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From single chemicals to mixtures—Reproductive effects of levonorgestrel and ethinylestradiol on the fathead minnow

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ABSTRACT

The aquatic environment is polluted with thousands of chemicals. It is currently unclear which of these pose a significant threat to aquatic biota. The typical exposure scenario is now represented by a widespread blanket of contamination composed of myriads of individual pollutants—each typically present at a low concentration. The synthetic steroids, 17α-ethinylestradiol and levonorgestrel, have been widely reported to be present in the aquatic environment in the low ng to sub-ng/l range. They are widely used in contraceptive formulations, both individually and in combination. Our research employed the fathead minnow (*Pimephales promelas*) 21 day 'pair-breeding' assay to assess reproductive output when pairs of fish were exposed to the single chemicals at low environmentally relevant concentrations, and then to a binary mixture of them. A variety of endpoints were assessed, including egg production, which was inhibited in a concentration-dependent manner by both the individual chemicals and the mixture. Significant, sex specific effects were also seen with both chemicals, at differing levels of biological organisation. Plasma concentrations of EE2 and levonorgestrel were predicted and in the case of levonorgestrel measured, and compared with the human therapeutic plasma concentrations (Read-Across approach) to support the interpretation of the results. A novel quantitative method was developed for the data analysis, which ensured a suitable endpoint for the comparative mixture assessment. This approach compares the reproductive performance from individual pairs of fish during chemical exposure to its pre-treatment performance. The responses from the empirical mixture study were compared to predictions derived from the single substance data. We hypothesised combined responses which were best described by the concept of concentration addition, and found no clear indications against this additivity expectation. However, the effect profiles support the current knowledge that both compounds act in different ways to reduce egg production in fish, and suggest that probably response addition (also called Independent action) is the more appropriate mixture model in this case.

1. Introduction

The presence of a whole range of different pharmaceuticals in the aquatic environment has become more obvious as analytical techniques have improved. Recent research (Lindberg et al., 2014; Borova et al., 2014; Hughes et al., 2013) has shown that very high numbers of pharmaceuticals (and their transformation products) are present simultaneously (Cwiertny et al., 2014). The concentrations of individual compounds detected in the environment are generally too low to cause adverse effects; however, this simultaneous presence of large numbers of chemicals in a given place at any given time suggests that mechanistic and modelling approaches are needed to achieve a more realistic environmental risk assessment, by predicting the risk that chemical mixtures may elicit.

The effects that many of these chemicals may have on aquatic organisms are mostly unknown, particularly when complex mixtures are considered. The greatest amount of evidence for the potential impact of pharmaceuticals on the aquatic environment (via endocrine disruption) currently comes from one of the ingredients used in oral contraceptives, the synthetic estrogen ethinylestradiol (EE2). EE2 is used in oral contraceptive formulations and hormone replacement therapy, and has been previously shown to be of environmental concern because of its effects on fish reproduction and sexual development at extremely low con-

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centrations (reviewed in Sumpter and Jobling, 2013). Along with EE2, synthetic progestins (e.g. levonorgestrel) are also used as contraceptives, both on their own or in combination with EE2. Progestins are chiefly responsible for the contraceptive effect in humans, due to their inhibitory effects on follicular development and ovulation, whereas the estrogens act on the endometrium as well as contributing to the prevention of ovulation via feedback responses (Erkkola and Landgren, 2005). In fish the disruption of the HPG (hypothalamic–pituitary–gonadal) axis is likely the primary and first cause of reproductive failure. EE2 is only partially metabolised and removed by sewage treatment plants, and can therefore be detected in surface waters of many countries, albeit at extremely low concentrations. Predictive modelling of European rivers (Johnson et al., 2013) suggests that EE2 concentrations in sewage effluent and receiving waters are likely to be 0.2–2 ng/l; concentrations in most American rivers are probably lower (Hannah et al., 2009). Levonorgestrel has been one of the most widely used synthetic progestins since its development in 1972, and has been previously shown to have very significant effects on fathead minnow reproduction at very low concentrations (below 1 ng/l; Zeilinger et al., 2009). At slightly higher concentrations levonorgestrel has been shown to masculinise female fish, probably due to its androgenic properties (Zeilinger et al., 2009; Runnalls et al., 2013; Overturf et al., 2014). Due to its partial metabolism and incomplete removal during sewage treatment, levonorgestrel has been found to be present in the environment in the low nanogram per liter range. It has been detected in effluents at concentrations up to 30 ng/l, and in surface and ground waters up to 11 ng/l (Petrovic et al., 2002; Creusot et al., 2014; Vulliet et al., 2008). However, a representative picture of the concentrations of levonorgestrel, or any other synthetic progestin, in surface waters is not currently available; typical river concentrations are likely to be significantly lower than those mentioned above.

As a result of these environmental findings, we became interested in investigating the combined action of EE2 and levonorgestrel on fish. Although it has been widely acknowledged that not only the risk of individual chemicals on the environment should be assessed but also of their combinations, the risk assessment of environmental mixtures is still one of the most demanding, yet important, issues in ecotoxicology. Here the so-called "bottom-up" approach is the most accepted method, which utilizes the concentration response information from the individual chemicals and predicts their most likely combination effect. All empirical evidence suggests that the concept of concentration addition (CA) generally provides the best approximation, with the best accuracy achieved if the compounds act via the same mode of action. If the mechanistic assumptions are not fulfilled, CA is often proposed as a "worst-case" assumption, i.e. mixture responses are expected to be overestimated (Kortenkamp et al., 2009). Early progress has been made studying similarly acting chemicals on fish (for example, Brian et al., 2007), and the specific nature of the chosen (biomarker) endpoints in these studies probably ensured the excellent agreement between observed mixture responses and those predicted by CA. However, there are few data to date regarding mixtures of chemicals which have more diverse modes of action. Studying these mixtures requires an endpoint that is able to integrate independent effects originating from different sites and modes of action, leading to a common endpoint. In the present study, the test chemicals act via different mechanisms. EE2 is a potent estrogen receptor (ER) agonist, whereas levonorgestrel binds to the progesterone receptor (PR). However, levonorgestrel is also a potent androgen receptor agonist (AR). The combination of the two chemicals leads therefore to the modulation of three key targets of the endocrine system: the ER, PR, and AR. In spite of the different mechanisms of actions (MoA), both chemicals can lead to reproductive effects, in particular decreased egg production. The latter endpoint can be accurately quantified using a fathead minnow 21-day reproduction assay (Harries et al., 2000), which we selected for our investigations. This short-term reproduction assay uses reproductive output as the integrated measure of toxicant response by measuring egg production of individual pairs over several weeks, and these data can be supported by other endpoints that reflect responses at different levels of biological organisation associated with (anti-) estrogens and androgens. To our knowledge, no validated biomarkers for (anti-) progestogens are known.

Here we present the results of three independent studies performed to investigate the effects of levonorgestrel and EE2, alone and in combination, on the reproductive performance of fathead minnow (*Pimephales promelas*). From a modelling perspective, we applied the concept of concentration addition (CA) for the mixture prediction. Although both chemicals were not expected to fulfil the mechanistic requirements for CA per se, both induce the common apical effect (e.g. inhibition of egg production) via different mode of actions. We were interested to see how well CA approximates mixture responses, and whether the pragmatic "worst-case" assumptions of using CA for mixture responses still hold true. The following approach was conducted: (i) a new, quantitative statistical method was developed to analyse egg production data with the aim of increasing its use in mixture ecotoxicology, (ii) both compounds were tested individually in order to provide sufficient concentration-response data for the mixture modelling, (iii) the combined responses from levonorgestrel and EE2 were then predicted by the concept of concentration addition (CA), and (iv) finally, the mixture was tested and responses were compared to the predictions. In addition, we also predicted and measured drug plasma concentrations in one of the single chemical studies (levonorgestrel) to support the quantitative interpretation of the observed reproductive effects, as proposed by the Read-Across approach (Rand-weaver et al., 2013). The latter states that similar plasma concentrations of pharmaceuticals will have similar mode of action-related effects at similar level of biological organisation in both humans and fish.

2. Materials and methods

2.1. Research organisms

Adult fathead minnow (*Pimephales promelas*) (8–11 months old) were obtained from a breeding stock maintained at Brunel University. Fish were fed four times per day, twice each with adult brine shrimp (Tropical Marine Centre, Gamma irradiated) and flake food (Tetramin, Tetra, Southampton, UK). Fish were not fed on sampling days. These studies were carried out at Brunel University, London under both Project and Personnel Licences granted by the UK Home Office under the United Kingdom Animals Act (Scientific Procedures), and also in accordance with Brunel University's ethical policies.

2.2. Experimental design

Three independent in vivo pair-breeding studies were conducted, with the first two studies consisting of individual concentration responses to EE2 and levonorgestrel. Data from these studies were used to design the third study, which consisted of a mixture of the two chemicals in a fixed ratio over three concentrations. Each in vivo study employed a continuous flow-through system, incorporating 8 L glass tanks, which ensured a complete change of dechlorinated tap water (5 and 10 μm carbon filtered) at least every 2 h. Every tank contained a pair of fish (1 male and 1 female), and there were 8 pairs of fish for each concentration of the test chemical and 16 pairs of controls. Each tank contained a glass

dish, grid and tile for the fish to spawn on. The pair-breeding assay consisted of a 21 day pre-exposure period, a 3 day transition (when dosing of the chemical started), and a further 21 days of exposure to the test chemicals, when specific endpoints were measured. Parameters monitored within the tanks throughout the studies were temperature (25 ± 1 °C) and dissolved oxygen (8 ± 1 mg/l), nitrite, nitrate, ammonia, pH, carbonate hardness and general hardness. Tank concentrations of each test chemical were also measured. The photoperiod was maintained at 16 h light:8 h dark throughout, incorporating 20 min dawn:dusk transition periods. All tubing was medical grade silicone. The initial studies (individual chemicals) were carried out using nominal concentrations of 0.5, 5 and 25 ng/l of either EE2 (study 1) or levonorgestrel (study 2). The subsequent mixture study (study 3) involved a fix ratio (1:1) of the chemicals, with concentrations of 0.25, 2.5 and 12.5 ng/l of each individual chemical in the mixture and therefore the combined concentrations were 0.5, 5 and 25 ng/l.

