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Anti-androgens act jointly in suppressing spiggin concentrations in androgen-primed female three-spined sticklebacks – prediction of combined effects by concentration addition

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ABSTRACT

Increasing attention is being directed at the role played by anti-androgenic chemicals in endocrine disruption of wildlife within the aquatic environment. The co-occurrence of multiple contaminants with anti-androgenic activity highlights a need for the predictive assessment of combined effects, but information about anti-androgen mixture effects on wildlife is lacking. This study evaluated the suitability of the androgenised female stickleback screen (AFSS), in which inhibition of androgen-induced spiggin production provides a quantitative assessment of anti-androgenic activity, for predicting the effect of a four component mixture of anti-androgens. The anti-androgenic activity of four known anti-androgens (vinclozolin, fenitrothion, flutamide, linuron) was evaluated from individual concentration-response data and used to design a mixture containing each chemical at equipotent concentrations. Across a 100-fold concentration range, a concentration addition approach was used to predict the response of fish to the mixture. Two studies were conducted independently at each of two laboratories. By using a novel method to adjust for differences between nominal and measured concentrations, good agreement was obtained between the actual outcome of the mixture exposure and the predicted outcome. This demonstrated for the first time that androgen receptor antagonists act in concert in an additive fashion in fish and that existing mixture methodology is effective in predicting the outcome, based on concentration-response data for individual chemicals. The sensitivity range of the AFSS assay lies within the range of anti-androgenicity reported in rivers across many locations internationally. The approach taken in our study lays the foundations for understanding how androgen

receptor antagonists work together in fish and is essential in informing risk assessment methods for complex anti-androgenic mixtures in the aquatic environment.

Keywords: Anti-androgen; *Gasterosteus aculeatus*; Mixture effects; Concentration addition; Pesticides; Endocrine disruption.

1. Introduction

Considerable attention and concern has been focused on contaminants in the aquatic environment that interfere with the functioning of the vertebrate reproductive system (endocrine disrupting chemicals: EDCs), the most-documented of which are EDCs that target estrogen-dependent pathways (Sumpter and Johnson, 2008). However, chemicals that interact with other elements of the reproductive endocrine system are of equal interest. In particular, EDCs with anti-androgenic properties are believed to be ubiquitous within the aquatic environment (Hill et al., 2010; Johnson et al., 2007; Urbatzka et al., 2007) and may be important contributors to reproductive dysfunction in aquatic animals (Jobling et al., 2009). Nonetheless, the biological significance of anti-androgenic contaminants is not yet fully understood. Relatively little is known about the disposition and identity (although see Rostkowski et al., 2011) of anti-androgenic EDCs or the extent of their effects on aquatic wildlife. These knowledge gaps highlight a need for further investigation and assessment.

The aquatic environment is a chemically complex medium in which individual contaminants may be present at low concentrations yet still contribute to joint effects on organisms as part of the overall assemblage of chemicals. In this context, without the ability to extrapolate likely combined effects, reference data derived from single-agent exposure studies are uninformative regarding the overall risk and potential adverse effects for exposed animals (Kortenkamp, 2007). Thus, there is a need to develop and refine methods that allow the prediction of effects of chemical mixtures on target organisms in the aquatic environment. To date, most studies using fish as an environmentally relevant model target organism to investigate mixture effects of EDCs, have focused on chemicals with estrogenic modes of action (Brian et al., 2005; Correia et al., 2007; Jukosky et al., 2008; Thorpe et al., 2001; Zhang et al., 2010). The purpose of the present study was to extend this approach to investigate the use of single agent concentration-response data to predict the effects on a relevant fish model of a mixture of chemicals with anti-androgenic properties.

In order to retain relevance to real-world exposure scenarios we adopted an *in vivo* assay system that utilises unique features of the three-spined stickleback (*Gasterosteus aculeatus* L.). The stickleback is ubiquitous in northern latitudes and widely employed in ecological, ecotoxicological and behavioural investigations (Katsiadaki et al., 2007; Pottinger et al., 2002, 2011, 2013; Sanchez et al., 2008). Male sticklebacks synthesize an androgen-dependent glycoprotein (spiggin) which is used to glue together the structural components of the nest (Jakobsson et al., 1999; Jones et al., 2001). Androgen-inducible spiggin is also present in the kidney of females but

normally at very low levels and this feature has been exploited to provide a bioassay for EDCs with anti-androgenic activity (Katsiadaki et al., 2002). Priming females by exposure to a standardised concentration of androgen in order to stimulate the synthesis of spiggin provides a sensitive *in vivo* quantitative assay system for the detection and evaluation of anti-androgenic EDCs (Jolly et al., 2009; Katsiadaki et al., 2006). The use of females, in which spiggin levels are normally low, provides a relatively constant baseline from which consistent androgen-induced spiggin levels can be achieved. This would not be possible using males in which the annual cycle of endogenous androgen causes large inter-individual fluctuations in kidney spiggin content. The use of females also reduces the likelihood that non-receptor mediated mechanisms, for example those acting on steroid synthesis, might affect spiggin levels; in females the synthesis and accumulation of spiggin is primarily a direct consequence of an androgen receptor-mediated process (Olsson et al., 2005). Because of this, the AFSS is an *in vivo* assay with a sound mechanistic basis that specifically identifies androgen receptor antagonists.

This series of studies was designed to evaluate whether the joint effects of a mixture of anti-androgens on spiggin synthesis in female sticklebacks could be predicted accurately from knowledge of the individual potencies of each component of the mixture. The concept of concentration addition (CA), which is applicable to mixtures of chemicals with a common mode of action (Drescher and Boedeker, 1995), was favoured as the prediction model. In the first instance our intention was to validate the usefulness of CA, rather than to study environmentally relevant mixtures.

