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The consequences of exposure to mixtures of chemicals: Something from 'nothing' and 'a lot from a little' when fish are exposed to steroid hormones

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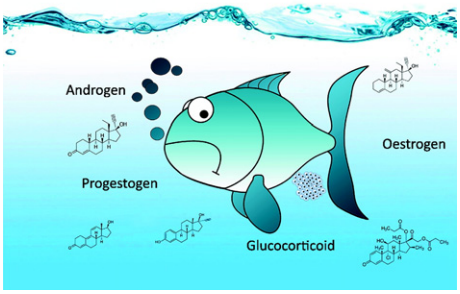
HIGHLIGHTS

- The effects of 5 synthetic steroidal pharmaceuticals on egg production of fish were investigated.
- A mixture of all 5 was tested twice.
- The additive effect of the mixture was best predicted by the model of independent action.
- A something from 'nothing' effect was demonstrated.
- Multiple steroids can be analysed for their potential combined environmental risk.

GRAPHICAL ABSTRACT



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ABSTRACT

Ill-defined, multi-component mixtures of steroidal pharmaceuticals are present in the aquatic environment. Fish are extremely sensitive to some of these steroids. It is important to know how fish respond to these mixtures, and from that knowledge develop methodology that enables accurate prediction of those responses. To provide some of the data required to reach this objective, pairs of fish were first exposed to five different synthetic steroidal pharmaceuticals (one estrogen, EE2; one androgen, trenbolone; one glucocorticoid, beclomethasone dipropionate; and two progestogens, desogestrel and levonorgestrel) and concentration-response data on egg production obtained. Based on those concentration-response relationships, a five component mixture was designed and tested twice. Very similar effects were observed in the two experiments. The mixture inhibited egg production in an additive manner predicted better by the model of Independent Action than that of Concentration Addition. Our data provide a reference case for independent action in an *in vivo* model. A significant combined effect was observed when each steroidal pharmaceutical in the mixture was present at a concentration which on its own would produce no statistically significant effect (something from 'nothing'). Further, when each component was present in the mixture at a concentration expected to inhibit egg production by between 18% (Beclomethasone diproprionate) and 40% (trenbolone), this mixture almost completely inhibited egg production: a phenomenon we term 'a lot from a little'. The results from this proof-of-principle study suggest that multiple steroids present in the aquatic environment can be analysed for their potential combined environmental risk.

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1. Introduction

A very high number of anthropogenic chemicals are present in the environment across the entire world. Few, if any, of these chemicals were present a century or more ago; some, such as nanomaterials, have appeared in the environment very recently. The range of chemicals present is vast, including as it does metals, surfactants, pesticides, persistent organic pollutants, plasticizers, pharmaceuticals, nutrients, endocrine disrupting chemicals and many other groupings of chemicals. Any specific environment will probably contain a unique range and concentrations of chemicals. Thus, it is clear that probably all living organisms on earth are presently exposed to highly complex, ill-defined mixtures of anthropogenic chemicals, as well as many natural chemicals that have probably been present in the environment for a very long time. Hence, if we are to understand the effects of anthropogenic chemicals on wildlife, we need to know if these complex mixtures of chemicals have any effects, and if they do, what these effects are, and how adverse (or not) they are. Only if we can adequately answer that question can we then put exposure to chemicals as a stressor into context with the many other stressors wildlife face (e.g. habitat loss, introduced diseases, alien species), and use that knowledge wisely to focus our attention on the greatest threat(s) (Sumpter, 2009; Johnson and Sumpter, 2014).

It is generally accepted that the aquatic environment is the environment most at risk from contamination by anthropogenic chemicals. This is because the aquatic environment receives (usually treated) effluent from wastewater treatment works which contains a plethora of 'down-the-drain' chemicals (Schwarzenbach et al., 2006; Loos et al., 2009), as well as a large number of agricultural chemicals (Moschet et al., 2014). Thus, essentially all aquatic organisms are exposed to highly complex mixtures of chemicals, most of them present at very low concentrations. Therefore, assessing the (potential) effects of these chemical mixtures requires an understanding of mixtures toxicity (EC COM, 2012). Despite this being obvious, and known for a long time, chemical risk assessment still relies on investigation of the toxicity of individual chemicals, although whole effluent screening (the assessment of the toxicity of an effluent) is also sometimes employed (Grothe et al., 1996). As the number of different mixtures of chemicals in the environment is essentially infinite, it will be necessary to rely on model predictions to protect aquatic organisms from chemical mixtures. Intuitively one might expect chemicals with similar modes of action (MoA) to act additively according to the concept of concentration addition when present as a mixture, and indeed this has been shown to be the case (e.g. Brian et al., 2005, 2007). It is less clear what overall effect to expect when an organism is exposed to a mixture containing chemicals with diverse MoAs; the research that has been conducted has been limited to predicting and assessing the toxicity of mixtures of dissimilarly-acting chemicals on bacteria and algae (Backhaus et al., 2000; Faust et al., 2002). Such mixtures could be comprised of chemicals that affect the same apical endpoint (e.g. growth, reproduction), but do so via different pathways, or chemicals that affect different apical endpoints.

To begin to provide robust data that might aid in the prediction of the effects of complex mixtures of chemicals on aquatic organisms, we have been conducting in vivo experiments in which fish are exposed to well-characterised mixtures of chemicals. We chose to study sex steroid hormones, because (1) mixtures of these chemicals are widely present in the aquatic environment, (2) they are often extremely potent, (3) although they all affect reproduction, they do so via different, and well-characterised, MoAs, and (4) they can be present in the environment at concentrations that affect fish, often causing intersexuality (Jobling et al., 1998). Recently we showed that a very simple (binary) mixture of an estrogen and a progestogen led to reproductive effects (reduced egg production) that did not deviate from those predicted by the model of concentration addition; that is, they were additive (Runnalls et al., 2015). Given the fact that steroid hormones are considered a high priority for environmental research (Runnalls et al., 2010), it is not surprising that many other research groups have recently begun to study how fish respond to simple mixtures of sex steroid hormones. Various mixtures, including those containing natural and synthetic progestogens (Zucchi et al., 2014; Rossier et al., 2016), only synthetic progestogens (Siegenthaler et al., 2017), an estrogen and an androgen (Chen et al., 2016; Velasco-Santamaria et al., 2010; Orn et al., 2016) and a progestogen and an estrogen, the latter to mimic the constituents of the oral contraceptive pill (Hinfray et al., 2016; Hua et al., 2016), have been tested. All these studies tested only binary (two component) mixtures; to date, no studies assessing the effects of more complex mixtures of steroids have been reported. Most often alterations in gene expression in embryos have been the endpoints, although a few studies have been conducted with adult fish, and gonadal histology or egg production utilized as the key endpoints. Relatively few studies have assessed the reproductive performance of fish exposed to mixtures of steroid hormones (Hua et al., 2016; Zhao et al., 2015; Runnalls et al., 2015), although assessing this apical endpoint is crucial from a hazard assessment perspective.