The mixture was tested according to a fixed-ratio mixture design, in which both compounds were present at mixture ratios proportional to their expected individual potencies, and the predictive power of CA was assessed by comparing the predicted reduction on egg reproduction of the two compounds with that observed. All levonorgestrel ((−) norgestrel, Sigma–Aldrich, UK. CAS: 797-63-7) and EE2 (Sigma–Aldrich, UK. CAS: 57-63-6) stock solutions were prepared weekly, in 2.5 L amber bottles, using double distilled water. Concentrated stock solutions (Masters) were made up in analytical grade ethanol and stored at 4 °C, and these were used each time the dosing stock solutions were made, to ensure reproducibility between dosing stocks. These dosing stock solutions were pumped at 12 ml/h (0.2 ml/min), using a Watson Marlow (Cornwall, UK) multi-channel peristaltic pump, into glass mixing vessels (each supplying 8 individual tanks). In these, it mixed with dilution water before delivery (at 125 ml/min) to each fish tank to produce the desired concentrations. Ethanol concentrations within each fish tank were no greater than 0.00003%. Flow rates and dosing efficiency were monitored daily, to ensure that the chemicals entered the fish tanks at the expected rates to produce concentrations close to those desired. Each day the spawning tiles, grids and dishes were removed from each tank and the number of eggs laid (including those stuck to the tile, attached to the grid and those that had fallen through into the glass collection tray) were counted and recorded.

At termination of the study, fish were sampled after overdose of anaesthesia using buffered MS-222 (Sigma, Poole, UK). The order in which the fish were sampled was defined using a random number generator (random.org) to ensure no sampling bias—particularly for quantification of the secondary sexual characteristics. Morphometric data were collected (length, weight, ovipositor length, abdominal girth, liver and gonad weight) along with individual blood samples. These samples were collected via the caudal peduncle using 75 μl heparinised capillary tubes and then decanted into Eppendorf tubes containing aprotinin (Sigma). These were then stored on ice until centrifugation at 7000 g for 5 min. The resulting plasma was withdrawn, snap frozen, and stored at −80 °C until analysis for sex steroid hormones (11-ketotestosterone (11KT) for males and 17β-estradiol (E2) for females), vitellogenin (VTG) and plasma levonorgestrel concentrations. Male secondary sexual characteristics present on any of the female fish (normally a specifically male characteristic) were also visually quantified. These included the presence/absence of tubercles, dorsal fin spot, and fatpad in both sexes.

Analysis of plasma 11KT and E2 was carried out using RIAs (radioimmunoassay), as previously described in Runnalls et al. (2013); where prior to assaying, individual plasma samples (10 μl) were placed in 1.5 ml Eppendorf tubes to which 100 μl distilled water and 1 ml ethyl acetate were added. The tubes were sealed, mixed thoroughly and then centrifuged to separate the two phases. The water phase was frozen by placing each tube for a brief interval on a block of dry ice and the ethyl acetate then poured into a borosilicate glass tube. The ethyl acetate was blown down under a stream of nitrogen in a heating block at 45 °C. The residue (containing the steroids) was redissolved in 1 ml buffer and 100 μl aliquots were used for each of the RIAs.

Plasma vitellogenin (VTG) concentrations were measured using an ELISA (enzyme-linked immunosorbent assay) kit designed specifically for the fathead minnow (Biosense Laboratories AS, Bergen, Norway). The detection limit was 2.5–5 ng/ml. Plasma samples were diluted 1:50, 1:5000 and 1:500,000 and assayed in duplicate according to the manufacturer's protocol. Inter-assay variation was between 5.1 and 21.0%.

2.3. Quantification of EE2 and levonorgestrel in water and plasma

2.3.1. EE2

Tank water samples (1L) were collected weekly for chemical analysis in (Winchester) amber glass bottles: 4 representative samples from each concentration were taken, at days 0 (day before dosing started), 7, 14 and 21. All samples were extracted after collection using solid-phase extraction (SPE) for sample cleanup and preconcentration. Extracts were eluted into methanol, which were dried down under a stream of nitrogen. The extracts were then resuspended in ethanol, and the EE2 concentration was determined using an established radioimmunoassay technique (Lange et al., 2001).

2.3.2. Levonorgestrel

As with EE2, levonorgestrel concentrations in tank water samples were quantified using solid phase extraction (SPE) followed by a commercially available radioimmunoassay (RIA) from Immunometrics UK Ltd, adapted for use with water samples. Tank water samples (1 L) were collected at termination of the experiment and were immediately frozen at −20 °C until extraction. They were primed and eluted using MTBE (MTBE: MEOH; 9:1), ethyl acetate, and MEOH. Levonorgestrel-spiked MilliQ water was also extracted in parallel to check extraction efficiencies/recoveries. Extracts were subsequently dried by Genevac (1 h at 32 °C and a further 2 h with no heat) and re-suspended in ETOH at different dilutions to bring them within the standard curve of the RIA. The RIA was carried out according to the manufacturer's instructions (Immunometrics UK Ltd). The levonorgestrel assay had a detection limit of 0.12 ng/l and cross-reactivity of 26% with 5α-dihydrolevonorgestrel, 12% with 3β, 5α-tetrahydrolevonorgestrel and 0.6% with 3α,5β-tetrahydrolevonorgestrel.

2.3.3. Mixture of EE2 and levonorgestrel

Tank water samples (1L) from fish tanks were collected weekly, and analysed for levonorgestrel using the Immunometrics RIA (as described above). Water samples were also collected at termination of the experiment and immediately frozen at −20 °C for EE2 measurement. EE2 concentrations were measured analytically by Anglian Water Services Ltd, (Cambridge, UK), where the steroid was extracted by solid phase extraction (SPE) and desorbed with ethyl acetate. The extracts were then cleaned up using normal phase chromatography followed by an additional clean up using gel permeation chromatography, then analysed by high performance liquid chromatography with mass spectrometer detection (triple quadrupole). The LOD was 0.03 ng/l.

2.3.4. Prediction of EE2 and levonorgestrel plasma concentrations

The human therapeutic plasma concentrations (H_TPCs) of EE2 and levonorgestrel were used to support the interpretation of the

observed reproductive effects in fish (Read-Across approach; Randweaver et al., 2013). This approach assumes that similar plasma concentrations of pharmaceuticals will have similar MoA-related effects at similar levels of biological organisation in both humans and fish. Specifically, in the case of levonorgestrel, with its potent contraceptive action, we hypothesised that drug plasma concentrations in fish equal to the H_TPCs would lead to a complete halt in egg production. For EE2, precise prediction of the effect magnitude was not possible as EE2 administration in humans is not intended to directly stop ovulation. Drug plasma concentrations of both EE2 and Levonorgestrel in fish were predicted as steady state plasma concentration (F_{SS}PC) using the Fish Plasma Model (Huggett et al., 2003). Model parameters were the measured water concentrations on day 21, and the Log $D_{7.4}$ values (predicted by ALOGPs) of EE2 (3.87) and levonorgestrel (3.32). The range of H_TPCs were derived from C_{max} values 0.04–0.16 ng/mL for EE2 (Reif et al., 2013; Cawello et al., 2013; Van den Heuvel et al., 2005), and 2.0–9.0 ng/mL for levonorgestrel (Cawello et al., 2013; Carol et al., 1992; Kuhnz et al., 1992).

2.3.5. *Quantification of levonorgestrel in fish plasma*

Levonorgestrel was isolated from plasma by liquid/liquid separation through the addition of ethyl acetate, as previously described for the extraction of 11KT, E2 and T. Levonorgestrel concentrations were then quantified by radioimmunoassay using a commercial kit (Immunometrics UK Ltd) and following the manufacturer's instructions. These measured concentrations were used to assess the reliability of the predictions generated by the Fish Plasma Model.

2.4. *Data treatment for reproductive performance*

Individual pairs of fish differ in both how often they spawn (spawning frequency) and the number of eggs they produce each time they spawn (fecundity), and consequently their time-course concentration-response data can be analysed in various ways. For the mixture assessment we considered an endpoint as appropriate if the following criteria were fulfilled: (i) clear and stable concentration-response pattern, (ii) sensitivity, (iii) unambiguous interpretation, and (iv) robustness, i.e. small changes within the time course data will not lead automatically to gross changes in the overall endpoint.

Fig. 1A is a bar chart showing cumulative egg production of control fish (±SEM) during the pre and post exposure periods, as it would have traditionally been presented (e.g. Paulos et al., 2010; Runnalls et al., 2013). The addition of the red dots (individual pair data) highlights the amount of data that is hidden by these types of plots. It has been very recently recommended that data in figures are presented in a way that provides maximum information to the reader (Weissgerber et al., 2015). We will present our data in this way, including data from individual pairs, along with the 95% confidence limits around the concentration-response relationship and enables EC20 and NOECs etc. to be more easily determined.

Fig. 1B shows the cumulative number of eggs over the entire experiment for each individual pair of fish from the control group. For a better visualisation the spawning events (dots) are connected by a smoothing line, revealing how consistent most pairs are in their spawning frequency and fecundity over time. An absolutely consistent pair would lay the same number of eggs in the second 21-day period as in the first 21-day period; thus its Npre:Npost ratio, expressed as a percentage, would be 100%. However, even with control fish reproductive performance can vary appreciably, some fish laying significantly few eggs in the second 21-day period, making reproductive performance <100%, whereas others lay significantly more, making their reproductive performance >100%. This suggests a nonlinear regression model would be best to describe these data, and here a second order polynomial expres-