Accordingly, the following androgen receptor antagonists (Kang et al., 2004; Lambright et al., 2000; Sebire et al., 2009; Tamura et al., 2001; Wong et al., 1995) were selected: fenitrothion [0,0-dimethyl 0-(4-nitro-m-tolyl) phosphorothioate] an organophosphate insecticide; vinclozolin [(RS)-3-(3,5-dichlorophenyl)-5-methyl-5-vinyl-1,3-oxazolidine-2,4-dione] a non-systemic dicarboximide fungicide, linuron [3-(3,4-dichlorophenyl)-1-methoxy-1-methylurea] a substituted urea herbicide, and flutamide [2-methyl-N-[4-nitro-3-(trifluoromethyl) phenyl] propanamide], a non-steroidal anti-androgenic therapeutant. The potency of each anti-androgen in countering androgen-induced spiggin synthesis in female sticklebacks was evaluated singly and these data were used to predict the outcome of a series of combined exposures in which all four anti-androgens were present in a mixture at ratios proportional to their expected individual potencies. Using this fixed-ratio mixture design, the predictive power of CA was assessed by comparing the predicted anti-androgenicity of the four compounds with that observed. Because differences between nominal and measured concentrations of the anti-androgens in the test mixtures changed the original mixture composition, in each mixture concentration the assumption of a common mixture ratio between the compounds and test concentrations was unavoidably violated. This would have resulted in restricting the comparative mixture assessment to only the analytically determined mixture concentrations, thereby discarding one of the biggest advantages of fixed-ratio mixture designs - the capacity to assess concentration ranges of the mixture that were not directly tested. We overcame these limitations in this study by estimating varying mixture ratios that allowed us to expand the traditional concentration-

response analysis established for fixed-ratio mixtures to more complex mixture compositions.

2. Materials and methods

2.1. Chemicals

Analytical grade flutamide (FL), fenitrothion (FN), vinclozolin (VZ) and dihydrotestosterone (DHT) were obtained from Sigma-Aldrich (Gillingham, UK) and linuron (LN) was purchased from QMX Laboratories (Thaxted, UK). All chemicals used in the study were matched across laboratories by batch number and were of high purity (≥99%). All other chemicals were obtained from Sigma-Aldrich unless otherwise stated.

2.2. Fish

Sticklebacks were obtained from a supplier (Moore & Moore Carp, Reading, UK; CEH Lancaster) or captured by beach seine in Oslo fjord (Drøbak Research Station; University of Bergen). At both Lancaster and Bergen, the fish were subsequently kept in glass aquaria supplied with a constant flow-through of water and fed five times weekly with frozen bloodworm. Because of the requirement that the test fish exhibit low levels of endogenous spiggin, only female fish were selected for these studies. Males were identified by inspection of iris and oesophageal colour (immature males exhibit traces of blue and red respectively) and separated from the females. For a

period of at least one month prior to the exposure studies the sticklebacks were acclimated to the temperature (Lancaster: 15 ± 2°C; Bergen: 16 ± 2°C) and photoperiod (12h light:12h dark) under which the studies were conducted.

2.3. Experimental design

Single agent and mixture studies were performed in parallel at two laboratories (Centre for Ecology & Hydrology, Lancaster, UK, and Department of Biology, University of Bergen, Norway) over a period of three years. The *in vivo* exposures closely followed procedures outlined in the OECD Guidance Document 148 (OECD, 2011). The exposure system comprised the required number of 30 L (working volume) glass aquaria each supplied with a constant inflow of untreated raw water (100 mL/min; PVC tubing, Portex; 5 mm i.d.; Lancaster: lake water; Bergen: seawater) via peristaltic pumps (Watson Marlow 505S; Marprene tubing, 6.4 mm i.d.) with twin head cassettes. The performance of each of the pumps was checked twice weekly by timing the delivery of 100 mL of water into a volumetric flask. Each tank was aerated throughout the study period via a single airstone. Working solutions of the test compounds were formulated in methanol and held in 1.0 L glass bottles. A multi-channel peristaltic pump (Watson Marlow 205U; 0.76 mm i.d. PVC manifold tubing) delivered the test compound solution from the stock bottle to the aquaria via silicone tubing through a three-way connector inserted immediately downstream of the raw water pump head. A pumped delivery rate of 100 µL/min for the chemical stock was maintained resulting in a concentration of methanol in the

exposure tanks of 0.1%. The stock solutions of test compound, either single chemicals or mixtures, were formulated at 1000-fold the nominal concentration required in the exposure tanks. The flow rates of the multi-channel pump were validated twice-weekly by determining the weight of solvent delivered into pre-weighed vials during a defined period of time. Water temperature was held within the range required using thermostatically controlled water heaters. The temperature within each exposure tank was logged at 30 min intervals via temperature probes attached to a 10-channel data logger, downloaded at weekly intervals to a computer. Water quality measurements (pH, dissolved O_2) were taken at weekly intervals with portable metering systems to ensure that study conditions met the requirements laid out in the OECD Guidance Document (OECD, 2011).

2.4. In vivo exposure studies

For each study, the exposure system was set up and run with the test chemical for one week to equilibrate the system before the fish were added. Each tank was populated with 10 - 15 female sticklebacks (according to the test requirements) from a stock population that had been acclimated to the experimental conditions of temperature and photoperiod. A series of single chemical concentration-response exposures were conducted first, the results of which were used to design the final four component mixture exposure study.