Although experiments involving binary mixtures are ideal to test certain hypotheses, such as whether or not an androgen can negate the effect of an estrogen, or an antagonist negate the effect of an agonist, they are only the first step in understanding the effects of 'real world' mixtures of steroid hormones, which are very likely to contain many different steroid hormones from different classes of steroids (e.g. androgens, estrogens, progestogens, glucocorticoids), as recent analytical studies demonstrate (e.g. Liu et al., 2011; Goh et al., 2016; Zhou et al., 2016). As the next step towards determining how fish respond to multi-component mixtures of sex steroid hormones, here we report the combined effects of five compounds (EE2, levonorgestrel, beclomethasone dipropionate, desogestrel and trenbolone) on the reproductive performance of the fathead minnow. Of particular interest, and importance, is the issue of whether or not such a mixture could inhibit reproduction even when each individual steroid is present in that mixture at a concentration below which it would have any statistically significant effect if tested alone: this is the so-called "something from 'nothing'" phenomenon (Silva et al., 2002).

2. Materials and methods

2.1. Chemicals

Aqueous stock solutions of Ethinyl estradiol (EE2) (Sigma-Aldrich, UK. CAS: 57-63-6, purity, ≥98%), Levonorgestrel (Sigma-Aldrich, UK. CAS: 797-63-7, purity, ≥99%), Desogestrel (Sigma-Aldrich, UK. CAS: 54024-22-5, purity, analytical standard), 17β-Trenbolone (Sigma-Aldrich, UK. CAS: 10161-33-8, purity, ≥93%), and Beclomethasone dipropionate (Sigma-Aldrich, UK. CAS: 5534—09-8, purity, ≥99%) were prepared weekly using 2.5 L Winchester amber glass bottles and double-distilled water. Dosing stock solutions were made at 5000 times concentrate to achieve desired tank concentrations. These master stocks were made up in ethanol and stored at 4 °C. Ethanol concentrations in the experimental tanks were no >0.00003%.

During the exposure period, medical grade silicone tubing delivered the chemical stock solutions to glass mixing vessels, where the stock was mixed, diluted and delivered to the tanks (8 replicate tanks per mixing vessel). The same procedure was applied to the mixture. Stock solutions of the mixture were prepared at the start of the experiment from five stock solutions, one of each of the individual compounds.

2.2. Experimental animals

Adult fathead minnow (*Pimephales promelas*) were obtained from a breeding stock (AstraZeneca, UK), and held in a recirculation system at the aquatic facility at Brunel University London until 12 months old. Fish were fed four times daily, twice with defrosted adult brine shrimp

(Tropical Marine Centre, Gamma irradiated) and twice with dry flake food (Tetramin, Tetra, UK).

2.3. The basic experimental set-up

The effects of five steroid pharmaceuticals and their combined mixture were investigated in vivo on fish reproduction. Independent experiments were conducted for each of the five single pharmaceuticals and the pharmaceutical mixture using the well-established pair breeding assay (Harries et al., 2001). Egg production of paired fish was quantified daily in 21 day pre-exposure and 21 day exposure periods. The cumulative number of eggs of each breeding pair was compared between these two periods. Concentration-response curves were generated by the single chemical experiments, some of which have been reported previously (EE2 and levonorgestrel: Runnalls et al., 2015; desogestrel: Runnalls et al., 2013). These single chemical concentration-response curves were used to design a five chemical mixture experiment.

Prior to the start of each experiment, a large group of fish (200 plus) were sexed and separated into male and female holding tanks. Individual fish were then selected from these tanks 14 days before the start of an experiment and paired at random (one female and one male per tank). During this pairing period, pairs were assessed daily for their reproductive compatibility. Replacements and exchanges were made where necessary until the desired number of compatible breeding pairs had been established. In all experiments there were at least 8 pairs of control fish and 8 pairs of fish for each concentration of the chemical or mixture of chemicals being tested.

After the pairing assessment period, a 2 × 21 day experiment was carried out. Fish were first subjected to a 21-day pre-exposure period, during which none of the pairs of fish were exposed to any chemical, and egg production quantified. A 3-day acclimation period followed, when chemicals dosing began. Previous research has demonstrated that after 3 days the concentrations of test chemical in the fish tanks have reached their desired levels. This 3-day acclimation period was followed by a 21-day exposure period, during which fish were exposed continuously to defined, stable concentrations of the chemicals. This basic experimental design, which we refer to as the pair-breeding assay, is illustrated in Fig. 1.

Throughout the experiments fish pairs were held in 8 L glass tanks under a continuous flow-through system of dechlorinated carbon-filtered tap water (5 and 10 μm filters), with a flow rate of 60 L/h (per 8 replicate tanks), resulting in a complete change of water at least every 2 h. Each tank was equipped with a tile, grid and dish as a spawning substrate. The photoperiod was maintained throughout on a 16 h light, 8 h dark cycle, with 20 min dawn and dusk periods. Temperature and dissolved oxygen were checked daily and maintained between 24.5 and 25.5 °C, and 7 and 8 mg/L, respectively. Nitrite, nitrate, pH and ammonia levels were checked once per week. Tanks were syphoned and cleaned weekly to remove uneaten food and faeces, and mixing vessels were cleaned on a regular basis. Flow rates of the test chemical, or mixture, into the mixing vessels were controlled using a multichannel peristaltic pump set at 0.2 mL/min (Watson Marlow, Cornwall, UK). Flow rates and dosing efficiency were monitored on a daily basis.

The reproductive capacity of fish pairs was assessed daily by recording the spawning frequency and the total number of eggs spawned. All eggs were counted manually using a hand-held counter. Dishes, tiles and grids were removed from each tank, checked and replaced with a fresh set if eggs were present. If no eggs were present, spawning substrates were rinsed and then returned to the tank. Fecundity was quantified daily throughout both the pre-exposure and the exposure period, allowing reproductive performance to be compared between these two periods for each individual pair (see Runnalls et al., 2015 for a detailed description of the methodology used to assess the effects of a chemical on each individual pair of fish). The condition, health and behaviour of the fish were continuously assessed throughout all experiments.

On day 21 of the exposure period, fish were euthanized with tricaine methanesulfonate (MS-222) (Sigma-Aldrich, UK) and measured for wet weight (g) and fork length (mm).

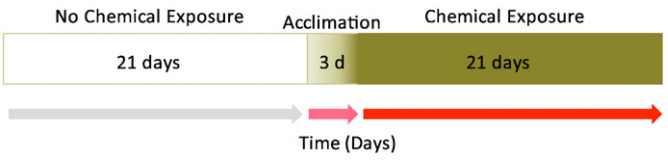

Fig. 1. Design of the pair-breeding assay. It consists of a 21 day period when the fish were not exposed to any test chemical followed by a 3 day acclimation period when the test chemical was delivered and concentrations increased to the desired level, followed by a further 21 days of exposure to the test chemicals.

2.4. Design of the mixture experiments

For the comparative assessment of mixture effects in biological assays, particularly in vivo studies, a well-defined robust endpoint with clear interpretation potential is needed (Runnalls et al., 2015). This can often be difficult with endpoints at the level of the whole organism. For predictive mixture modelling, compounds in any given mixture must affect the same end point. Fish reproduction studies are a successful example of a quantifiable endpoint, namely egg production, at the organism level that is ecologically important and can be robust and well-defined enough to use in assessing mixture effects. Egg production in a single study of paired fathead minnows can exhibit a degree of biological variability, for example in spawning frequency and batch size (Runnalls et al., unpublished data). However, this end point is considered robust enough for mixture studies. A novel means of representing egg production data was developed by Runnalls et al. (2015), confirming that this endpoint fulfils the necessary criteria for assessing mixture effects.

The endpoint for egg production (R) was defined as the ratio between the cumulative number of eggs estimated in the second 21-day period ($\hat{N}_{post}$) and that estimated in the first 21-day period ($\hat{N}_{pre}$):

$$R = \frac{\hat{N}_{post}}{\hat{N}_{pre}}. \quad (1)$$

The statistical unit was therefore the relative change of the cumulative number of eggs per pair and tank after exposure.