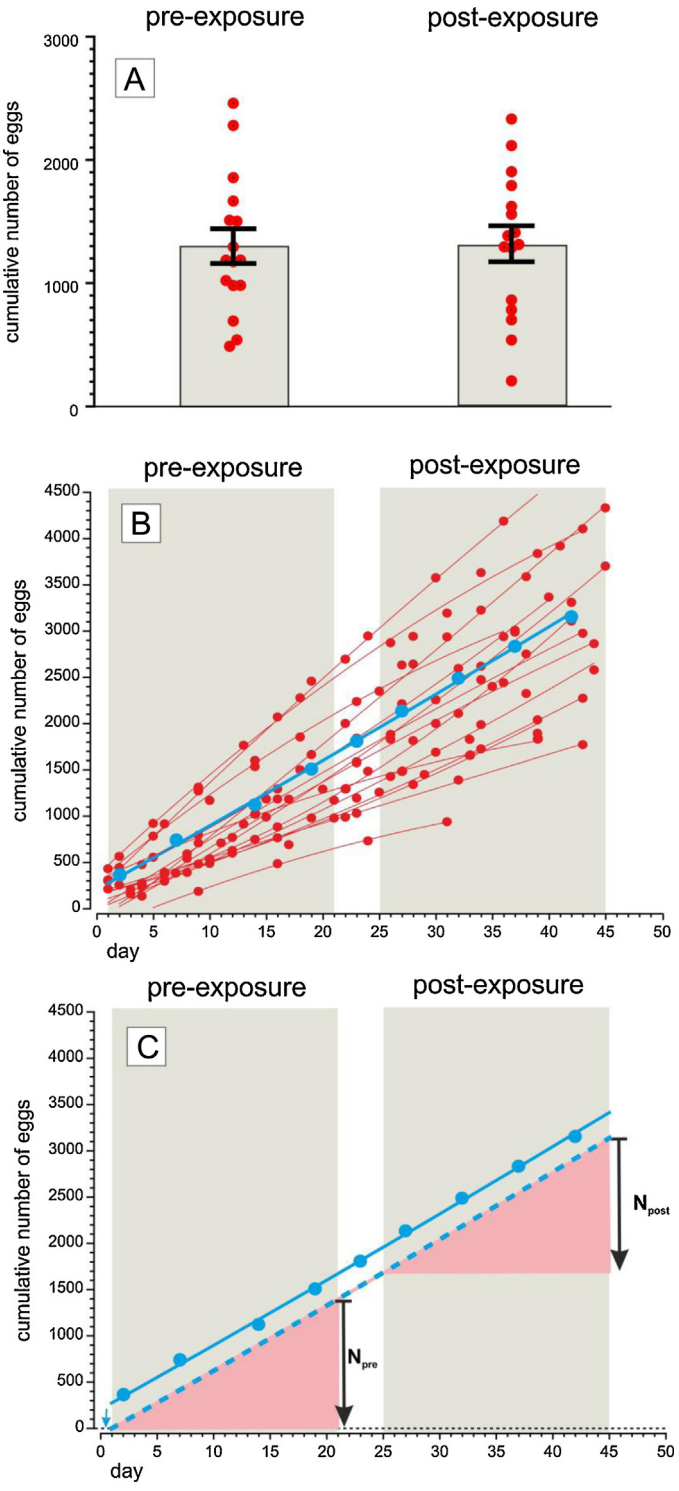

Fig. 1. Fecundity of fathead minnows in the pair breeding assay. Fig. A is a bar chart showing mean cumulative egg production of pairs of control fish in the pre- and post-exposure periods (±SEM) presented in the traditional way, with the addition of the red dots, highlighting the individual pair data. Fig. B shows cumulative egg production from the same individual control pairs measured over the duration of 45 days (21 days pre-exposure, 3 days transitional phase, 21 days post-exposure). Fig. C demonstrates how the egg production data from one individual control pair (blue dots) were analysed: the cumulative number of eggs is estimated for both the 21 days pre- and post- exposure periods by fitting a nonlinear regression curve to the time-course data (blue solid line), with the curve shifted to the zero origin (blue dotted line). Corresponding cumulative number of eggs are estimated for each period as the difference between the egg numbers estimated at the beginning (N_{pre}) and end (N_{post}) of the 21-day periods. The ratio of both estimates (N_{post}/N_{pre}, expressed as percentage) was used for data analysis, describing the relative change of the total number of eggs produced in the post-exposure period to the total number of eggs produced in the pre-exposure period. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

sion (parabolic curve) was deemed as sufficient (but in principle any other suitable regression function can be used). This function covers also linear relationships. Typically, the data variability is high and mainly biologically motivated (Fig. 1B), and thus cannot be influenced. However, we identified two experimental design factors which may be responsible for increased variation: (i) the data are not synchronised—in the pre-exposure period not all individual egg production lines start at day zero, and (ii) if the reproductive performance is poor in the first 21-day period, then it is likely that the pair will not maintain the same performance in the second 21-day period, and in the worst-case scenario, may even stop spawning. The latter not only enhances data variability, but also produces biased estimations. These asynchronicities were addressed by shifting the regression curve to the zero origin, i.e. all spawning starts on day 0 with zero eggs (Fig. 1C). Exclusion criteria for a potentially unsuccessful reproductive pairs were derived from simulation studies performed on historical control data, and on that basis the data were only accepted from an individual pair where the estimated cumulative number of eggs in the first 21-day period was above 500 and from at least 3 spawnings.

The last observed spawning within the second 21-day period can be interpreted in two different ways: either spawning has stopped completely (e.g. in response to the exposure), which would suggest estimating the reproductive performance until this day, or that the next spawning was not recorded because of it happening after the end of the study, which would suggest using the estimate from day 21. Again, simulation studies were conducted to determine the best compromise between both scenarios, and here we concluded that if the last observed spawning was recorded before day 16 of the exposure period, then a further spawning was unlikely to happen and the cumulative egg numbers were only estimated until this day, otherwise we used the regression fit to estimate the cumulative egg number from the last day of the study.

Thus 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 estimated in the first 21-day period ($\hat{N}_{pre}$):

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

with $\hat{N}_{post}$ either estimated until the day of the last recorded spawning (if observed before day 40) or until day 45 (day 21 of the exposure period) (Fig. 1C). All statistical analysis for this endpoint is based on this expression, and data are expressed as percentage only in the figures.

2.5. Statistical dose-response analysis

2.5.1. Testing for statistical differences

Data from specific 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 (e.g. estradiol), data were analysed by Tobit regression, otherwise by ANOVA. All egg count data were examined by GLM (Poisson or logit link), and categorical data (tubercle prominence, fatpad grade) by the nonparametric Exact Wilcoxon test. For all endpoints, 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).

2.5.2. Regression modelling

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, which ignores the fact that data are censored as values below zero are not possible. However, the error in overestimating these severe effects was considered as non-relevant for the purpose of these studies. This approach was implemented using the NLMIXED function of the SAS statistical software package (SAS Institute, Cary, USA).

2.6. 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 as:

$$EC_X(\text{mixture}) = \left(\frac{p_{EE2}}{EC_X(EE2)} + \frac{p_{levonorgestrel}}{EC_X(levonorgestrel)} \right)^{-1}, \quad (2)$$

where EC_X (mixture) is the mixture concentration that produces the effect X for a combination of C_{EE2} and $C_{levonorgestrel}$ (i.e. the concentration of EE2 and levonorgestrel in the mixture, respectively), p_{EE2} and $p_{levonorgestrel}$ are the ratios of EE2 and levonorgestrel in the mixture (i.e. the sum of p_{EE2} and $p_{levonorgestrel}$ equals 1), and EC_X (EE2) and EC_X (levonorgestrel) are the effect concentration of EE2 and levonorgestrel producing the same effect level X. Both effect concentrations are derived from the inverse of the nonlinear regression function which describes best the observed concentration effect data of the components (Table 2). To account for the statistical uncertainty in the CA prediction, we used the bootstrap method (Efron and Tibshirani, 1993) to produce approximate 95% confidence limits around the mean predicted effect.

3. Results

All single substance and mixture studies ran to completion. No atypical behaviour was observed during any of the studies. Mortality during the studies did not exceed 3.8% and there was no evidence of disease or parasite infections.

3.1. Single substance effects

Measured water concentrations are displayed in Table 1, and the statistical concentration-response descriptors for egg production can be seen in Table 2.

EE2 concentrations from the first study (measured by RIA; Table 1) indicated that actual tank concentrations were between 52 and 74% of nominal values. Week 0 samples and all control samples were below the limit of detection for the assay. Levonorgestrel concentrations from the second study (also measured by RIA; Table 1) indicated that the actual tank concentrations were between 63.2 and 105% of nominal values.

Fig. 2 shows the results from the single chemical exposure studies. Controls pairs in both studies had slightly better reproductive performance in the second half of the study (Fig. 2, Table 2), however, these were not statistically significant. Levonorgestrel reduced egg production in a concentration-dependent manner (Fig. 2A). The highest nominal concentration, 100 ng/l, stopped the fish reproducing after only a few days of exposure. A concentration of 5 ng/l reduced egg production by nearly 40%, with some of the pairs of fish ceasing spawning completely by the end of the exposure period. At the lowest concentration, 0.5 ng/l, egg production was lowered, although not significantly, by about 20%. The estimated EC20 was 0.9 ng/l. A similar concentration-dependent response was observed for EE2 (Fig. 2B), with a potency (EC20 = 0.4 ng/l) comparable to that of levonorgestrel (EC20 = 0.9 ng/l). A concentration of 5 ng/l reduced egg production by about 50%, and 0.5 ng/l reduced it by 31% ($p < 0.03$). In both studies the coefficient of variation for the control responses

Table 1
Measured water concentrations of EE2 (from 4 randomly chosen tanks) sampled weekly and levonorgestrel (all 8 tanks per treatment group were sampled) at week 4. All data are from the single chemical exposure studies. DL—detection limit.

Nominal	EE2 (ng/l)							Levonorgestrel (ng/l)			
	Week 0	Week 1	Week 2	Week 3	Week 4	Mean	% of nominals	Week 4		Mean	% of nominals
Control	<DL	<DL	<DL	<DL	<DL	<DL		<DL	<DL	<DL	
		<DL	<DL	<DL	<DL			<DL	<DL		
		<DL	<DL	<DL	<DL			<DL	<DL		
		<DL	<DL	<DL	<DL			<DL	<DL		
0.5 ng/l	<DL	0.34	0.395	0.33	0.32	0.37	74.7%	0.25	0.49	0.42	84.0%
		0.42	0.49	0.43	0.23			0.38	0.54		
		0.21	0.39	0.4	0.42			0.43	0.53		
		0.37	0.53	0.3	0.4			0.30	0.42		
5 ng/l	<DL	2.1	4.59	2.03	3.87	3.18	63.7%	5.31	5.46	5.25	105.0%
		2.69	3.31	1.92	2.95			4.30	7.83		
		1.61	3.8	2.8	2.65			4.48	4.54		
		3.2	4.17	4.87	4.38			5.22	4.90		
25 ng/l	<DL	9.3	15.8	11.87	8.72	13.20	52.8%	9.01	14.78	15.80	63.2%
		8.21	19.3	8.97	13.84			14.89	15.52		
		11.59	13.47	14.51	12.97			7.99	18.53		
		10.34	24.8	12.76	14.75			14.58	31.11		

Table 2
Statistical descriptors for EE2 and levonorgestrel on relative egg reproduction of individual compounds.