2.4.1. Single chemical exposures

All studies were carried out using the same protocol in which groups of female sticklebacks were exposed to DHT (5 μg/L), a non-aromatizable androgen, to stimulate spiggin synthesis both in the presence of a range of concentrations of the test chemical and in the absence of the test chemical (positive control). In addition, a single tank received the highest concentration of test chemical in the absence of DHT (negative control) and water-only (absolute control) and methanol-only (solvent control) control tanks were also included. Each single compound was tested at a minimum of seven concentrations, with nominal concentrations ranging from 0.1 to 250 μg/L for FN, 5 to 250 μg/L for FL, 0.25 to 250 μg/L for LN and 0.25 to 500 μg/L for VZ. Single compound tests for FN were conducted only at Lancaster. FL was tested at both Lancaster and Bergen and VZ and LN were tested only at Bergen. In each individual study, one tank per treatment group was used based on the assumption that the standardised and closely controlled experimental conditions would minimise between-tank variation, other than that arising from the treatment. Additional confidence was provided by the replication of studies across two laboratories. All single compound data were then pooled for further data analysis.

2.4.2. Four component mixture exposures

The mixture study comprised a series of tanks receiving each chemical singly at both the IC50 and IC50/10 together with tanks receiving a mixture of all four anti-androgens at mixture ratios proportional to their individual potencies and ranging from the IC50 to the IC50/100 (fixed-ratio mixture design, see Table S1 for details).

Water-only (absolute control), methanol-only (solvent control) and DHT-only (positive control) treatments were also included. The range of dilutions was based on the concentration range described by the additivity prediction, such that the mixture was expected to inhibit completely the androgenic effect of DHT. The four-component mixture study was conducted in both the Bergen and Lancaster laboratories.

2.5. Sampling procedure

At the end of each study (day 21) the fish were killed immediately by immersion in a lethal dose of sedative (2-phenoxyethanol; 1:1000), and stored individually at -20°C in labelled 12 mL polypropylene centrifuge tubes (Sarstedt). All the fish from a single tank were processed before disturbing the second tank. Kidneys were dissected from part-thawed carcasses, placed in 2 mL screw-capped cryovials (Nalgene, VWR International), and stored at -20°C until required for assay. Water samples (1000 mL) were taken from each tank at time 0 and at 7, 14 and 21 days after the start of the study. These were collected by immersing bottles (Nalgene HDPE; VWR International) directly in the tanks and were stored at -20°C before extraction. Extraction was accomplished by pumping the water sample (at 10-20 mL/min) through a methanol-conditioned, distilled water-washed, solid phase extraction (SPE) cartridge (Sep-Pak C18; Waters Ltd, UK) with an inline 0.45 µm pre-filter (Pall Gellman Acrocap, Pall Life Sciences). Air-purged cartridges and filters were labelled,

241 wrapped in parafilm, and stored at -20°C until being despatched (on dry ice) to the
242 receiving laboratories for chemical analysis.

243

244 *2.6. Analytical procedures*

245 Water sample extracts and kidney spiggin concentrations were analysed at the Cefas
246 laboratories. Kidney spiggin content was measured using a specific ELISA and is
247 reported as arbitrary spiggin units per gram body weight (U/g bw; Katsiadaki et al.,
248 2002). Concentrations of DHT in aquarium water extracts were determined with an
249 established radioimmunoassay procedure (Katsiadaki et al., 2002; Scott et al., 1984).
250 Concentrations of FL, LN, FN and VZ in the extracts of exposure tank water were
251 determined as described previously (Katsiadaki et al., 2006). In brief, concentrated
252 methanolic SPE derived extracts were analysed by high performance liquid
253 chromatography with electro spray ionization and selected ion mass spectrometry
254 (HPLC-ESI-SIM-MS). Quantities of the target chemicals were determined by external
255 calibrations, using a series of calibration (n=6) solutions prepared from the same
256 stock chemicals used for the exposure studies. The performance of the SPE
257 procedure was assessed prior to the start of the studies by extracting six replicate
258 solutions each containing a mixture of FL, LN, FN and VZ at 100 µg/L each. The
259 extracts were then analysed as described above and percent recoveries calculated.

260

261 *2.7. Concentration-response analysis*

262 To account for inter-study variation in absolute spiggin levels, spiggin concentrations
263 (U/g bw) were log-transformed and then standardised to the mean values of the
264 positive DHT-stimulated controls and the solvent controls (unstimulated baseline
265 spiggin concentration). By this scaling approach, the absolute effects scale was
266 normalised to relative effects between 0 and 1. The median inhibitory concentration
267 (IC50) of DHT was that which produced a $\log_{10}$-transformed spiggin inhibition which
268 was median in relation to the DHT controls (maximum spiggin concentration) and
269 solvent controls (minimum spiggin concentration). Concentration response data
270 analysis was based on the geometric mean of concentrations of the test chemicals
271 that were measured at intervals (7d, 14d, 21d) during the three-week exposure
272 period. We determined concentration–response curves for each of the four
273 chemicals using pooled data from the exposures conducted by the two participating
274 laboratories. To account for the intra- and inter-experimental variability associated
275 with this nested data scenario, we used the generalised, nonlinear mixed modeling
276 approach in which both fixed and random effects are permitted to have a non-linear
277 relationship with the effect end point (Vonesh and Chinchilli, 1997). A shift
278 parameter was included in the non-linear regression model as a random effect which
279 accounts for a shift of the whole curve based on the $\log_{10}$-transformed concentration
280 scale. Furthermore, a best-fit approach was adopted, in which different regression
281 models were fitted independently to the same pooled data set, and the best fit was
282 selected on the basis of statistical criteria (Scholze et al., 2001). This approach was
283 implemented using the NLMIXED function of the SAS statistical software package
284 (SAS Institute, Cary, USA).