A five component mixture was designed based on the concentration responses of the individual compounds. A fixed equipotent mixture ratio was used, proportional to the concentration of each compound that produced a 10% effect level, or EC10 (interpreted from the single concentration response data). Three mixture concentrations were chosen in order to assess mixture effects at a range of concentrations. The lowest concentration was chosen so that the effects of the individual compounds were all below the expected statistical detection limit of the experimental design, and would therefore be expected to be judged as having no significant effect on reproductive performance. What we define as the statistical detection limit refers to the minimal effect magnitude that can be detected as statistically significant by hypothesis testing methods. This detection limit is expected to refer to egg production R approximately 20–30% below control level. This means that egg productions R of ca. 70–80% (and less) of control values are detectable with reasonable confidence ($\alpha = 5\%$, power = 80%). Mixture effects according to both CA and IA would be predicted to exceed this detection limit. Two other mixture concentrations were chosen whereby the model predictions were easily differentiated from one another and observed effects could be easily aligned to one of the models. The actual concentrations of the five chemicals making up the mixture are provided in Table 1.

Two independent mixture studies were conducted. The first one contained four treatments: control, and low, medium and high

Table 1
Composition of the mixture.

		Concentration of components in the mixtures (ng/L)		
	Fraction p_i [%]	Low	Medium	High
EE2	0.043	0.16	0.5	1
Levonorgestrel	0.078	0.28	0.9	1.8
Beclomethasone dipropionate	5.479	19	63	127
Trenbolone	8.974	32	105	208
Desogestrel	85.426	300	990	1980
Total	100	351.44	1159.4	2317.8

concentrations of the mixture (Table 1). Due to the current concern about the quality of some ecotoxicology research (e.g. Harris et al., 2014), and evidence showing that the repeatability of a significant proportion of that research has not been demonstrated (Harris and Sumpter, 2015), we conducted a second mixture experiment in order to be able to determine the repeatability of our results (i.e. to assess their robustness). In this second mixture experiment only the low and medium concentrations of the mixture, plus a control group, were included.

2.5. Measurement of exposure concentrations in the mixture experiments

Due to the workload and costs of measuring the actual concentrations of all five chemicals in the mixture experiments, we chose to measure only three of them. Our reasoning was that if the actual concentrations of these three chemicals were reasonably close to their nominal concentrations, then it was very likely that the concentrations of the other two chemicals would also be close to their nominal values. We chose to measure the concentrations of EE2, levonorgestrel and trenbolone because commercial assays are available for these chemicals, which we had considerable experience of, and had found to be very reliable (see Runnalls et al., 2015).

Water samples (2 L) were collected weekly from alternate tanks (4 out of 8 tanks) for each treatment during the exposure period on days 0, 7, 14 and 21. The same tanks were sampled each week. An additional sample set was collected before the start of the chemical exposure period, on the final day of the pre-exposure period. Samples were extracted immediately over the following 2 days. Sample volumes were divided in half, with 1 L extracted on day one and 1 L extracted on day 2. Solid Phase Extraction was used for sample clean up and pre-concentration. Two separate methods were used for the extraction of the 3 compounds: EE2, 17β-Trenbolone, and Levonorgestrel.

In one method, 1 L samples were extracted and eluted to measure EE2 and 17β-Trenbolone (TB) concentrations in tank water. EE2 and TB-spiked MilliQ water samples were also extracted to test extraction efficiencies and recoveries. Samples were extracted by Solid Phase Extraction using a Visiprep SPE Vacuum Manifold (Supelco). Samples were extracted on to Sep Pak C18 cartridges (Waters, UK), and stored at −20 °C until analysis. Immediately prior to steroid measurement, cartridges were removed from the freezer and allowed to warm to room temperature. Extracts were eluted with 2 × 5 mL methanol (MEOH). Samples were dried in a centrifugal vacuum concentrator (miVac Quattro and miVac SpeedTrap) at 30 °C at 2 h intervals until completely dry. They were then re-suspended in ethanol at various dilutions to achieve final concentrations within the range of the standard curve of each assay. Concentrations of 17β-trenbolone and EE2 were measured using commercial ELISAs (5081TREN from Europroxima and L22000405 Ethinylestradiol EIA from Biosense, respectively). In the other method, Levonorgestrel concentrations were quantified using a commercially-available radioimmunoassay from Immunometrics UK Ltd. after its adaptation for use with aqueous samples. One liter water samples were extracted and eluted as described above and stored at −20 °C until use. Levonorgestrel-spiked MilliQ water samples, to provide quality control, were also extracted and analysed. Extracts were eluted from C18 cartridges with 1 × MTBE (90% MTBE, 10% MeOH), 1 × ethyl acetate and 1 × MeOH. Samples were dried as described above and re-suspended in ethanol at various concentrations appropriate to the range of the standard curve. Further details are provided in Runnalls et al., 2015.

2.6. Concentration-response analysis

Data from continuous endpoints were examined for normal distribution and homogeneity of variance, and if relevant, transformed. If data were censored due to values below the limit of quantification, data were analysed by Tobit regression, otherwise by ANOVA. All egg count data were examined by generalized linear modelling (Poisson or logit link). Statistical significance between control and treatment means was assessed using multiple contrast tests (Dunnett contrasts, global error rate $\alpha = 5\%$, two-sided) (Bretz et al., 2005).

We adopted a best-fit approach for describing the relative egg production parameter (Eq. (1)) in response to the exposure, in which different regression models were fitted independently to the same data set, and the best fit was selected on the basis of statistical criteria (Scholze et al., 2001). The model parameters were estimated by least squares, and potential data censoring at zero effect levels was considered as irrelevant for our mixture assessment and not implemented in data analysis. This approach was applied using the NLMIXED function of the SAS statistical software package (SAS Institute, Cary, USA).

2.7. Mixture prediction and assessment

As described by Faust et al. (2001), under the assumption of CA a mixture concentration producing an effect X can be calculated for a n-compound mixture as

$$EC_X(mixture) = \left(\sum_{i=1}^{n} \frac{p_i}{EC_{X,i}}\right)^{-1}, \quad (2)$$

where EC_X(mixture) is the mixture concentration that produces the effect X for a combination of n individual concentrations c_i, $EC_{X,i}$ are the concentrations of the individual components that on their own produce the same effect X as the mixture, and p_i is the ratio of the ith component in the mixture ($p_i = c_i/(c_1 + .. + c_n)$, (see Table 1). The individual effect concentrations (EC10, EC50) are derived from the inverse of the nonlinear regression function which describes best the observed concentration effect data of the components (Table 2).

The basic version of IA has been formulated under the simple assumption that the susceptibilities of the individuals of an at-risk-population to different dissimilarly acting mixture components are not correlated with each other (Faust et al., 2003). For a n-compopund mixture and a relative effect endpoint that describes reductions of responses (descending dose-response curves) in relation to the average control level (Eq. (1)), IA is commonly defined by the equation as

$$E(c_{mixture}) = \prod_{i=1}^{n} E(c_i), \quad (3)$$

where $E(c_i)$ denote the effects produced by the individual compounds c_i, and $E(c_{mixture})$ is the total effect of the mixture. The main assumption is that the effect endpoint is normalised to an effect range 0 to 1, i.e. control and exposure mean estimated outside this range would violate the use of Eq. (3). Ideally, all control estimates in the pair-breeding assay would be identical to a common reference value of 1, with the condition that the egg production is stable over the entire study duration of nearly 7 weeks. However, occasionally we observed deviations from this optimal condition, with mean control estimates slightly above or below 1. Although too small to be judged statistically different from this reference value, it is nevertheless biologically plausible that the egg performance is on average slightly better at the beginning or end of the

Table 2
Statistical analysis of egg production by pairs of Fathead Minnows exposed to both individual steroid pharmaceuticals and their mixtures, together with the corresponding mixture responses predicted by Concentration Addition (CA) and Independent Action (IA).