Substance	Concentration response function					EC20 [ng/l]	NOEC [ng/l]	Detection limit[a]
	RM	$\hat{\theta}_1$	$\hat{\theta}_1$	$\hat{\theta}_{min}$	$\hat{\theta}_{max}$			
					Nominal			
Levonorgestrel	Logit	1.101	−1.25	0	1.05	0.90 [0.11;7.06]	0.5	74%
EE2	Weibull	0.044	−0.75	0	1.06	0.39 [0.06;2.60]	<0.50	78%
					Measured			
Levonorgestrel	Logit	1.136	−1.36	0	1.05	0.93 [0.12;7.48]	0.42	
EE2	Weibull	−0.097	−0.82	0	1.07	0.30 [0.06;1.64]	<0.37	

EC20: concentration reducing egg production by 20%. 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); $\hat{\theta}_1, \hat{\theta}_2, \hat{\theta}_3, \hat{\theta}_{min}$ estimated model parameters, given for concentrations expressed in ng/l (rounded values), θ_{min} were not estimated, but set to 0. θ_{max} equals the estimated control level.

[a] Detection limit (sensitivity) based on alpha = 5% (one-sided), beta = 20% (i.e. power = 80%), *t*-test (no correction for multiplicity) and control sample variability.

was around 30%, leading to a statistical detection limit of approximately 75% using the 106% control level, 16 controls and 8 pairs of fish per exposure group (false-negative rate > 20%). For modelling purposes, the moderate uncertainty of the EC20 estimations suggests that a 20% reduction is probably the best low effect descriptor for this specific endpoint and experimental design. As both compounds produced clear concentration-responses for egg reproduction, effect ranges between 75 and 20% were deemed appropriate for the mixture prediction and comparative mixture assessment.

Assessment of the effects of levonorgestrel on other endpoints revealed a very sex-specific pattern; whereas there were no significant effects on any parameters in males, there were very wide-ranging effects on females (Table 4). The highest concentration (25 ng/l) led to an increase in the weight, length and condition factor of the fish, as well as an increase in abdominal girth. Secondary sexual characteristic in females were also very sensitive to levonorgestrel exposure: all three concentrations induced dorsal fin spots and fatpads, as well as facial tubercles appearing in 2 females at the highest concentration—all are normally male-only characteristics. Assessment of the effects of EE2 on the other hand, highlighted minimal effects on parameters measured in females; only the plasma vitellogenin concentration was increased significantly ($p < 0.05$) at the highest concentration, and the plasma estradiol was significantly lower than that of the control at 0.5–25 ng/l (Table 5). EE2 had much more obvious effects on males (Table 5). For example, it significantly increased ovipositor length, reduced the prominence of secondary sexual characteristics such as tubercle number and prominence, and reduced plasma 11-ketotestosterone concentrations. The gonadosomatic index was also significantly decreased at the highest concentration. A summary table (Table 7) highlights statistically significant effects on different endpoints after the single chemical and the mixture exposures.

3.1.1. Drug plasma concentrations and reproductive effects

EE2 and levonorgestrel concentrations in fish plasma were predicted from measured concentrations in the tank water (Table 1), and were then compared with human therapeutic plasma concentrations. EE2 fish steady state plasma concentrations (F_{SS}PCs) were predicted to be within or above the H_TPCs range (0.04–0.16 ng/ml, Fig. 3A) both in the single and mixture exposure studies, with the exception of the lowest exposure group of the mixture study, which was predicted to have F_{SS}PC (0.013 mg/ml) below the H_TPCs. In both studies, EE2 led to significant inhibition of egg production only at plasma concentrations 3- to 11-fold above the higher value of the H_TPCs range (Fig. 3A).

In contrast, concentrations of levonorgestrel in fish plasma were predicted to be lower than H_TPCs in all treatment groups, in both the single and mixture exposure studies, with the exception of fish exposed to the high concentration in the single exposure study (100 ng/l) where levonorgestrel F_{SS}PC were predicted to be within the H_TPCs range. As levonorgestrel is the main disruptor of ovulatory processes in humans, it was hypothesised that drug F_{SS}PCs equal to the H_TPCs would inhibit egg production in fish completely. This agrees well with the experimental findings of

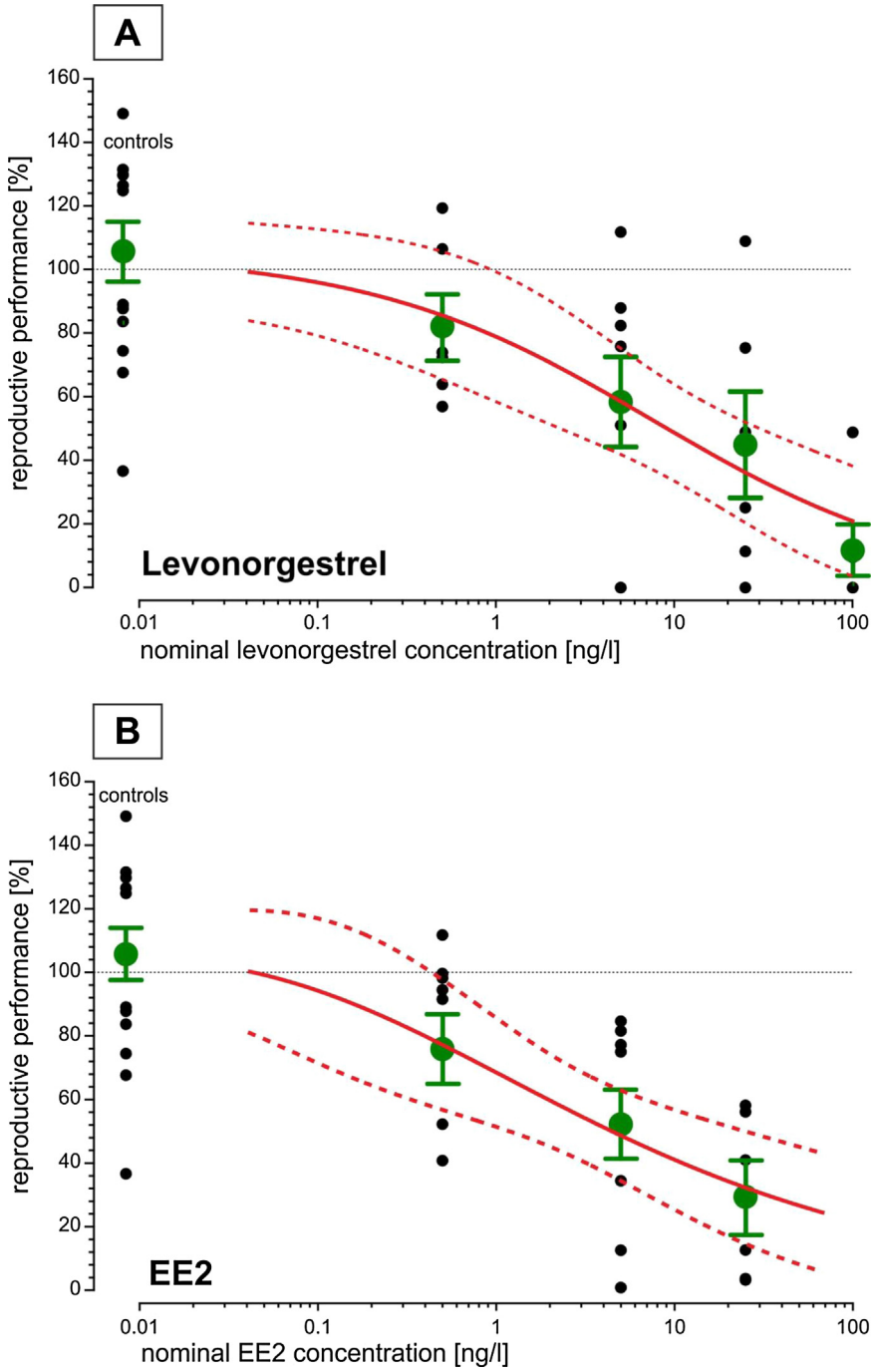

Fig. 2. Fecundity of fathead minnows in the pair breeding test. Concentration-response data and best-fit regression curves are shown for levonorgestrel (A) and EE2 (B). Each point represents the cumulative number of eggs produced by one pair of fish after 3 weeks of exposure compared to the cumulative egg number observed during a 3 week pre-exposure period. Error bars indicate the SEM around the mean response. The solid line represents the best-fit regression curve, the dashed lines the 95% confidence interval. Additional data from 100 ng/l levonorgestrel comes from an earlier experiment, the results of which are reported in Runnalls et al. (2013).

an approximately 85% reduction in egg production (Fig. 2A). At median effect concentrations (0.5 and 5 ng/l) the predicted $F_{SS}PC$ were 3.3-fold and 10-fold below the lowest H_TPC value, respectively (Fig. 3B).

To assess the reliability of the fish plasma prediction model for levonorgestrel we measured plasma concentrations in male fish exposed to 5.2 ng/l ($n=7$) and 15.8 ng/l ($n=6$) of levonorgestrel (measured concentrations) in the single exposure study (Fig. 3C). Measured plasma concentrations were 0.36 ± 0.1 ng/ml in fish exposed to 5.2ng/l and 1.51 ± 0.5 ng/ml in fish exposed to 15.8 ng/l. Control fish ($n=3$) were analysed to establish any potential unspecific-binding of the antibody with endogenous steroids and levonorgestrel concentrations were all <LOD, confirming the high specificity of the assay. Observed inter-individual variability was approximately 2-fold. The average measured plasma concentrations were predicted by the FPM with high accuracy based on the Log Kow (3.72). Whereas, when Log D7.4 was used (3.32), predicted values were underestimated by 56% at 5.2 ng/l and by 40% at 15.8 ng/l. The different degrees of accuracy reflect the uncertainty in the computational prediction of partitioning factors.