2.8. Mixture prediction

Following the logic of Berenbaum (1985), and as described by Faust et al. (2001), under the assumption of concentration addition (CA) contours of constant effect X are planar such that

$$\sum_{i=1}^{n} \frac{c_i}{EC_{Xi}} = 1, \qquad (1)$$

where, for a combination of *n* components, c_i is the concentration of the ith component in the mixture concentration $c_{mixture} = (c_1,\ldots,c_n)$ that produces the effect X, and EC_i^X is the concentration of the ith component that produces the same magnitude of effect. The effect concentration EC_i^X is derived from the inverse of the regression function which describes the observed concentration effect data of the ith component (Table 1). The nonlinear regression models used in the best-fit approach assumes that the expected mixture effect *X* at given mixture concentration $c_{mixture}$ can only be calculated by solving iteratively Equation 1. A fixed-ratio mixture design simplifies this implicit equation by re-arranging Equation 1 into an explicit form that allows the calculation of the effect concentration at given mixture concentration (Faust et al. 2001):

$$c_{mixture} = \left(\sum_{i=1}^{n} \frac{p_i}{EC_{Xi}} \right)^{-1}, \qquad (2)$$

where p_i is the is the ratio of the ith component in the mixture and the sum of all p_i equals 1. This equation also allows interpolative calculations for untested concentration ranges, similar to the common concentration-response regression analysis for single components. The prerequisite is that the relative composition remains unchanged at every mixture concentration, i.e., all test concentrations of the mixture have something in common functionally. However, a common problem with exposures in aquatic flow-through test systems is that the measured concentrations do not always closely correspond to nominal concentrations. Consequently, an identical relative composition at every mixture concentration can *a priori* not be assumed, and therefore the calculation of effect concentrations according to Equation 2 is *a priori* not feasible. For the mixture assessment this means, in the worst-case, that only observed and predicted responses at mixture concentrations for which measured concentrations are available can be compared. Effect concentrations corresponding to values between measured concentrations cannot be assessed. To overcome this limitation, we developed a methodology that under certain assumptions about the functional relationship between measured and nominal concentrations allows the prediction of mixture effects at untested concentrations. To better understand the fixed-ratio design and its meaning for our proposed method, we have illustrated in Figure 1 how concentrations of two single components can be combined in a mixture: showing the range of all possible combinations of test concentrations that can be tested as a mixture. The fixed-ratio design limits them to those pairs which follow a straight line with zero origin (Figure 1, Line A), i.e. the compounds in the mixture are characterised by a consistently

326 identical ratio. If the measured concentrations are not the same as those planned,
327 three cases can be identified that would still allow a mixture assessment for untested
328 concentrations: (i) all single compound concentrations measured in the mixture
329 deviate at all test mixtures roughly by the same factor from the planned
330 composition, in which case the relative mixture composition is unchanged and can
331 be described by the same line (Figure 1, line A) and no corrections to Equation 2 are
332 required; (ii) the concentrations of compounds measured in the mixture deviate in
333 all test mixtures by approximately the same factor, but for each compound a
334 different ratio is estimated. This still maintains a straight line (Figure 1, line B), but
335 would require the correction of the mixture ratio by replacing the original fractions p_i
336 in Equation 2 with fractions estimated from the measured concentrations; (iii) for at
337 least one mixture compound the ratio between nominal and measured
338 concentrations is not constant over all tested mixtures, but follows a functional
339 relationship (e.g., recovery rate decreases with increasing test concentrations).
340 Consequently, each mixture concentration has a different composition and the
341 fraction p_i in Equation 2 depends on the measured mixture composition. The
342 functional relationship is non-linear (Figure 1, line C). However, in this case Equation
343 2 cannot be used as each effect level X requires its own mixture composition, and
344 mixture effects can only be calculated according to Equation 1. In the present study
345 we were challenged with a mixture that deviated from linearity as illustrated in
346 Figure 1 (line C). We used a second-order regression function to describe the
347 measured concentrations in relation to the nominal values, which also allowed the
348 prediction of mixture effects for concentration ranges along the non-linear plot for

349 which analytically determined concentrations of anti-androgen were not available. 350 Details of the regression model can be found in the Appendix. As the measured 351 concentrations were replaced by smoothed regression estimates, we additionally 352 calculated the mixture effect at the measured mixture concentration.

353 All effect concentrations of the single components are estimates and are therefore 354 subject to stochastic variability. This meant that the predicted effect concentration 355 of the mixture also had to include a measure of statistical uncertainty. This was 356 achieved by using the bootstrap method (Efron and Tibshirani, 1993), which enabled 357 approximate 95% confidence limits to be derived for the mean predicted effect. It 358 should be noted that the variation of measured concentrations observed over the 359 exposure period was not taken into account, i.e. the geometric mean of the 360 measured exposure concentration was used as a fixed value in the resampling 361 approach. Therefore the confidence limits might slightly underestimate the true 362 uncertainty.

363

364 **3. Results**

365 All single-agent and mixture studies ran to completion. No atypical behaviour was 366 observed among the fish during any of the studies. Mortality among the test fish did 367 not exceed 1.5% overall and there was no evidence of disease or parasite infections. 368 At Lancaster there were no significant differences between spiggin concentrations in 369 fish from the water control tanks (73 ± 10 U/g bw, n = 20), solvent control tanks (105 370 ± 17 U/g bw, n = 22) and negative control tanks (58 ± 8 U/g bw, n = 21). In studies

conducted at Bergen, there was a small but significant difference between spiggin concentrations in fish from solvent tanks (43 ± 8 U/g bw, n = 60) and negative control tanks (66 ± 6 U/g bw, n = 63) but neither were significantly different from the water controls (46 ± 5 U/g bw, n = 61) (One-way ANOVA, Tukey's Test). There were no significant differences between studies or laboratories in the DHT-stimulated spiggin concentration measured in positive controls (overall mean = 43730 ± 3064 U/g bw, n = 94). The extraction efficiency of the SPE procedure was found to be high for all compounds (LN: 83.5 ± 2.2 %, FN: 94.0 ± 3.2 %, FL: 84.3 ± 3.9 %, VZ: 86.0 ± 2.0 %; mean ± SEM, n = 6). For the single agent studies mean recoveries of the test compounds from the exposure tanks were: LN: 43.3 ± 4.8 %, n = 12, FN: 53.9 ± 6.3 %, n = 15, FL: 49.6 ± 4.2 % n = 24, VZ: 6.7 ± 1.4 %, n = 9 (mean ± SEM). The % recovery values for each test compound in the mixture study are provided in Table 2 and Fig. 4.