Substance (in order of their EC50s)	Concentration response function							
	RM	$\hat{\theta}_1$	$\hat{\theta}_2$	θ_{min}	$\hat{\theta}_{max}$	EC10[a] [ng/L]	EC50* [ng/L]	NOEC* [ng/L]
EE2	Weibull	0.044	-0.75	0	1.06	0.16 [0.01;1.90]	4.44 [1.26;15.6]	<0.5
Levonorgestrel	Logit	1.101	-1.25	0	1.05	0.28 [0.02;4.85]	9.11 [2.96;28.1]	0.5
Trenbolone	Weibull	1.829	-0.78	-0.5	1.01	13.4 [2.31;84.3]	176 [49.6;624]	16
Beclomethasone	Logit	4.181	-1.52	0	1.02	25.7 [3.36;197]	596 [269;1320]	<100
Desogestrel	Logit	5.956	-1.51	-1	1.05	300 [41.5;2170]	2253 [880;5771]	<10
Mixture	Logit	8.344	-3.35	0	0.97	n.d.	283 [171;468]	n.d.
Predicted by CA							977 [488;1545]	
Predicted by IA							258 [40;564]	

EC10, EC50: concentration reducing egg production by 10% and 50%, respectively; NOEC: No observed effect concentration. Values in brackets denote the upper and lower limits of the approximate 95% confidence interval; the column "RM" indicates the mathematical regression function as defined by Scholze et al. (2001); $\hat{\theta}_1, \hat{\theta}_2, \hat{\theta}_{max}$ estimated model parameters, given for concentrations expressed in ng/L (rounded values), θ_{min} were not estimated, but set to the reported values. $\hat{\theta}_{max}$ equals the estimated mean control level.
n.d. = not determined.
[a] All values are derived on the basis of nominal concentrations.

study. For this reason we always estimated a baseline control level in regression modelling (see Table 2). To account for the variation between the individual baseline control estimates in the calculation of IA mixture responses, we further normalised the endpoint to its study control mean, and Eq. (3) was corrected to

$$E(c_{mixture}) = mean\,control_{mixture} * \prod_{i=1}^{n} \frac{E(c_i)}{mean\,control_i}. \quad (4)$$

Here, control means of the individual mixture components and mixtures were estimated from their corresponding regression model parameters $\hat{\theta}_{max}$ (Table 2). This correction assumes that data from studies with different control responses are proportionally the same, and from our experience with repeated studies this seems to be justified for reasonably small differences between control levels. This correction only has a measurable impact on the low effect estimates that are needed for accurate IA predictions. Since the prediction of mixture effects according to CA is based on effect concentrations that correspond to the same defined response magnitudes, and not on the responses themselves, we did not use this approach for CA calculations, but only for IA calculations.

To account for the statistical uncertainty in the CA and IA predictions, we used the bootstrap method (Efron and Tibshirani, 1993) to produce approximate 95% confidence limits around the mean predicted effect. Differences between predicted and observed effect concentrations were deemed statistically significant when the 95% confidence belts of the prediction did not overlap with those of the experimentally observed mixture effects.

3. Results

No evidence of acute toxicity was observed in any of the experiments. Fish appeared healthy throughout and behaved normally. The mortality rate was below 1.5% in all experiments (often 0%), and within the range of normal survival rates recorded in our laboratory for this fish species.

3.1. Single chemicals

The single chemical data for all five chemicals are shown in Fig. 2. Data from previous studies of EE2 and Levonorgestrel on egg production have already been demonstrated to be suitable for mixture studies (Runnalls et al., 2015), and the remaining three chemicals, namely trenbolone, beclomethasone dipropionate and desogestrel, all inhibited egg production in a clear concentration-dependant manner, which allowed the estimation of concentration-response regression curves (Fig. 2). In the case of trenbolone, data from two separate experiments were combined for data analysis. As a consequence, the updated statistical estimates for this chemical differ slightly from the original values (based only on the first experiment) used for the mixture planning; the trenbolone concentration present in the lowest mixture concentration is now expected to produce a slightly higher degree of inhibition of egg production of about 20% compared to the original estimate of 10% inhibition (see Fig. 2).

Potencies of the five chemicals varied over a range of 500-fold, with EE2 the most potent at reducing egg production with an estimated EC50 of 4.44 ng/L, and the synthetic progestin desogestrel the least potent with an estimated EC50 of 2253 ng/L. All regression curves are relatively flat, indicated by the low estimates of their regression steepness parameters (Θ, Table 2). The uncertainty of a median effect concentration (EC50) can be assessed by its 95% confidence belt, which were estimated for all chemicals to be around a factor of 10. As a mathematical consequence, an EC50 predicted by CA has to be of lower uncertainty, which we considered as sufficient for the comparative mixture assessment (Table 2).

In conclusion, the pair-breeding test provides robust, repeatable data that are suitable for mixture studies.

3.2. Mixture of all five chemicals

All data analyses are based on nominal concentrations. The reason for this is that the measured concentrations of EE2, trenbolone and levonorgestrel were all close to the nominal concentrations (see Table 4). Considering the error involved in the measurement of these low concentrations of pharmaceuticals, it was not possible to conclude that the measured concentrations were any more accurate than the nominal concentrations. As a consequence, the mixture assessments were performed on nominal concentrations, and this is valid only if the measured concentrations of the individual chemicals are not only reproducible between studies, but also similar in the mixture as when tested alone. Based on our experience from repeated studies and a previous binary mixture study with EE2 and levonorgestrel (Runnalls et al., 2015), we expected similar robust analytical findings for the five chemical mixture, as was indeed obtained (see below).

The fish tank concentrations of three of the five chemicals in the mixture, namely EE2, 17-β trenbolone and levonorgestrel, were measured as part of mixture experiment 1 (Table 4). Recoveries from water samples spiked with either EE2 or 17-trenbolone were between 79 and 100%, suggesting accurate measurement of the spikes (Table 3). Recoveries of levonorgestrel spikes were around 200%, possibly suggesting that our procedures were overestimating the actual concentrations of this chemical (Table 3). Similarly, measured water concentrations of both EE2 and 17β-trenbolone taken during the exposure period of experiment 1 were