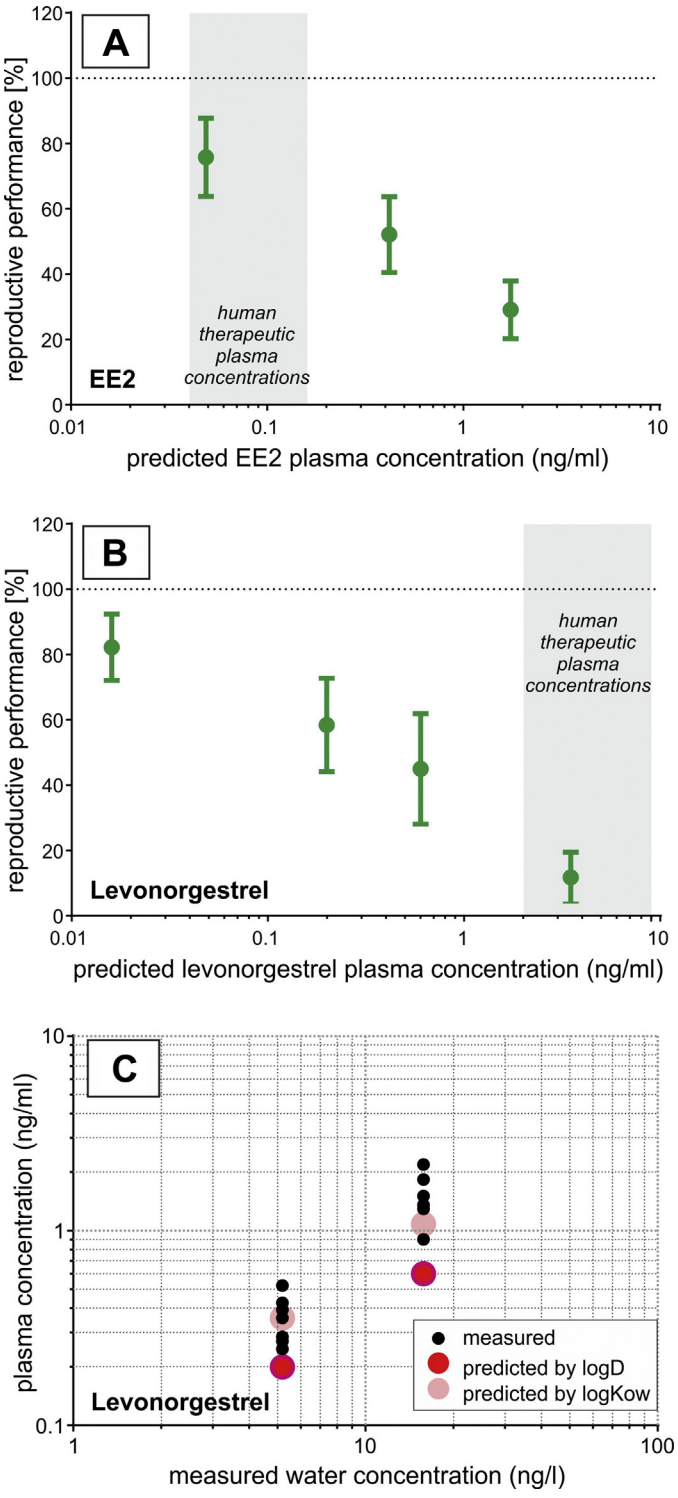

Fig. 3. The relationship between the predicted and measured plasma concentrations of EE2 and levonorgestrel and their effects on egg production (mean ± SEM, $n = 7$ and 6, respectively). A and B investigate the relationship between egg production and the predicted fish plasma concentrations of EE2 and levonorgestrel, respectively. The range of Human Therapeutic Plasma Concentration (H_TPC) is shown as a grey shaded area. Note that predicted fish plasma concentrations of levonorgestrel below the human plasma therapeutic range reduced egg production appreciably (B), whereas the predicted fish plasma concentrations of EE2 were in, or above, the human plasma therapeutic range when reduced egg production occurred (A). Due to the potent contraceptive action of levonorgestrel, it was hypothesised that drug plasma concentrations in fish equal to the H_TPC range would cause complete inhibition of egg production. A preliminary determination of the actual (i.e. measured) fish plasma concentrations of levonorgestrel (C) demonstrated that these were slightly, but not markedly, above, (using Log D7.4) and very close (using Log Kow) to the predicted concentrations in male fish maintained at the two highest concentrations of levonorgestrel. The red and pink dots are the predicted concentrations using Log D and Log Kow respectively, and the black dots are measured plasma levonorgestrel concentrations in individual fish. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 3
Measured weekly water concentrations of levonorgestrel from 4 randomly chosen tanks per dose from the mixture study. EE2 concentrations from 4 randomly chosen tanks, determined at termination of the study by Anglian Water. All data are from the binary mixture study.

Nominal	Levonorgestrel (ng/l) in the mixture							EE2 (ng/l) in the mixture		
	Week 0	Week 1	Week 2	Week 3	Week 4	Average	% of nominals	Week 4	Average	% of nominals
Control	<DL	<DL	<DL	<DL	<DL	<DL		<DL		
		<DL	<DL	<DL	<DL			<DL		
		<DL	<DL	<DL	<DL			<DL		
		<DL	<DL	<DL	<DL			<DL		
0.25 ng/l	<DL	0.28	0.35	0.28	0.35	0.34	136.0%	0.13	0.15	60.0%
		0.28	0.38	0.3	0.36			0.16		
		0.35	0.36	0.45	0.4			0.22		
		0.34	0.37	0.4	0.34			0.12		
2.5 ng/l	<DL	1.94	1.95	2.82	2.81	2.51	100.0%	1.67	1.49	59.6%
		1.92	2.19	2.93	2.13			1.31		
		2.11	2.08	2.39	4.4			1.57		
		2.97	2.52	2.35	2.64			1.43		
12.5 ng/l	<DL	8.69	10.62	8.73	11.6	9.21	73.0%	9.96	8.64	69.1%
		10.1	8.94	10.66	9.06			9.39		
		5.82	7.88	10.05	11.83			8.35		
		8.38	6.75	7.71	10.55			6.87		

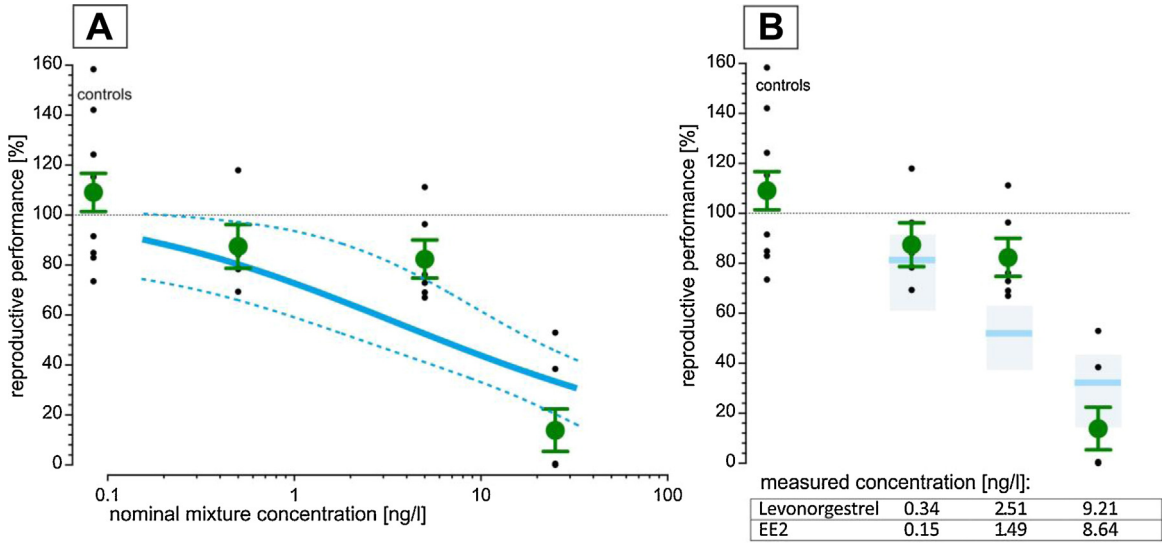

Fig. 4. Comparison between the observed and CA-predicted mixture effects of EE2 and Levonorgestrel on cumulative egg reproduction of the fathead minnow, based on nominal (A) and measured concentrations (B). Each point represents the cumulative number of eggs produced by one fish pair after 3 week exposure compared to the cumulative egg number observed during a 3 week pre-exposure period. Error bars indicate the SEM around the mean response, the solid blue line represents the CA prediction, and the dashed lines (A) and blue areas (B) the corresponding 95% confidence bootstrap interval. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

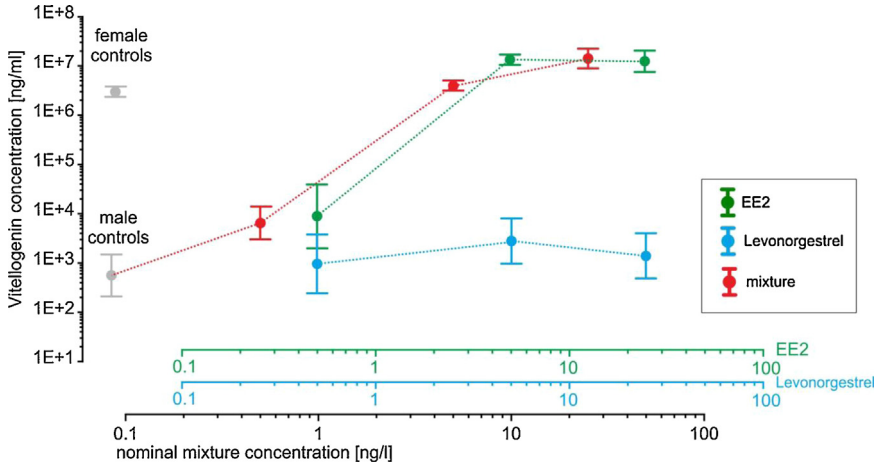

Fig. 5. VTG induction in fathead minnow by EE2, levonorgestrel and their combination. Error bars indicate 95% confidence belts around the mean, individual compounds were rescaled to the mixture concentration. Control data are shown from the mixture study.

Table 4
Effects of levonorgestrel on fathead minnow after 21 days exposure.