3.1. Single compound studies

Repeated studies with the four compounds were performed in two separate laboratories (Lancaster and Bergen) over a period of three years. Concentration-response data were always in good agreement, and differences in response between laboratories or time trends were not statistically significant. Each of the chemicals that were tested inhibited spiggin induction in a concentration-dependent manner, confirming that the AFSS effectively detects androgen receptor antagonists (Fig. 2). It was possible to determine a concentration that elicited full inhibition of spiggin

393 production (relative to the negative control) for three chemicals FL, FN, and VZ and
394 for these, the lowest tested concentration did not evoke effects significantly
395 different from the untreated controls. This allowed the estimation of near-complete
396 concentration response curves without needing to extrapolate to untested effect
397 levels (Fig. 2; Table 1). Based on the measured concentrations, the most potent anti-
398 androgen was VZ (IC50 = 8.57 µg/L), and the least potent was LN (IC50 = 172 µg/L).

399

400 Between-study differences in absolute spiggin concentrations (U/g bw) were
401 relatively small. For example, mean female spiggin values for the DHT control were
402 within one order of magnitude (interquartile range: 34,000 - 63,000 U/g bw). The
403 normalisation approach we adopted for the spiggin data further improved the
404 comparability of concentration-response data from different studies (Fig. 3). Here
405 data obtained from two LN exposure studies are plotted as absolute (Fig. 3a) and
406 transformed (Fig. 3b) spiggin values. Because the means of the DHT controls differed
407 slightly between studies better agreement of the data at low effect concentrations
408 was achieved following normalisation.

409

410 *3.2. Mixture studies*

411 The mixture of anti-androgenic chemicals was tested at both Lancaster and Bergen,
412 and the actual and nominal concentrations for each component of the mixture are
413 given in Table 2. The average variation of the single component measurements
414 between the sampling days was found to be random in nature, i.e. trends across the

testing period could not be detected (data not shown). As was the case for the single substance studies, we found no consistent agreement between nominal and measured concentrations of the test compounds, with average recovery in most cases of less than 100%. The estimated concentrations which were derived from the regression method outlined in the Appendix are given in Table 2. The entirety of the curves estimated are shown in Figure 4. The corresponding model parameters can be found in Table S2. There were marked differences in the actual chemical concentration relative to the nominal concentration not only for compounds within the mixture, but also between laboratories. Data for FL were most consistent in terms of both recovery and agreement between laboratories (Fig. 4a), and both FL and LN (Fig. 4c) exhibited a mostly linear relationship between nominal and measured concentrations. However, for FN (Fig. 4b) and VZ (Fig. 4d) more complex relationships between nominal and actual concentrations were evident. Nevertheless, in all cases it was possible to establish a clear functional relationship between nominal and measured concentrations. For most compounds the second-order model parameter θ_3 was not statistically significant (Table S2), and a linear nominal-measured model assumption would have led to nearly identical estimates. However, a significant non-linearity term was estimated for VZ. This approach yielded a significant improvement for VZ where the non-linearity arose mainly because the lower concentrations of VZ showed better recovery rates than the higher concentrations. Overall, the analytically determined chemistry data indicated that the relative composition differed significantly between both mixtures and that therefore separate data analysis for each mixture was appropriate. The chemical

data also showed that not all mixture concentrations can be described by a common ratio between the component concentrations, which justified our decision to estimate mixture concentrations through variable mixture ratios.

For studies in both laboratories the mixture of VZ, FL, LN and FN produced a concentration-dependent inhibition of spiggin induction in DHT-primed female sticklebacks (Figure 5). The lowest tested mixture concentration induced no statistically significant changes, and the highest tested mixture concentration produced a maximal spiggin inhibition, equivalent to spiggin levels in non-DHT-exposed controls, in both studies. Data variability was comparable with that from the single component studies (Table 3). The concentration-response data for the single components, pooled from all studies (see Figure 2 and Table 1), were used to compute predicted concentration-additive combination effects covering the entire range of effects (Figure 5a and 5b; solid lines). For both studies, the anticipated combination effects fell within the range of the effects that were observed experimentally. The pooled data sets provided sufficient information for predictions of low statistical uncertainty (Figure 5; dotted lines), and were therefore a good basis for the comparative mixture assessment. The comparison of the observed spiggin induction with the prediction curve yielded a good agreement for most effect levels. No statistical deviation could be detected, with the average spiggin induction lying within or close to the narrow 95% confidence limits along the full length of the curve. The only exceptions to this trend were the responses we observed at 142.8 µg/L (nominal), which were significantly overestimated by CA in the studies conducted at

both laboratories. The observed responses were surprising inasmuch as they were similar to those observed at the preceding concentration (nominal 57.1 µg/L), i.e. a concentration-dependent decrease could not be detected between these two successive dilutions. Nevertheless, and notwithstanding this anomaly, these findings provided overall evidence that anti-androgenic chemicals act in an additive manner *in vivo* and that their effects can be predicted accurately using CA.