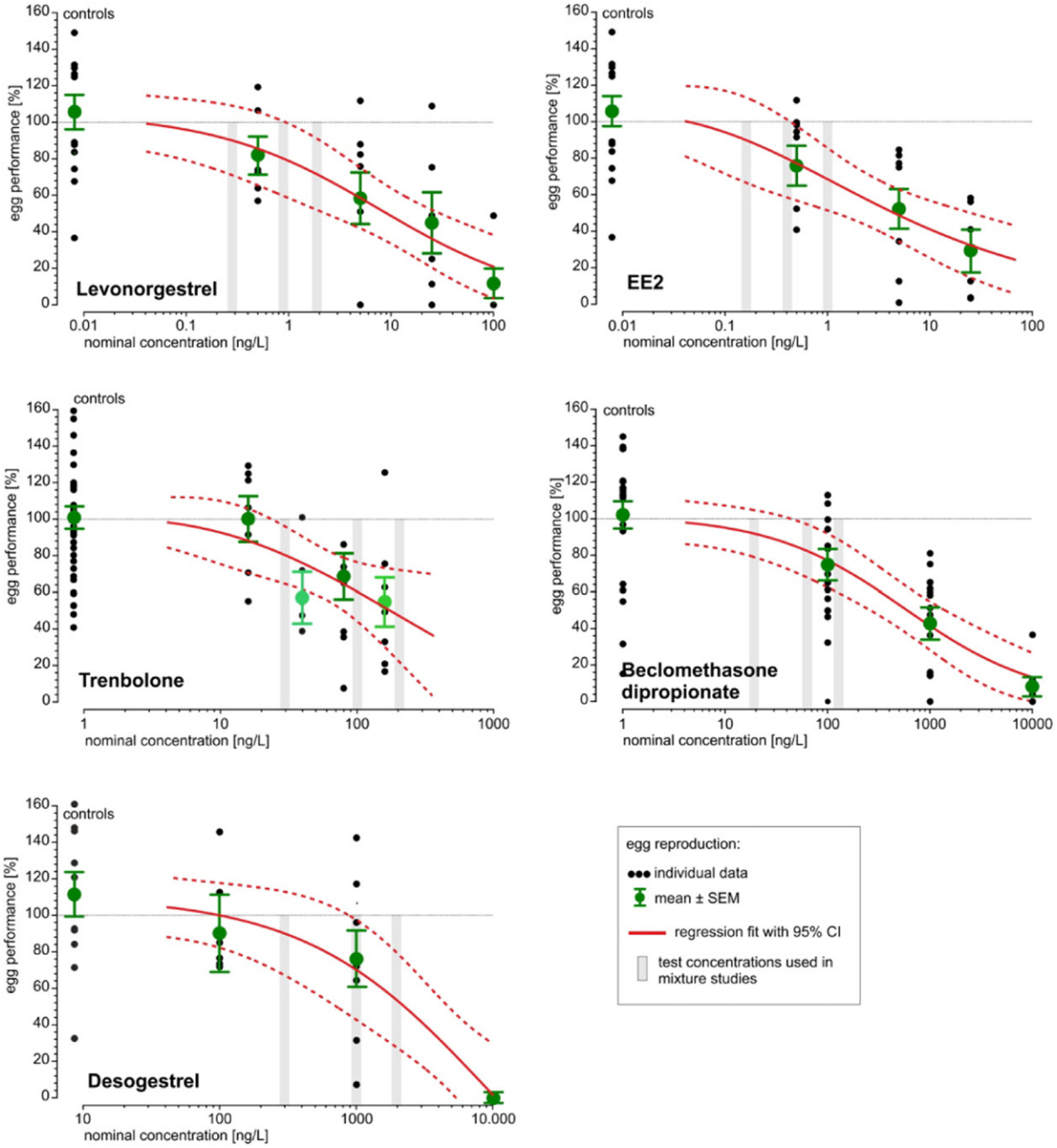

Fig. 2. Concentration-related inhibition of egg production of pairs of Fathead Minnow by the synthetic steroids Levonorgestrel, EE2, Trenbolone, Beclomethasone dipropionate and desogestrel. Trenbolone data tested in parallel to the mixture study are coloured in light green. Shown are individual data points (black dots), means and SEMs (error bars) and the best-fit regression models with their 95% confidence intervals (red lines and red dots, respectively). Data for EE2, Levonorgestrel and desogestrel are from previous studies (Runnalls et al., 2013; Runnalls et al., 2015).

close to nominal concentrations, ranging from 72 to 110%, whereas measured concentrations of levonorgestrel were higher than nominal concentrations, ranging from 158 to 167%. None of the test chemicals could be detected in water from the control tanks. Given the difficulty of accurately measuring such low concentrations of these three chemicals, we consider that the results demonstrate that the actual concentrations in the fish tanks were very similar to the nominal concentrations. The fact that the levonorgestrel concentrations in both spiked water samples and water samples from mixture experiment 1 were over-estimated by about 2-fold suggest that the over-estimate is an artifact of the procedure used to measure the concentrations, rather than the concentrations in the mixture experiment actually being higher than intended. Measured tank water concentrations of EE2 (the only chemical measured in mixture experiment 2) were, as with mixture experiment 1, close to nominal concentrations. Overall, these findings meant that (1) nominal concentrations could be used in the analysis of the data from the mixture experiments, and (2) the egg production data from the two mixture experiments could be combined. The observed degrees of inhibition of egg production were also comparable between the two mixture experiments (see below), further supporting the pooling of data. Mean control egg production levels were also similar between the two mixture experiments, with an estimated average of 2060 eggs

Table 3
Recoveries of 17α-Ethinylestradiol, 17β-Trenbolone and levonorgestrel from spiked water samples.

Nominal [ng/L]	Measured [ng/L]				Mean [ng/L]	Recovery [%]
	Week 1	Week 2	Week 3	Week 4		
17α-Ethinylestradiol						
0.16	0.11	0.13	0.15	0.12	0.13	82.5
0.52	0.40	0.42	0.46	0.42	0.42	82.5
1.046	0.77	0.90	0.81	0.83	0.83	79.3
17β-Trenbolone						
31.62	9.86	47.2	36.1	34.4	31.8	99
104.4	54.0	104.5	112.4	107.6	94.6	90
208.7	126.7	183.0	179.8	203.3	173.2	83
Levonorgestrel						
0.28	0.75	0.70	0.67	0.63	0.69	245
0.93	2.19	2.11	2.0	1.94	2.06	221
1.86	1.97	4.17	4.33	5.03	3.87	208

Table 4
Measured concentrations of 17α-Ethinylestradiol, 17β-Trenbolone and levonorgestrel in the water of the tanks containing the fish.

Nominal [ng/L]	Measured [ng/L]					Mean [ng/L]	% of nominal
	Week 0	Week 1	Week 2	Week 3	Week 4		
17α-Ethinylestradiol							
Control	<DL	<DL	<DL	<DL	<DL	<DL	–
		<DL	<DL	<DL	<DL		
		<DL	<DL	<DL	<DL		
		<DL	<DL	<DL	<DL		
0.16	<DL	0.10	0.13	0.16	0.17	0.14	87.9
		0.10	0.18	0.15	0.21		
		0.11	0.14	0.15	0.16		
		0.19	0.08	0.10	0.09		
0.52	<DL	0.36	0.44	0.45	0.54	0.41	79.9
		0.30	0.45	0.53	0.67		
		0.31	0.48	0.62	0.28		
		0.23	0.30	0.33	0.34		
1.046	<DL	0.68	0.86	1.04	1.06	0.75	72.1
		0.72	0.97	1.06	0.05		
		0.59	0.60	1.20	0.84		
		0.45	0.50	0.61	0.82		
17β-Trenbolone							
Control	<DL	<DL	<DL	<DL	<DL	<DL	
		<DL	<DL	<DL	<DL		
		<DL	<DL	<DL	<DL		
		<DL	<DL	<DL	<DL		
31.62	<DL	17.53	31.48	33.43	43.08	33.72	106.6
		19.99	44.43	44.78	42.71		
		23.89	43.74	42.41	30.83		
		12.21	34.73	42.88	31.41		
104.4	<DL	91.78	139.95	113.48	130.95	115.24	110.4
		44.91	132.05	132.05	135.44		
		50.73	137.24	144.23	148.86		
		68.14	100.56	125.61	147.88		
208.7	<DL	110.53	233.67	278.10	219.09	194.87	93.4
		107.45	290.41	180.84	101.96		
		130.11	141.54	273.96	267.56		
		178.89	160.81	225.65	217.60		
Levonorgestrel							
Control	<DL	<DL	<DL	<DL	<DL	<DL	–
		<DL	<DL	<DL	<DL		
		<DL	<DL	<DL	<DL		
		<DL	<DL	<DL	<DL		
0.28	<DL	0.31	0.46	0.46	0.59	0.44	158
		0.30	0.46	0.46	0.60		
		0.29	0.46	0.43	0.59		
		0.25	0.43	0.45	0.57		
0.93	<DL	1.09	1.70	1.59	1.88	1.55	167
		1.08	1.61	1.56	1.91		
		1.17	1.59	1.63	1.87		
		1.67	1.64	1.06	1.90		
1.86 ng/L	<DL	1.99	3.35	4.54	3.63	3.11	167
		2.27	3.15	3.49	3.57		
		0.98	3.28	3.46	3.53		
		1.56	3.31	3.62	3.70		