	Treatment (ng/l)															
	Survival	Length (mm)	Weight (g)	Condition factor	Abdominal girth (mm)	Liver somatic index [%]	Gonad-somatic index [%]	Ovipositor length (mm)	Tubercle number	Tubercles prominence	Fin spot	Fatpad presence	Fatpad index [%]	Fatpad height (mm)	Vitellogenin (ng/ml)	Estradiol (ng/ml)
Females																
Control	15/16	51.5 ± 0.93	1.94 ± 0.105	1.41 ± 0.02	7.5 ± 0.26	3.93 ± 0.26	17.3 ± 1.16	1.96 ± 0.053	None	–	0/15	0/15	–	–	2.0E6 (1.7E6–2.4E6)	4.2 (2.7–6.5)
0.5	7/8	51.1 ± 0.94	1.79 ± 0.08	1.34 ± 0.05	7.0 ± 0.31	3.99 ± 0.30	15.3 ± 1.06	1.90 ± 0.101	None	–	6/7*	1/7*	–	–	1.4E6 (1.0E6–1.9E6)	5.1 (3.0–9.0)
5	8/8	49.5 ± 1.35	1.91 ± 0.19	1.54 ± 0.06	7.8 ± 0.42	3.00 ± 0.35	22.0 ± 1.94	1.97 ± 0.100	None	–	5/8*	2/8*	–	–	2.0E6 (1.3E6–3.0E6)	2.5 (1.3–5.1)
25	8/8	56.4* ± 1.28	2.82* ± 0.13	1.58* ± 0.08	8.9* ± 0.61	2.68* ± 0.32	19.6 ± 3.04	1.93 ± 0.119	7[a],*	1[a],*	8/8*	4/8*	–	–	3.6E5* (1.1E5–1.2E6)	1.8* (0.8–3.9)
Males																
												Fatpad index[%]	Fatpad grade			11 ketotestosterone (ng/ml)
Control	16/16	66.3 ± 0.97	5.25 ± 0.225	1.80 ± 0.06	6.3 ± 0.17	1.95 ± 0.09	1.8 ± 0.13	0.26 ± 0.26	18.8(16.5–21.4)	3.88 ± 0.22	16/16	0.008 ± 0.0008	2.44 ± 0.16	4.3 ± 0.34	2.5E2 (1.6E2–3.9E2)	17.1 (9.2–31.9)
0.5	8/8	66.5 ± 2.50	5.20 ± 0.38	1.77 ± 0.08	6.5 ± 0.24	1.92 ± 0.16	1.8 ± 0.24	0.11 ± 0.38	15.1(12.5–18.3)	3.63 ± 0.42	8/8	0.007 ± 0.0013	2.25 ± 0.16	3.9 ± 0.66	1.2E2 (3.0E1–4.8E2)	13.0 (5.4–31.4)
5	8/8	64.9 ± 2.11	4.93 ± 0.45	1.80 ± 0.11	6.4 ± 0.42	1.97 ± 0.16	2.1 ± 0.16	0.54 ± 0.32	17.5(14.6–20.9)	3.88 ± 0.35	7/8	0.008 ± 0.0019	2.50 ± 0.27	4.7 ± 0.92	3.5E2 (1.2E2–1.0E3)	27.9 (11.5–67.4)
25	8/8	66.6 ± 2.23	5.32 ± 0.44	1.80 ± 0.12	6.6 ± 0.33	2.11 ± 0.23	1.8 ± 0.06	0.08 ± 0.39	18.0(15.1–21.5)	3.63 ± 0.38	8/8	0.008 ± 0.0017	2.5 ± 0.33	4.5 ± 0.83	1.7E2 (6.0E1–5.0E2)	12.1 (5.0–29.4)

Data represent means ± SEM, or means with 95% confidence belts (in brackets).
* Statistical significant different compared to controls ($p < 0.05$).
[a] Two females responded.

Table 5
Effects of 17α-ethinylestradiol on fathead minnow after 21 days exposure.

	Treatment (ng/l)															
	Survival	Length (mm)	Weight (g)	Condition factor	Abdominal girth (mm)	Liver somatic index [%]	Gonad-somatic index [%]	Ovipositor length (mm)	Tubercle number	Tubercles prominence	Fin spot	Fatpad index [%]	Fatpad grade	Fatpad height (mm)	Vitellogenin (ng/ml)	Estradiol (ng/ml)
Females																
Control	15/16	51.5 ± 0.93	1.94 ± 0.105	1.41 ± 0.02	7.5 ± 0.26	3.93 ± 0.26	17.3 ± 1.16	1.96 ± 0.053	None	–	0/15	–	–	–	2.0E6 (1.7E6–2.4E6)	4.2 (2.7–6.5)
0.5	8/8	52.0 ± 0.93	1.99 ± 0.099	1.42 ± 0.08	7.7 ± 0.42	3.85 ± 0.25	16.8 ± 1.50	2.12 ± 0.081	None	–	1/8	–	–	–	1.9E6 (1.3E6–2.9E6)	1.5* (0.7–3.2)
5	8/8	51.0 ± 1.12	2.07 ± 0.153	1.54 ± 0.06	8.3 ± 0.48	3.48 ± 0.18	18.4 ± 2.51	2.12 ± 0.084	None	–	2/8	–	–	–	3.0E6 (2.1E6–4.4E6)	2.3 (1.1–4.8)
25	8/8	50.4 ± 0.82	1.94 ± 0.163	1.51 ± 0.08	7.8 ± 0.52	4.04 ± 0.27	14.0 ± 2.42	2.15 ± 0.069	None	–	2/8	–	–	–	9.9E6* (5.3E6–1.8E7)	0.82* (0.5–1.3)
Males																
																11 ketotestos-terone (ng/ml)
Control	16/16	66.3 ± 0.97	5.25 ± 0.225	1.80 ± 0.06	6.3 ± 0.17	1.95 ± 0.09	1.8 ± 0.13	0.42 ± 0.17	18.8(16.5–21.4)	3.88 ± 0.22	16/16	0.008 ± 0.0008	2.44 ± 0.16	4.3 ± 0.34	2.5E2 (1.6E2–3.9E2)	17.1 (10.7–27.3)
0.5	8/8	63.6 ± 1.25	4.96 ± 0.321	1.92 ± 0.11	6.3 ± 0.27	1.95 ± 0.12	2.2 ± 0.33	0.47 ± 0.23	18.0(14.9–21.7)	4.25 ± 0.25	6/8	0.011 ± 0.0015	2.89 ± 0.30	5.0 ± 0.52	8.9E3* (2.0E3–4.0E4)	13.9 (7.2–26.9)
5	6/8	63.0 ± 2.68	4.66 ± 0.523	1.83 ± 0.05	6.4 ± 0.29	2.30 ± 0.23	1.6 ± 0.31	1.39* ± 0.23	18.2(14.6–22.5)	3.63 ± 0.32	6/6	0.007 ± 0.0010	2.17 ± 0.17	3.7 ± 0.40	1.3E7* (1.1E7–1.7E7)	6.9 (3.2–14.7)
25	7/8	66.6 ± 1.96	5.67 ± 0.333	1.97 ± 0.21	9.2* ± 0.57	1.85 ± 0.10	0.8* ± 0.06	1.59* ± 0.21	8.0* (5.9–10.8)	1.29* ± 0.18	7/7	0.005 ± 0.0010	2.14 ± 0.34	3.8 ± 0.94	1.2E7* (7.6E6–2.0E7)	All below DL*

Data represent means ± SEM, or means with 95% confidence belts (in brackets).
* Statistical significant different compared to controls ($p < 0.05$).

The average measured bioconcentration factor water:plasma ($BCF_{water:plasma}$) was 70 at the lowest concentration of levonorgestrel and 96 at the higher concentration. These BCF values are in agreement with those that would be predicted for levonorgestrel by the FPM (35–68, using Log D7.4 and Log Kow, respectively), and are similar to the measured BCFs of other synthetic progestogens (Nallani et al., 2012), but are very much lower than the BCF of 12,000 reported by Fick and colleagues (Fick et al., 2010).

3.2. Binary mixture effects

Water concentrations of levonorgestrel were determined from 4 randomly chosen (but used consistently) tanks, with EE2 levels being measured only at termination of the mixture study. Levonorgestrel concentrations in the mixture were between 73 and 139% of nominal concentrations, and EE2 concentrations at the end of the experiment were between 59 and 69% of nominal (Table 3), and these were in good agreement with the findings from the single substance data. Concentrations in control samples were always below detection limits. The relative composition of the mixture determined at the end of the experiment appeared to indicate changes with respect to the mixture ratio: for levonorgestrel, from ca. 70% at the lowest concentration to 52% at the highest mixture concentration. Consequently, each measured mixture concentration may have had its own slightly different mixture ratio, and in a strict qualitative sense the concentration response data from the mixture cannot be plotted on a common concentration scale, where only a well-defined fixed-ratio design would define the mixture composition even at untested ranges. It is also possible that the apparent change in the mixture ratio is an artefact caused by the variability associated with measuring the water concentrations. Whether or not the ratio actually changed, we present the concentration effect data for the reproductive performance endpoint based on both nominal and measured concentrations, in the latter case only for the three tested mixture concentrations. Similarly, mixture predictions were generated either on nominal or measured concentrations from data obtained in the single substance studies, based on regression models shown in Table 2. It should be noted that for varying mixture ratios under certain circumstances it is possible to estimate interpolative predictions on a one-dimensional mixture concentration scale (Pottinger et al., 2013), but to fulfil the methodological requirements more data would have been required.

The mixture of EE2 and levonorgestrel reduced egg production in a concentration-dependent manner (Fig. 4), with only the highest concentration having produced a statistical significant response. Observed responses were compared with the predictions generated by concentration addition model using both nominal and measured water concentrations. The latter were considered to reflect the real exposure levels in the experiments more closely and thus were more suitable for the comparison. The predicted mixture responses were nearly identical in both cases. Whereas the responses of the lowest and highest mixture concentrations were in good agreement with the CA prediction, with deviations of ca. 10% and 18%, respectively, the response to the medium concentration of the mixture (2.5 ng/l of each chemical) were less than expected, with a deviation of ca. ~40%.

As with the single chemical exposures, other specific endpoints were also investigated at the end of the exposure period and the data are shown in Table 6. All of the positive responses to the mixture could be explained by the observed effects of one or other of the individual compounds, or occasionally both (Tables 6 and 7). Essentially, the responses to the mixture can be characterised as the sum of the responses of the individual chemicals. There was little evidence that one of the chemicals had antagonised the response

Table 6
Effects of a mixture of 17α-ethinylestradiol and levonorgestrel on fathead minnow after 21 days exposure.