4. Discussion

The results of this study confirm the utility of the female three-spined stickleback as a model organism for evaluating the *in vivo* anti-androgenicity of compounds in an aquatic environment (Katsiadaki et al., 2006, 2012; OECD, 2011). In addition they established that spiggin is an endpoint for anti-androgenic activity with sufficient resolution and sensitivity to permit complex mixture effect analysis.

4.1. Mechanistic basis of the spiggin response

Spiggin synthesis in sticklebacks is assumed to be regulated by a renal androgen receptor (Olsson et al., 2005) and therefore an obvious route by which the inhibition of spiggin production can occur is via antagonism of androgen binding to androgen receptors in the kidney by competing ligands. The four anti-androgens used in this study exhibit anti-androgenic activity in mammalian systems via the competitive inhibition of androgen binding to the androgen receptor (Lambright et al., 2000;

Tamura et al., 2001; Wong et al., 1995) and three of the compounds (VZ and metabolites, LN and FL) have also been demonstrated to displace androgens from a teleost androgen receptor *in vitro* (Wilson et al., 2007). In the current study the degree to which the selected anti-androgens interfered with androgen-stimulated synthesis of renal spiggin in female sticklebacks was broadly consistent with earlier reports of the relative activity of these antagonists *in vitro*. We found LN to be the least potent of the compounds tested and this is consistent with its activity relative to FN and VZ in a human breast cancer reporter cell line (Orton et al., 2011). We also found VZ to be more potent than FL in suppressing androgen-stimulated spiggin production, which conforms to reports of the relative anti-androgenic activities of these compounds in the same cell line (Aït-Aïssa et al., 2010). However, in the present study the apparent potency of VZ may have been inflated due to the degradation of VZ to active derivatives, which were not measured directly. The low recoveries reported for VZ may be due to the fact that via hydrolysis, photolysis and/or microbial metabolism, VZ yields several degradation products. Compounds 2-[[(3,5-dichlorophenyl)-carbamoyl]oxy]-2-methyl-3-butenoic acid (M1) and 3',5'-dichloro-2-hydroxy-2-methylbut-3-enanilide (M2) ultimately degrade further to the terminal degradation product 3,5-dicholoraniline (M3; Dhananjeyan et al., 2006). Metabolites M1 and M2 are androgen receptor antagonists (Wong *et al*, 1995). Metabolite M3 requires at least 21 days of reaction time to appear (Szeto et al., 1989) in an aqueous medium and may therefore have contributed less to the observed effects. Concentrations of the parent compound VZ only (rather than the metabolites M1 and M2) were determined in this study.

505

506 *4.2. Sensitivity of spiggin production to anti-androgens*

507 The IC50s identified for VZ and FL in the single agent calibration exposures in the 508 present study fall within or below the lower range of the concentrations used in 509 previous studies with fish. However, previous reports in which immersive exposure 510 to anti-androgens was employed, rather than direct dosing via the food, have not 511 always employed range-finding studies to set exposure levels. Consequently, the 512 range of concentrations of anti-androgens reported to be bioactive in fish is wide. 513 For VZ, effects in fish have been reported for concentrations of 100 µg/L (medaka, 514 *Oryzias latipes*; León et al., 2008), 60 - 450 µg/L (Martinović et al., 2008), 600 µg/L 515 (zebrafish, *Danio rerio*; Martinović-Weigelt et al., 2011) and 2500 µg/L (medaka; 516 Kiparissis et al., 2003). Between-study comparisons are to some degree confounded 517 by variation in exposure conditions, endpoints, species, and developmental stage of 518 the test fish so, for example, exposure to VZ at 90 – 1200 µg/L evoked no significant 519 effects in embryos whereas in the same study adults exposed to VZ at 700 µg/L did 520 exhibit adverse outcomes (fathead minnow, *Pimephales promelas*; Makynen et al., 521 2000). Flutamide at a concentration of 412 µg/L was found to elicit only minor 522 phenotypic alterations in exposed fish accompanied by more pronounced effects on 523 gene expression (fathead minnow; Filby et al., 2007). At levels of 651 µg/L (fathead 524 minnow; Jensen et al., 2004), 1000 µg/L (medaka; León et al., 2008) and 1700 µg/L 525 (zebrafish; Martinović-Weigelt et al., 2011), significant effects were observed. Fewer 526 data are available describing sub-lethal endocrine disruptive effects on fish exposed 527 to FN and LN but the IC50s identified within the present study for each are

consistent with other investigations in which anti-androgenic activity was detected in sticklebacks at concentrations of between 15 and 200 µg/L (FN) and 150 - 250 µg/L (LN) (Hogan et al., 2012; Katsiadaki et al., 2006; Sebire et al., 2009). Overall, the concentration-response data presented here underline the effectiveness of spiggin as an endpoint for detection of anti-androgenicity and suggest that sticklebacks may be more sensitive to environmental anti-androgens than has hitherto been evident.

