fecundity measurements. Thus, the effort placed in quantitative pre-exposure fecundity measurements would be better served if used to increase the sample size of exposure replicates. Since this increase may be impractical for many laboratories relying on proportional diluters for toxicant delivery, the qualitative approach used in the 14-day protocol is a reasonable, yet cost effective alternative. It is recommended that additional development of the abbreviated 14-day spawning protocol be performed to evaluate how well the assay performs in comparison to the full 21-day protocol with weaker acting and/or high log-P compounds.

When comparing the results between the non-spawning protocol and those incorporating reproduction, it is clear that all protocols would provide similar conclusions with strong-acting endocrine disruptors tested at high-exposure levels. However, comparison of the results from the weak-acting compounds and results from all low-exposure treatments suggests the non-spawning protocol is less sensitive and may falsely conclude that a chemical is not an endocrine disruptor. For example, all measured endpoints were negative in the non-spawning assay during the flutamide exposure, yet the high-exposure level clearly reduced fecundity and elevated androgen levels in both sexes in the spawning protocols. In the low-fadrozole exposure, the non-spawning assay gave completely negative results, whereas the spawning protocols exhibited a pattern of effects typical of anti-estrogens. Thus, we cannot recommend the non-spawning protocol, as the potential exists for a high false-negative rate with weak-acting compounds and negative results due to testing an insufficiently low concentration.

7.6 Transferability

Comparison of the results from this study with previous EPA-MED efforts using the 21-day spawning protocol suggest the protocols are readily transferable, in that similar conclusions regarding mode of action can be made from the experimental results. The consistency in the results between the current and past studies is most obvious at the high-treatment rates and with measurements of fecundity, sex steroids, and histology. In a few instances in the present study, notably with the methoxychlor results, a lack of response was observed in some endpoints, whereas a significant change was noted in a previous study. However, unusually high variation in some parameters, such as fecundity during the methoxychlor exposures, reduced the sensitivity of this and other measurements. In general, higher variability (and hence, reduced statistical power) was a greater problem at lower exposure levels throughout the study and in particular with weak-acting chemicals. Thus, for acceptable transferability of the protocol, proper dose selection is critical. Greatest reproducibility of the results would be expected to occur at the highest possible dose level that does not cause unacceptable lethality during the course of the study.

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