Treatment (ng/l)	Survival	Length (mm)	Weight (g)	Condition factor	Abdominal girth (mm)	Liver somatic index [%]	Gonad-somatic index [%]	Ovipositor length (mm)	Tubercle number	Tubercles prominence	Fin spot	Fatpad presence	Fatpad grade	Fatpad height (mm)	Vitellogenin (ng/ml)	Estradiol (ng/ml)
Females																
Control	8/8	52.0 ± 1.00	2.05 ± 0.124	1.44 ± 0.02	6.78 ± 0.287	4.36 ± 0.34	15.7 ± 1.24	1.97 ± 0.06	None	-	0/8	0/8	-	-	3.0E6 (2.4E6–3.6E6)	5.5 (3.0–9.9)
0.5	7/8	55.3 ± 1.46	2.39 ± 0.179	1.40 ± 0.05	7.30 ± 0.389	4.00 ± 0.45	14.5 ± 1.39	2.06 ± 0.06	None	-	5/7*	0/7	-	-	2.7E6 (1.6E6–4.5E6)	4.3 (2.3–8.1)
5	8/8	51.6 ± 0.75	2.10 ± 0.101	1.52 ± 0.05	7.86 ± 0.239	4.25 ± 0.24	16.2 ± 1.01	2.15 ± 0.07	None	-	4/8*	0/8	-	-	3.4E6 (2.5E6–4.6E6)	1.6* (0.9–3.0)
25	7/8	56.4* ± 0.95	2.73* ±0.141	1.52 ± 0.07	8.03 ± 0.600	3.34 ± 0.23	14.3 ± 2.38	2.22 ± 0.09	None	-	6/7*	7/7*	1[a,*]	-	1.3E7* (5.3E6–3.0E7)	0.7* (0.4–1.4)
Males																
												Fatpad index[%]				11 ketotestosterone (ng/ml)
Control	8/8	68.5 ± 0.93	6.13 ± 0.320	1.91 ± 0.09	7.33 ± 0.338	1.92 ± 0.13	1.9 ± 0.13	0.86 ± 0.16	21.8 (18.5–25.6)	3.88 ± 0.358/8		0.010 ± 0.0016	2.75 ± 0.25	5.1 ± 0.56	5.6E2 (2.1E2–1.5E3)	17.8 (7.6–41.7)
0.5	8/8	64.4* ± 1.31	5.33 ± 0.340	1.99 ± 0.10	7.21 ± 0.413	2.35 ± 0.25	2.3 ± 0.26	0.57 ± 0.17	19.4 (16.3–23.0)	4.25 ± 0.378/8		0.009 ± 0.0017	2.50 ± 0.33	4.5 ± 0.64	6.4E3* (3.0E3–1.4E4)	18.0 (7.6–42.2)
5	8/8	63.9* ± 0.67	5.05* ± 0.216	1.94 ± 0.08	6.78 ± 0.277	2.13 ± 0.16	2.1 ± 0.07	1.25 ± 0.15	20.1 (17.0–23.8)	4.1 ± 0.35 8/8		0.012 ± 0.0011	3.25 ± 0.25	5.9 ± 0.37	4.0E6* (3.3E6–4.8E6)	26.6 (11.3–62.6)
25	7/8	64.0* ± 1.05	5.55 ± 0.347	2.12 ± 0.13	7.74 ± 0.630	2.28 ± 0.25	1.9 ± 0.16	1.68* ± 0.16	14.4* (11.6–17.9)	2.86 ± 0.607/7		0.009 ± 0.0021	2.29 ± 0.36	4.8 ± 1.00	1.4E7* (9.0E6–2.2E7)	1.9* (0.7–5.0)

Data represent means ± SEM, or means with 95% confidence belts (in brackets).
[a] One female responded.
* Statistical significant different compared to controls ($p < 0.05$).

Table 7
Summary table of statistically significant effects on different parameters taken at the end of the exposure periods to EE2, levonorgestrel and a mixture of both.

Endpoints	EE2	Levonorgestrel	Mixture
Length		↑ ♀ at 25	↑ ♀ at 25 ↓ ♂ at 0.5/5/25
Weight		↑ ♀ at 25	↑ ♀ at 25 ↓ ♂ at 5
Condition factor		↑ ♀ at 25	
Abdominal girth	↑ ♂ at 25	↑ ♀ at 25	
HSI		↓ ♀ at 25	
GSI	↓ ♂ at 25		
Ovipositor length	↑ ♂ at 5/25		↑ ♂ at 25
Tubercle number	↓ ♂ at 25	↑ ♀ at 25 (2 fish)	↓ ♂ at 25
Tubercle prominence	↓ ♂ at 25	↑ ♀ at 25 (2 fish)	
Fin spots		↑ ♀ at 0.5/5/25	↑♀ at 0.5/5/25
Fatpad		↑ ♀ at 0.5/5/25	↑ ♀ at 25
11KT	↓ ♂ at 25		↓ ♂ at 25
VTG	↑ ♂at 0.5/5/25 ↑ ♀ at 25	↓ ♀ at 25	↑ ♀ at 25 ↑ ♂ at 0.5/5/25
E2	↓ ♀ at 0.5/25	↓ ♀ at 25	↓ ♀ at 5/25
Summary	**Males markedly affected. Minimal effects on females at any concentration of EE2**	**Females markedly affected. No significant changes in males at any concentration of levonorgestrel**	**Significant effects of the mixture on both sexes**

of the other chemical, except in the case of the fatpads and fin spots in females. Only the highest mixture concentration induced fatpads and fin spots, whereas levonorgestrel on its own induced them at all three concentrations.

In the case of vitellogenin, as expected, control females had very high plasma concentrations, whereas males had very much lower levels, by about 10,000-fold (Tables 4, 5 and 6). Therefore, any stimulatory effects on vitellogenin are better studied in males (Fig. 5). EE2 stimulated an increase in the plasma vitellogenin concentrations in males in a concentration-dependent manner; even the lowest concentration tested, 0.5 ng/l, significantly increased the concentration (Fig. 5). The highest concentration, 25 ng/l, raised the plasma vitellogenin to above that of the control females. In complete contrast, levonorgestrel had no effect on the plasma concentration of vitellogenin in males, and might if anything have decreased it somewhat in females (Table 4). The mixture of EE2 and levonorgestrel increased the plasma vitellogenin concentration in a manner identical to that of EE2 alone (Fig. 5). Both 25 ng/l EE2 and the mixture of 12.5 ng EE2 plus 12.5 ng levonorgestrel /l increased the plasma vitellogenin concentration maximally.

4. Discussion

The results of these studies confirm the utility of the pair-breeding assay as an experimental platform for evaluating the combined responses of steroidal pharmaceuticals on fish reproduction. In addition they demonstrate that even with small-to-moderate sample size, sound concentration-response conclusions can be drawn, and by significantly improving the analysis and presentation of egg production data, more robust statistical data descriptors (NOECs, EC_X) can be obtained.

4.1. Data analysis

One of the major challenges for mixture ecotoxicology is the difficulty to identify relevant endpoints that can be consistently interpreted across studies. The 45-day pair-breeding assay produces a complex set of dose-response data over time, and until now it has been unclear how to analyse the data in the most effective way, especially when it comes to the specific demands of mixture assessments. It therefore became crucial to develop a new quantitative method to condense and analyse the data, such that an optimal mixture assessment was guaranteed. The endpoints from this novel approach were used to design the mixture study and to assess the additivity expectations.

Time-course dose-response data from the pair-breeding assay can be analysed in various ways, but our method comparing reproductive performance before and after addition of the chemical (i.e. comparing pre- and post-exposure egg production) results in a robust concentration-response analysis, especially for small sample size designs which are typical for this labour- and cost intensive assay. For example, if we had followed the traditional method of using only total egg number from the post-exposure period, the statistical detection limit would have dropped from a 25 to 30% reduction in egg number to ca. 50%, but also the regression analysis would have produced less reliable outcomes, and consequently more uncertain additivity predictions. The main reason is that information from the pre-exposure period are used more efficiently by assessing the performance of an individual pair over a larger time period, and by excluding those individual pairs which have poor egg production during the pre-exposure period. If possible, we suggest starting with a larger number of fish pairs, which then can be minimised to the favoured sample size for the post-exposure testing. We consider our endpoint as robust in the sense that individual fecundity events and characteristics, such as spawning frequency, eggs per spawn, etc. have only a minimal impact on the outcomes of the statistical dose-response data analysis (e.g. hypothesis testing, regression analysis), i.e. the chance of measurement errors influencing the data interpretation is minimal. Although data for individual pairs of fathead minnow can vary in both spawning frequency and spawning interval, it should be emphasised that each pair is, in general, fairly consistent (Fig. 1A), as has also been reported by others (Thorpe et al., 2009). An additional important factor for the comparative mixture assessment is reproducibility, and ideally all single and mixture studies should have been repeated at least twice. Due to cost and resource limitations this was not done, but from our past experience where we have repeated selected exposure concentrations in this assay, we have generally found that the inter-study variability was comparable to that typically observed in 21 day fish study designs.

4.2. The effects of individual chemicals

Ethinylestradiol inhibited egg production in a concentration-dependent manner (Fig. 2C) over the whole test range (LOEC = 0.5 ng/l; estimated EC20 = 0.4 ng/l). In a life-cycle experi-

ment performed by Lange et al. (2001) using fathead minnow, the NOEC for egg production was 1 ng/l. Given the regulatory interest in EE2–the European Union recently placed this pharmaceutical on the Watch List of the Water Framework Directive (COM (2011) 876)—it would be very beneficial to accurately define the no effect concentrations range for this chemical. However, given the fact that statistical detection limits always exist in experimental studies and hence small effect changes to controls can never be identified with sufficient certainty, discussions should be focused instead on what effect changes we are willing to accept. This, ultimately, requires a better understanding about the consequences of a reduction in egg production on fish population dynamics and the aquatic ecosystem, a link which at present is still unclear.