from controls in study 1 and 2330 eggs in study 2 (data not shown). These performances are in the range previously observed in the single substance studies.

The mixture responses from the two studies were tested for differences and this did not reveal any significant differences, making the pooling of data from the studies possible. The mixture reduced egg production in a clear concentration-dependent manner (Fig. 3A). The lowest mixture concentration reduced the average egg production by ca. 50%, with each individual fish performance in the range of 0%–75% and thus well below the average control performance. As the data pooling increased the number of fish per treatment group and thus lowered the statistical detection limit, the statistical identification of a 20% reduction of the egg performance as significant was very likely (at false-positive rate alpha = 5% and false-negative rate beta = 20%), and therefore we are confident that the observed reduced egg production at the lowest mixture concentration is significant and not due to biological variability. At the highest mixture concentration, egg production completely ceased in all fish pairs after 7 days of exposure.

Both CA and IA predicted the observed mixture responses reasonably well (Fig. 3B and C, respectively), although the average reductions in egg production at all test concentrations were closer to the IA prediction curve than they were to the CA prediction curve. CA slightly underestimated the mixture responses. Both mixture models predicted that the lowest mixture concentration would produce a clear reduction in egg production, according to CA by about 30% and based on IA by approximately 50%. Both these predictions exceeded the 20% statistical detection limit of our experimental study design. At the highest mixture concentration, both models predicted a more or less complete cessation of egg production, as was indeed observed.

As the widths of the uncertainty belts in Fig. 3 indicate, the statistical uncertainty of the IA prediction was higher than that of CA (see also Table 3). This was expected, because median IA mixture responses are calculated from low effect estimates of the individual compounds, which are far more uncertain than median effect estimates (see Confidence Intervals in Fig. 2 and Table 2). In contrast, the certainty associated with the CA prediction is greater as it is based effect concentration for the individual compounds which covers the same effect ranges as the mixture prediction. Consequently, only at effect ranges around 50% is there a sufficient discrimination between the two prediction models supported with a relatively high degree of statistical certainty.

The issue of whether or not a mixture can produce a significant effect when each of the compounds is present in the mixture at a concentration that on its own produces very little or no statistically significant effect is investigated in Fig. 4. The effects of each individual chemical at the concentrations present in the lowest concentration of the mixture were estimated from the individual best-fit regression models (Table 2, Fig. 2) and are shown in Fig. 4A. For all 5 chemicals, this analysis revealed that the effect expected to occur after single administration is not statistically significantly different from untreated controls (illustrated by the gray area around the control line). Consequently, in single chemical tests these concentrations would have been declared as the NOEC for this endpoint. However, the lowest mixture concentration produced a very significant effect; egg production was inhibited by over 50%, thus demonstrating a very marked "something from 'nothing'" effect. In the case of the medium mixture concentration (Fig. 4B), there was a nearly total suppression of egg production (by 90%), yet, had each compound been tested individually at the concentration it was present at in the mixture, egg production would have been estimated to be reduced by between 18% (Beclomethasone diproprionate) and 40% (trenbolone). Thus, one could call this additivity response a clear demonstration of 'a lot from a little'.

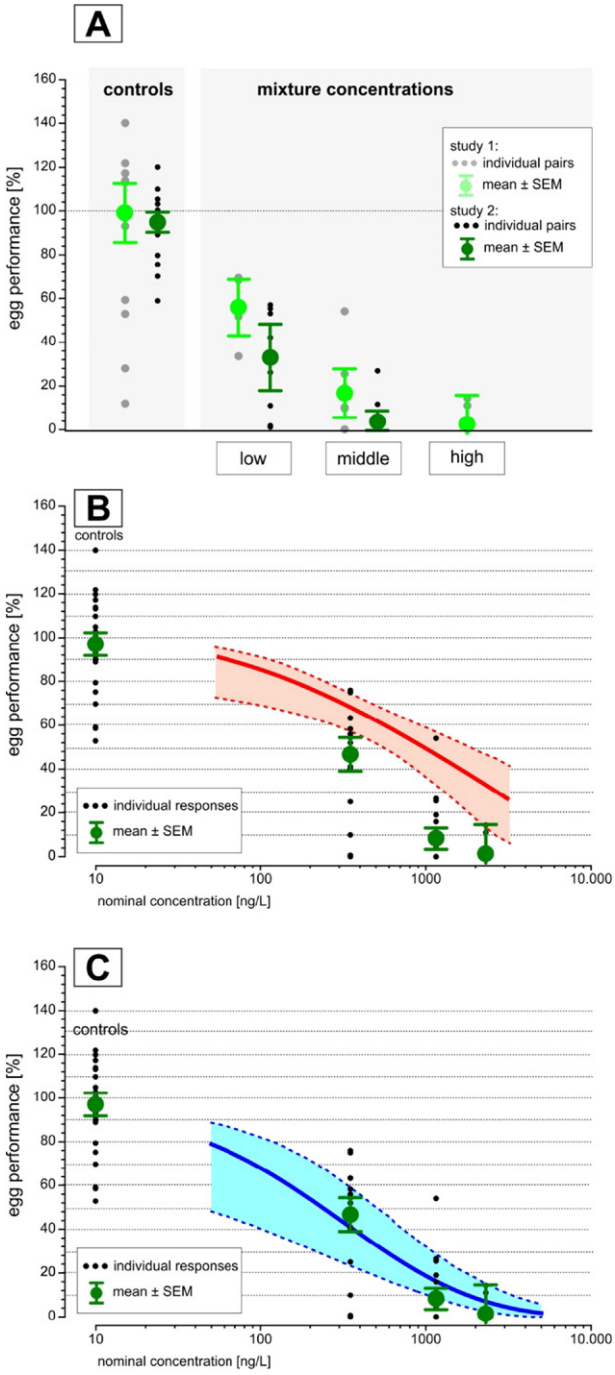

Fig. 3. Observed and predicted egg production to a mixture of 5 pharmaceuticals. Observed mixture effects from both studies are shown in (A), and predictions for Concentration Addition (B) and Independent Action (C) are shown as mean curves ±95% confidence belt, together with pooled data from both studies.