4.3. Specificity of the spiggin response to anti-androgens and compliance with the CA model

The model anti-androgen compounds deployed in this study are assumed to interfere in a specific manner with androgen receptor-dependent signaling pathways throughout the animal. Nevertheless, it is possible that collateral effects within the reproductive endocrine system contributed to the magnitude of the reduction in spiggin. In principle, this could have resulted in unpredictable interactions between the effects of each chemical in the mixture. Certainly, anti-androgenic compounds are reported to exert a wide range of phenotypic effects on the teleost reproductive system (Baatrup and Junge, 2001; Bayley et al., 2002, 2003; Jensen et al., 2004; Kinnberg and Toft, 2003; Kiparissis et al., 2003; Makynen et al., 2000; Martinović et al., 2008; Panter et al., 2004). More recent studies have highlighted the extent to which gene expression within the reproductive axis of fish is modulated by anti-androgens in fish (Filby et al., 2007; Garcia-Reyero et al., 2009; León et al., 2008; Martinović-Weigelt et al., 2011; Villeneuve et al., 2007). Given the wide range of

550 genomic and phenotypic responses that are reported to occur in fish exposed to
551 anti-androgens, it was not clear whether the assumption inherent in the CA model,
552 that the components of the mixture do not influence each others uptake,
553 distribution or metabolism, could be met (Backhaus and Faust, 2012). The mixture
554 composition used in the present study was formulated on the basis of the response
555 of sticklebacks to single agent exposures; each component was present in the final
556 mixture at a ratio proportional to their individual potencies. Interactive effects
557 between the anti-androgens which might be evident only during concurrent
558 exposure to several or all the components of the mixture could not be taken into
559 account during the planning stage of the experiment. For example, individual
560 components of a mixture may exhibit different abilities to induce biotransformation
561 enzymes which in turn may impact on the potency of the mixture overall (discussed
562 by Petersen and Tollefsen, 2011). In addition, VZ has been shown to upregulate the
563 expression of the androgen receptor gene in zebrafish and fathead minnow
564 (Martinović et al., 2008; Smolinsky et al., 2010) with uncertain consequences for the
565 interplay between androgen and anti-androgen. In principle, there might have been
566 disparity in the ability of the tested chemicals to displace or compete with other
567 ligands at the androgen receptor site arising from factors affecting access to the
568 receptor, susceptibility to biotransformation, interaction with other elements of the
569 endocrine system such as sex hormone binding globulin, or differences in the
570 breadth of effects, including non-receptor-mediated effects, exerted by each
571 compound. These are issues that hold greater significance in a whole animal
572 exposure system such as that employed here, than in *in vitro* test systems, and might

negatively impact upon the usefulness of CA to predict joint effects *in vivo*. However, the fact that substantial deviations from anticipated CA did not become apparent when the individual effects of all mixture components were used as the basis for the predictions, suggests that the importance of these intervening factors was minimal. In this context, it is noteworthy that in contrast with the findings of the current *in vivo* study in which VZ was shown to be the most potent anti-androgen tested, an *in vitro* stickleback kidney cell assay identified both FN and FL as more potent anti-androgens than VZ (Jolly et al., 2009). This may relate to the degradation of VZ to active metabolites which is more likely to occur in a large scale mixed solvent/aqueous tank-based exposure system than a small-scale *in vitro* system. The discontinuity in the mixture concentration-response curve (between 57.1 and 285.6 µg/L nominal), which was observed in the current study at both laboratories independently, and does not reflect the response profiles seen in the single agent exposures, is currently inexplicable. It might be due to a more complex response to the mixture exposure than that assumed by the model however the mechanism by which this might have occurred is unclear and requires further investigation.

4.4. Deviation of exposure concentrations from nominal and implications for the CA model

The flow-through system adopted for these exposures presented technical challenges, including maintenance of the desired concentrations of chemicals, both singly and in the mixture. Failure to maintain steady-state concentrations has

595 implications for the assumptions inherent in the CA model. In flow-through systems,
596 measured chemical concentrations can differ from the intended nominal values for a
597 number of reasons including: uptake by the fish, losses due to degradation,
598 adsorption to surfaces, evaporation, photolysis, hydrolysis, or simply by inaccuracies
599 in the preparation of stock solutions or the dosing of tanks. A sufficiently high flow
600 rate might overcome some of these problems, but there are physical limits to the
601 rate of flow that can be sustained through aquaria of the size employed in the
602 present study (30 L). High flows have practical and cost implications for the volumes
603 of chemical stock solutions that are required and the frequency with which they
604 must be prepared and replenished. Given that the measured concentrations for all
605 the chemicals in the test tanks were below the expected concentrations, the decision
606 to carry out the concentration-response analysis on the basis of measured exposure
607 concentrations was vitally important. Consequently, the originally intended
608 composition of the mixture, in a strict quantitative sense, varied along the
609 concentration profile and therefore affected our mixture assessment which was
610 based on data obtained from fixed-ratio mixture designs. This design is particularly
611 suited for multi-component mixtures as it allows, with a relatively small amount of
612 data, an accurate comparison between observed and predicted effect
613 concentrations. However, outcomes are limited to the relative composition of the
614 tested mixture. If data analysis is based on measured concentrations and these vary,
615 then each mixture concentration can be considered to have its own unique
616 composition and the tested concentrations are no longer sequential dilutions of each
617 other. In cases where the differences between nominal and measured exposures are

618 minimal and do not deviate significantly from the original fixed ratio composition,
619 then the applicability of the traditional data analysis for fixed-ratio mixtures is
620 unaffected (Brian et al, 2005). The same holds true if the concentrations of chemicals
621 comprising the mixture are changed, but across all the tested combinations retain
622 the same proportional relationship with each other (constant ratio). In that case the
623 relative composition of the mixture can be re-calculated on the basis of the
624 measured exposures. For example, Correia et al. (2007) studied the joint effect of
625 estradiol-17β, 17α-ethinylestradiol, and bisphenol A on vitellogenin in sea bass
626 (*Dicentrarchus labrax*) and faced the problem of very low recovery rates for the two
627 steroids. Adjustment of the mixture ratio to the measured exposures allowed these
628 authors to perform a comparative mixture assessment for a broad range of mixture
629 exposures for which measured concentrations were not available. In the current
630 study the components of the mixture were not at a constant ratio for all the tested
631 mixture exposures (see Figure 4) an issue which was particularly evident for FN.
632 Nevertheless, by regression modeling it was possible to smooth the measured
633 concentrations, which manifested varying mixture ratios that allowed predictions for
634 a broader range of mixture concentrations. Where exposure data fail to provide a
635 clear functional pattern between the measurements of the component within the
636 mixture, it is nevertheless desirable to investigate outcomes for exposures outside
637 the tested mixture concentrations. Thus all the single compound and mixture data
638 can then be used to estimate a so-called response surface (Gennings and Carter,
639 1995).