Concentrations of EE2 in some effluent-impacted rivers of many European countries may well reach 0.2 or 0.3 ng/l (Williams et al., 2003; Johnson et al., 2013), and thus may be high enough to have some inhibitory effect on egg production of wild fish. Concentrations of EE2 in most rivers in America are likely lower than European rivers, and hence this chemical seems unlikely to affect egg production of wild fish by itself, although it could still contribute to the overall estrogenic activity of rivers. Only when very accurate, reliable measurements of the concentration of EE2 in a range of rivers are available will it be possible to know how close these are to those that inhibit egg production of fish. Surprisingly, there is not a great deal of data in the literature addressing the issue of what concentration of EE2 is required to begin inhibiting egg production. It is well recognised, from both laboratory (e.g. Lange et al., 2001) and field (Kidd et al., 2007) studies that a concentration in the low ng/l range prevents fish from reproducing. However, sub-ng/l concentrations have not usually been studied, although Parrott and Blunt (2005) reported that a concentration as low as 0.32 ng/l, for only a brief period (48 h), adversely effected reproduction. Given the consensus that concentrations of only a few ng/l cause reproductive toxicity (reviewed in Caldwell et al., 2008), it is very surprising that Thorpe et al. (2009), in a series of three pair-breeding experiments, reported that a concentration as high as 15 ng/l produced no effect on reproduction. Those authors report that egg production in the post-exposure 3 week period was unchanged from that in the pre-exposure 3 week period.

Unlike the situation with EE2, the reproductive toxicity of natural and synthetic progestins to aquatic organisms has been known for only a few years. Zeilinger et al. (2009) first reported that the synthetic progestin levonorgestrel inhibited egg production of fish at very low concentrations; even the lowest concentration they tested, namely 0.8 ng/l, reduced egg production from pairs of fathead minnow. Subsequent independent studies (e.g. Runnalls et al., 2013; Kroupova et al., 2014; Svensson et al., 2013) have confirmed that levonorgestrel adversely affects reproduction of fish at low (ng/l) concentrations. However, many questions remain unanswered, such as whether some species are more sensitive to the chemical than are others (for example, the stickleback may be less sensitive than some other species; Svensson et al., 2013), whether some life stages are more sensitive than others, whether some endpoints are more sensitive than others, and what is the lowest concentration that causes an adverse reproductive effect. That final question is particularly important from a regulatory standpoint. Our data suggest that concentrations below 1 ng/l can lead to reduced egg production, although exactly how much below awaits further research. Levonorgestrel appears to be essentially equipotent to EE2 when egg production is the endpoint quantified.

Levonorgestrel is a PR agonist in humans, but it is also known to modulate the AR. This was confirmed by the strong androgenic effects observed in the present and previous studies. The drug has a high affinity for the human (Runnalls et al., 2013) and fish (Ellestad et al., 2014) AR. It induced spiggin production in female sticklebacks, which is strongly indicative of its androgenic activity in this species (Svensson et al., 2013), and it induced male secondary sexual characteristics in female fathead minnows (Zeilinger et al., 2009; Runnalls et al., 2013; this study), which again is indicative of its in vivo androgenic activity.

4.2.1. Drug plasma concentrations and reproductive effects

Until recently the concentration of pharmaceuticals in the water has been one of the key factors for the interpretation of ecotoxicology results. However, external concentrations are only one aspect of the exposure scenario, since it is the internal concentration of a chemical (e.g. in the blood or in a target tissue) that ultimately induces pharmacological or toxicological responses in the organisms (Margiotta-Casaluci et al., 2014). Here we applied the Fish Plasma Model and the Read-Across approach (Rand-weaver et al., 2013) to support the interpretation of the observed effects. In the single exposure studies, EE2 elicited significant effects on egg production only at predicted fish plasma concentrations at or above the human therapeutic range (Fig. 3A). In humans, EE2 at therapeutic concentrations does not directly inhibit ovulation; hence a quantitative comparison with the effects on egg production in fish is not possible. This highlights the importance of selecting appropriate endpoints when comparative approaches (i.e. Read-Across) are used to generate quantitative predictions. However, EE2 may contribute to the overall inhibitory effect via feedback mechanisms at the HPG axis (e.g. by suppression of FSH).

In contrast, the effects of exposure to levonorgestrel were in complete agreement with the predictions, both qualitatively and quantitatively. Plasma concentrations of levonorgestrel that fully inhibit ovulation in humans (i.e. are in the human therapeutic range) also caused an 85% inhibition of egg production in fish (Fig. 3B).

4.3. Binary mixture assessment

Many steroidal pharmaceuticals from different classes (e.g. oestrogens, androgens, progestins and glucocorticoids) have pronounced effects on fish when exposure is to very low (ng/l) concentrations. As rivers receiving effluent are likely to contain many different steroidal pharmaceuticals, if we are to understand the effects these pharmaceuticals might have on fish, it is necessary to understand how fish respond to these mixtures, rather than individual chemicals. Ideally, if some general principles could be established, it might become possible to predict the effects of different mixtures of steroidal pharmaceuticals without testing each and every one, which would be impossible.

We observed no indications for a significant deviation from additivity, and therefore we conclude there was no relevant antagonistic or synergistic interaction between EE2 and levonorgestrel on egg reproduction in fathead minnow. The statistical detection limit for our mixture data prevented identification of any significant differences below 30%, thereby neither confirming nor denying statistically significant effects of the mixture at the lowest concentrations. If present, we judge them as very close to the CA prediction. However, whether CA is the best additivity expectation is rather unclear. It is well-known that binary mixture studies are not ideal for judging which of the additivity concepts, CA or Independent action (IA), is better suited to describe the observed combinations effects: mathematically it has been demonstrated that for a fixed-ratio binary mixture, both concentration-response prediction curves can deviate along the concentration-axis only by a relatively small factor (maximally 2-fold) (Drescher and Boedeker, 1995; Junghans et al., 2006). The determining factors here are the mixture ratio, the effect level and, most important, the steepness of the individual concentration-response curves of the mixture compounds. In fact, in our case both of the prediction curves are nearly identical, which can be verified easily by using the concentration-

response regression parameter provided in Table 2. Considering the typical data variability in in vivo studies, it is therefore impossible for binary mixture designs with feasible animal numbers to decide statistically whether the observed mixture responses are closer to one of the mixture predictions, especially as the predictions are also subject to a stochastic uncertainty. Only a wider discrimination between both predictions can ensure which of the models is closer to the observed mixture responses, and this can only be achieved with mixtures containing many more chemicals (Faust et al., 2003; Altenburger et al., 2013; Junghans et al., 2006). However, given the practical difficulties associated with doing in vivo mixture studies, together with the uncertainties associated with how the chemicals might interact, we considered it appropriate to begin by using binary mixtures, before moving on to mixtures containing larger numbers of chemicals. Nevertheless, the two chemicals present in the mixture used in this study are thought to have different mechanisms of action (EE2 is an ER agonist, levonorgestrel a PR and AR agonist), suggesting that IA may be the better additivity model. In addition, the different concentration-related sex specific effect profiles at differing levels of biological organisation observed with both chemicals support the idea that the pharmacological assumptions of CA might be not fulfilled, at least not in the sense of strictly similarly-acting compounds.

Certainly more empirical evidence is needed before any generalisation can be drawn from our results. Both additivity expectations rest on the pharmacological assumption of strict similar or dissimilar modes of action, which are probably rarely, if ever, fulfilled for most real-life mixture scenarios. Occasionally it is possible to group the mixture compounds into common assessment groups and then predict their combined effect by hybrid CA/IA prediction models (Ermler et al., 2014), but if compounds have multiple modes of action, such as many steroidal pharmaceuticals probably do, statements based on strict similar or dissimilar modes of action are fraught with difficulties. Here all experimental evidence suggest that none of these models should be expected to describe the mixture toxicity accurately, but the mixture responses should always be between both predictions ('prediction window'; Kortenkamp et al., 2009). Therefore CA is often considered as the preferred additivity reference, as it usually predicts higher mixture responses ("worst-case" assumption). However, all these expectations rest on the assumption of non-interaction, i.e. the observed mixture effect is the result of all compounds exerting their effects without interfering with the way all other chemicals act. In contrast, interaction is thought to have occurred when one or several compounds are likely to have interacted with each other (e.g. by influencing each other's uptake, transport, metabolism or excretion), such that the observed mixture effects deviate from what was expected. As concluded before, no indications for a relevant interaction were observed for the main assessment endpoint, relative egg performance (Fig. 4).

Also for the other endpoints, there was no clear evidence that one of the pharmaceuticals inhibited any of the effects of the other when the mixture was tested. When comparing the individual responses with those from the mixture (Tables 4–6) we see that, in general, if an endpoint was affected by an individual chemical, then it was also similarly affected by the mixture. Put another way, no unexpected effects of the mixture occurred; they all could readily be explained by the effects of the individual chemicals. The results also demonstrate that additive mixture responses may not necessarily occur at all levels of biological organisation, which has also been observed by others (Säfholm et al., 2015; Zhao et al., 2015; Zucchi et al., 2014). This was clearly demonstrated by looking at a biochemical endpoint, vitellogenin synthesis, and the holistic endpoint, egg production. Only one of the chemicals, namely EE2, stimulates vitellogenin production, whereas both inhibit egg production. When tested as a mixture, there was no evidence of any combination effects on vitellogenin synthesis—the estrogenic activity of EE2 was unaffected by the co-exposure to levonorgestrel (as was also recently shown by Säfholm et al., 2015 to occur in amphibians)—however, an additive (i.e. combination) effect on egg production occurred. This lack of apparent effect of levonorgestrel on the plasma vitellogenin concentration is, nevertheless, surprising, as the high androgenic potency of levonorgestrel is thought to account for the adverse effects in fish. Exposure to androgens can reduce E2 concentrations as well as vitellogenin concentrations (Ankley et al., 2003). The probable explanation is that once intense vitellogenin synthesis was induced maximally in males after initial exposure to the EE2, it became difficult to observe any reductions within the relatively short period (21 days) of the experiment, because vitellogenin clears very slowly from the blood.

Research into the effects of dissimilarly acting chemicals in the aquatic environment is still in its infancy. The pair-breeding assay provides an ideal testing platform for studying the relevance of IA: in theory a large number of different molecular initiating events (Ankley et al., 2010) are possible, that can all inhibit egg performance, directly or indirectly, and this provides an excellent starting point to enable us to select compounds which can be considered as most "dissimilar". However, creating a clear reference case for IA can only be realised with large multi-component mixtures, a challenge which will require considerable effort and resources.

5. Conclusions

In conclusion, with the present studies we have demonstrated the potential of modelling approaches to bring data analysis beyond the horizon of traditional statistics and to maximise the informative value of ecotoxicological studies. The integration of these approaches in the Read-Across concept, based on internal exposure concentrations and cross-species extrapolation, can represent a valuable strategy to enhance the effectiveness and reliability of risk assessment for both single chemicals and their mixtures.

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