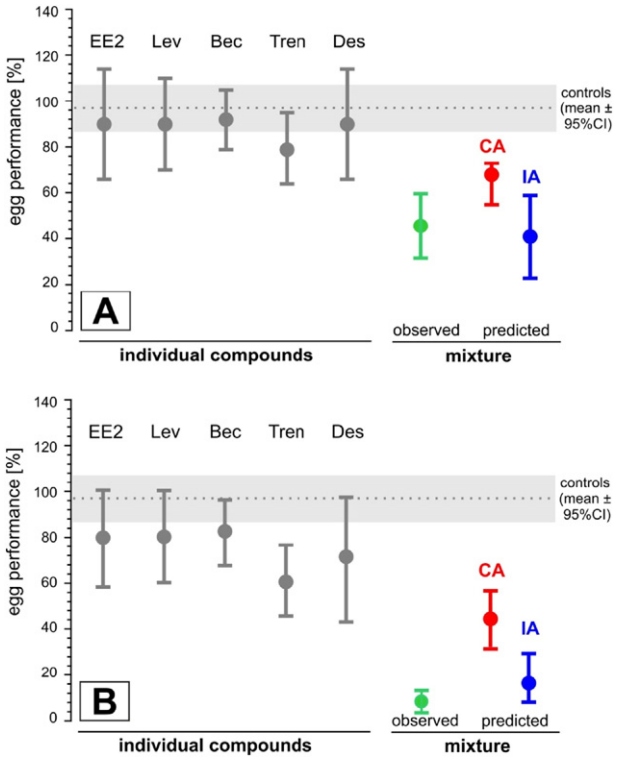

Fig. 4. Comparison of mixture effects (observed and predicted) with the individual effects of all 5 mixture compounds at low (A) and medium (B) concentrations of the whole mixture. Predictions for Concentration Addition (CA) and Independent Action (IA) are shown as mean ± 95% confidence belt, together with pooled data from both studies. Individual effects are estimated from the best-fit regression curves (Fig. 2).

4. Discussion

Mixture toxicity is extremely important, because essentially all wildlife (and humans), worldwide and in all habitats, are exposed to complex, ill-defined mixtures of chemicals, but it is very difficult to study in a manner likely to provide robust, generalizable results. This is especially true for in vivo experiments. This study took three years and cost in the region of 1.5 million euros (or dollars). Further, considerable experience and expertise are required, together with excellent facilities.

In this proof-of-principle study with five commonly used pharmaceuticals, which used egg production of fish as the apical endpoint, we hypothesized that their combined responses would follow expectations of additive effects. Here the responses from two independent but similar mixture studies were compared with predictions derived from two well-established mixture assessment concepts, CA and IA. Both concepts describe the joint effects on the basis of the concentration-response information of the individual compounds, albeit in different ways, and therefore they can produce different additivity predictions. From our mechanistic understanding about how these compounds act on egg production (see below), it was unclear which, if either, of these models would best describe the actual observations. For this reason, both concepts were utilized.

All five compounds, when tested individually, inhibited egg production in a concentration-dependent manner. This included a glucocorticoid, beclomethasone dipropionate, which is not a traditional reproductive toxicant (but see Kugathas et al., 2013). The potencies of the five compounds varied considerably. Levonorgestrel and EE2 were the most potent; effects occurred at sub-ng/L concentrations (see also Runnalls et al., 2015). Trenbolone was reasonably potent, as has been shown previously (Ankley et al., 2003), as was beclomethasone dipropionate. The synthetic progestogen desogestrel was the least potent of the compounds tested. The very potent nature of these steroid pharmaceuticals supports the concerns of Runnalls et al. (2010), who considered this class of pharmaceuticals as of high environmental concern.

Although each of the five steroid hormones used in this study probably has one major mode of action (MoA), none of them can be considered specific to a single receptor type. It is now widely recognized that pharmaceuticals (and probably all chemicals) do not have a single MoA; instead, they have a range of targets (receptors, enzymes, ion channels, etc.), showing different degrees of affinity for these different targets, a concept termed polypharmacology. Put another way, a variety of MoAs are exhibited by each of the steroids used in this study. For example, the synthetic progestins levonorgestrel, gestodene and norethindrone have high binding affinities not only for the human PR but also for

the human AR, probably because many synthetic progestins are 19 carbon steroids structurally related to nortestosterone (Runnalls et al., 2013). They can also bind to and activate the ER, albeit only at higher concentrations (Runnalls et al., 2013). Synthetic progestins can also activate the fish glucocorticoid receptor (Miyagawa et al., 2015). In fact levonorgestrel, which is thought of primarily as a synthetic progestin, probably exerts its adverse effects on the reproductive axis of fish via the AR, not the PR (Svensson et al., 2013). As fish synthesize very little progesterone, and instead utilize 17α,20β- dihydroxyprogesterone as their physiologically most important natural progesterone, their PR has a considerably different specificity to that of the mammalian PR (Chen et al., 2010), making it difficult presently to know to what extent synthetic progestins will bind to and activate the fish PR. Further, full characterization of the repertoire of steroid hormone receptors in fish, including the Fathead Minnow, has not yet been achieved. Fish do, like all vertebrates, possess ER, AR, PR and GR, but they often possess more than one form of each receptor class, and these different forms can have somewhat different specificities (e.g. Tohyama et al., 2015), and the same type can have a somewhat different specificity in different species of fish as a consequence of small differences in the ligand binding domain of the receptor (Miyagawa et al., 2014). To add even more complexity, metabolism of these hormones is likely to produce active metabolites with different MoAs to the parent hormone (Ojoghoro et al., 2017). All of these factors, and probably others, make it very difficult, if not impossible, to decide the exact MoAs of the steroid hormones we used that led ultimately to reductions in egg production. Hence from pharmacological and physiological points of view it is impossible to decide which of CA or IA is likely to be the most appropriate mixture model to apply.

Although each of these compounds has unique biological effects (e.g. EE2 stimulates production of vitellogenin, and beclomethasone dipropionate elevates the blood glucose level), nevertheless they acted additively on egg production, an apical endpoint used widely in ecotoxicology tests because it is ecologically very relevant. Both mixture concepts predicted the potency of the mixture well, albeit that IA produced a more accurate prediction than CA. To our knowledge this is the first time for a multi-component mixture tested on an apical parameter in a multi-cellular organism that IA not only predicted a higher response than CA, but where the observed responses of the mixture were also better described by IA. So far, all experimental in vivo evidence suggest that CA generally predicts higher toxicity than IA, independently of how well the mixture constituents fulfilled the pharmacological assumptions of the mixture models, and independently of how many compounds were included in the mixture studies (Kortenkamp et al., 2009). This common empirical observation has led to the suggestion that CA should be used to produce a worst-case estimation in mixture toxicology, and therefore it is often considered as the preferred default additivity assumption in the risk assessment of chemical mixtures (EFSA, 2013). Our mixture findings suggest that the worst-case assumption of CA might not always be fulfilled; for example, the EC50 predicted by IA was a factor of about 3.8 lower than the EC50 predicted by CA (Table 2). That factor could be considered relatively minor and probably largely irrelevant in the context of assessing the environmental risk of chemical mixtures. However, in the environment we can expect many more than 5 compounds that affect egg production to be present at the same time and location (see below), which raises the issue of whether or not the underestimation of additivity responses by CA can become more dramatic (i.e. factor > 10), and hence of more consequence, when the mixture consists of higher numbers of compounds.

Since the quantitative interrelationship between both prediction models is well known, with the determining factors being the mixture ratio, the effect level and the steepness of the individual concentration-response curves of the mixture compounds (Drescher and Boedeker, 1995; Junghans et al., 2006), it is possible to calculate for any mixture that mixture composition which leads to the maximum difference between the predictions of the two models. However, as sufficient data for our chosen endpoint (egg production) are available for only a small number of compounds, caution is required before it is assumed that the outcome can be generalized. Nevertheless, if we assume that the egg production data obtained during this study are representative for all chemicals that are able to reduce egg production, it is possible to use simulation techniques to calculate how many compounds would be needed to obtain a 10-fold lower EC50 by IA compared to CA. That simulation predicts that a 23-fold higher number of compounds (i.e. 115 compounds) would be required to achieve a 10-fold lower EC50. Although such an exposure scenario (115 different compounds, all able to inhibit egg production, are present simultaneously) seems intuitively unlikely, it is not out of the question, as discussed below. However, to fulfil this scenario it is necessary for all 115 compounds to inhibit egg production via different modes of action, as well as all compounds being present at approximately the same effect level, to produce a balanced mixture design (here roughly in proportion to the EC10 of each compound): both assumptions seem unlikely, because there is a physiological limit as to the number of truly independent modes of action by which egg production can be inhibited. Further, evidence from monitoring studies suggests that usually only a few compounds dominate the mixture response; that is, are drivers of mixture toxicity (Price and Han, 2011; Evans et al., 2015).

It is not easy to gauge the environmental relevance of our results because a full picture of what steroidal pharmaceuticals are present in the aquatic environment, and at what concentrations, is not available. Accurately measuring the extremely low (sub-ng/L) concentrations of many steroidal pharmaceuticals in rivers has proved very challenging, and only recently have analytical techniques been developed that have the required sensitivity. The picture that is emerging is that quite high numbers of steroidal pharmaceuticals are probably present simultaneously in rivers receiving wastewater effluent, albeit that their concentrations are very low. Early information on possible concentrations of sex steroid hormones in streams in the US suggested that concentrations of many different ones were high, being >100 ng/L in some cases (Kolpin et al., 2002). These initial estimates were soon challenged (Ericson et al., 2002) and are now recognized to be wrong (Johnson et al., 2008). As analytical techniques have improved, and more experience has been gained, reported concentrations have fallen steadily. Many reports provide data on the concentrations of the various classes of steroids, both natural and synthetic, in wastewater influent and effluent, but it is concentrations in river water that are relevant to risk assessment, because fish and other aquatic organisms live in rivers and lakes, not wastewater treatment works. The very low concentrations of steroid hormones in rivers have presented a real analytical challenge, but recent publications probably provide realistic estimates of concentrations. Even when detection limits are below 1 ng/L, it is often the case that few, if any, steroids can be detected and their concentrations quantified (Matejicek and Kuban, 2007; Zhou et al., 2016; Zhang et al., 2017). Given that Gardner et al. (2012), in an extremely comprehensive study of 162 wastewater effluents in the UK, report that EE2 concentrations were less than 1 ng/L in most effluents, this is not surprising. In one of the most thorough investigations reported to date, Zhang and Fent (2018) report that out of a wide range of different steroids (androgens, estrogens, corticosteroids and progestins), only two could be detected in rivers in Switzerland. As their detection limits for most of the steroids were less than 1 ng/L, this indicates that if steroid hormones are present in Swiss rivers, their concentrations are likely to be very low. In support of these findings, Zhou et al. (2016) attempted to measure androgens, estrogens, glucocorticosteroids and progestogens in a shallow Chinese lake receiving wastewater effluent, but found concentrations of most of the targeted steroids non-detectable; this included EE2 and levonorgestrel.

As far as environmental concentrations of the five steroids used in this study are concerned, it is probably possible to conclude the following. EE2 can be present in rivers, but its concentration is usually less than 1 ng/L; for example, Avar et al. (2016) report an average concentration of 0.084 ng/L in Hungarian rivers. However, such a low

concentration should not be dismissed, because it might adversely affect fish (Caldwell et al., 2012; Runnalls et al., 2015). The same is probably true of levonorgestrel, although much less information is available on this widely-used synthetic progestogen. Trenbolone is widely used in farming in the US, and as a consequence is detectable in rivers downstream of large animal holding facilities at low to sub-ng/L concentrations (e.g. Cavallin et al., 2014), although data are sparse currently. Trenbolone seems unlikely to be present in the environment in many parts of the world, such as the European Union, because it's use as a growth promoter in agriculture is banned, although there may nevertheless be illegal use in agriculture and aquaculture, and also by sportspeople wanting to strengthen their muscles. We are unaware of any reports of the presence of desogestrel and beclomethasone dipropionate in the aquatic environment. The later glucocorticoid is a pro-drug that is readily metabolised to its active form in patients (Margiotta-Casaluci et al., 2016) and hence is unlikely to reach the environment as beclomethasone dipropionate. In summary it can be concluded that the concentrations of EE2, and probably also levonorgestrel, used in this study are environmentally relevant. Those of trenbolone were probably above typical environmental concentrations. Those of beclomethasone dipropionate and desogestrel were probably much higher than typical environmental concentrations. However, it should always be kept in mind that there may be 'hot spots' in the environment where concentrations of steroid hormones are much higher than typical concentrations: one such location downstream of a pharmaceutical manufacturing facility has been identified in France (Creusot et al., 2014).

From a regulatory perspective, the 'something from 'nothing' and 'a lot from a little' results shown in Fig. 4 are probably by far the most important findings included in this paper. At a simplistic level, these results demonstrate that assessing risk based on the effects of individual chemicals can significantly underestimate the degree of risk. The concept of "something from 'nothing'" has previously been demonstrated in vitro (Silva et al., 2002), but to our knowledge has never been studied in vertebrates. The term 'nothing' refers to an often-occurring decision dilemma in toxicology, when effect magnitudes too small to be detected by statistical testing (statistical detection limit) declared as NOECs are misinterpreted as generally being without biological relevance. As a result, a NOEC is often confused with a threshold (in the sense of a concentration associated with zero effect) when in fact it only signifies a concentration associated with an effect magnitude too small to be distinguished from the effect variation seen with untreated controls. Due to the variation inherent in complex in vivo endpoints such as egg production in fish, the detection of low (and biologically often relevant) effects involves a high degree of uncertainty. Our proof-of-principle study demonstrates that small effects can add up to reach a statistically and biologically significant response when there is simultaneous exposure to multiple chemicals in fish. Furthermore, mixture prediction models are capable of anticipating such additive effects with a degree of certainty that we consider acceptable for risk assessment.

5. Conclusions

We have shown that a mixture of five steroidal pharmaceuticals considered to have distinctly different MoA's can inhibit egg production of fish in a manner accurately predicted by the additivity model IA. We further demonstrate that compounds present at low concentrations that on their own do not produce statistically significant effects can add up to elicit substantial mixture responses (something from 'nothing'). Egg production can even be suppressed entirely at concentrations of the individual mixture components which affect the egg performance only to a minor degree ('a lot from a little'). This proof-of-principle study also demonstrates that the existing compound-based mixture models can be used for more complex apical endpoints with inherently high biological variability. This evidence challenges the current regulatory framework as discussed by Kortenkamp et al. (2009). We are in full agreement with the conclusion of a very recent paper (Zhang et al., 2017) which was "Although steroid concentrations are low in Swiss rivers, the possibility of additive effects may be of concern". We would add only that these additive effects will not be confined to Swiss rivers; they could occur anywhere where steroids are present in the environment.

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