640

4.5. Environmental relevance of the findings

This study has confirmed that the inhibition of spiggin production in androgen-primed female sticklebacks provides a sound basis for evaluating the inhibitory potency of mixtures of similarly-acting anti-androgenic chemicals. The data available that describe the occurrence of anti-androgenic substances in the aquatic environment suggests that this class of chemicals may represent a significant developing wildlife and environmental issue, notwithstanding existing concerns about possible human health issues arising via other routes of exposure (Diamanti-Kandarakis et al., 2009). Anti-androgenic compounds appear to be present in most final effluent discharges from wastewater treatment works (WWTW) in the United Kingdom (Johnson et al., 2007). They are found in both the water column (Grover et al., 2011; Urbatzka et al., 2007; Zhao et al., 2011) and in sediments (Weiss et al., 2009; Zhao et al., 2011) at combined concentrations sufficiently high to raise concerns about effects on exposed biota. For example, total anti-androgenic activity (as FL equivalents; eq.) in effluents from forty one UK WWTW were found to range from 29.5 to 844 µg/L FL eq. (mean of two samples at each site) with a median value of 102 µg/L FL eq. and an overall mean of 201 µg/L FL eq. (Johnson et al., 2007). Given that WWTW discharges can contribute a large proportion of the total flow in many receiving waters, this suggests that the range of concentrations of anti-androgens deployed within the mixture studies described for the present study were environmentally realistic for UK rivers. This supposition is supported by the findings of Grover et al. (2011) who reported within-river measurements of anti-androgen concentrations in the R. Ray (southern England) as 206 to 1070 µg/L FL eq. at 100 m

664 downstream of a WWTW and as high as 200-400 µg/L FL eq. up to 10 km
665 downstream of the effluent discharge. Similar levels of anti-androgenicity have been
666 reported for rivers elsewhere, including southern China (935 µg/L FL eq., Pearl River;
667 Zhao et al., 2011) and Italy (460 µg/L FL eq., R. Lambro; Urbatzka et al., 2007). These
668 concentrations lie comfortably within the sensitivity range of the assay system
669 employed in the present study. The *in vivo* androgenised female stickleback screen
670 (AFSS) is thus fit for the purpose of the investigation of mixture issues concerning
671 chemicals with anti-androgenic properties, both with regard to the use of an
672 ecologically relevant test species and the sensitivity of the endpoint to anti-androgen
673 exposure. This is underlined by a recent study in which an increase in spiggin levels in
674 female sticklebacks downstream of a WWTW in southern England was detected after
675 remediation of the WWTW effluent, and removal of much of the anti-androgenic
676 activity (Grover et al., 2011; Katsiadaki et al., 2012).

677

678 The array of chemicals with anti-androgenic properties that enter the aquatic
679 environment via WWTW effluents appears to be extensive (Rostkowski et al., 2011)
680 and is likely to be augmented by anti-androgenic chemicals from both agricultural
681 and industrial sources. The methodological approach described here is likely to be
682 too targeted to play a direct role in assessing the risks to wildlife of specific effluents
683 in which the interactions of numerous, ill-defined components are responsible for
684 final effects. However, in order to inform regulatory decisions regarding complex
685 effluents over which some control can be exerted, or in which some components are
686 clearly quantitatively dominant, it is necessary that the manner in which individual

components act together is understood. In this context there is an urgent need for the methodology adopted in the present study.

Acknowledgements

This study was funded jointly by the European Commission and Natural Environment Research Council, U.K. and was conducted as part of the project "Endocrine Disrupters: Exploring Novel Endpoints, Exposure, Low Dose- and Mixture-Effects in Humans, Aquatic Wildlife and Laboratory Animals" (QLK4-CT-2002-00603). The authors acknowledge the technical assistance of Jonathan James and Angela Scott at the Cefas Weymouth laboratory.

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929 **Figure legends**

930 **Figure 1:** Graphical representation of a fixed- and variable-ratio for a mixture of two
931 compounds. The line A refers to the planned mixture composition based on nominal
932 concentrations, and the lines B and C represent scenarios in which both compounds
933 were measured with different recovery ratios: line B assumes constant ratios
934 between nominal and measured concentrations, and line C occurs when for one
935 compound at least the ratio varies independently of the tested mixture
936 concentration.

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938 **Figure 2.** Concentration-response data and fitted curves for (a) flutamide, FL; (b)
939 fenitrothion, FN; (c) linuron, LN; (d) vinclozolin, VZ. Data are pooled from
940 independent studies carried out in two laboratories (Lancaster and Bergen; see
941 section 2.4.1 for details). Each point is the mean ± standard error. The best-fitting
942 regression models (solid lines; see Table 1) are shown together with the
943 corresponding 95% confidence intervals (dotted lines).

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945 **Figure 3.** Between-study variability in the spiggin response and normalisation of the
946 absolute effect scale. Concentration-effect data for linuron (LN) from two
947 independent studies (denoted by circles and stars) conducted at Bergen are shown
948 based on (a) raw spiggin values, and (b) spiggin values normalized to solvent and
949 positive DHT controls. Each point represents the median value with the 25th and 75th
950 sample percentiles indicated. The horizontal lines correspond to the control means.

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48 49 50 51 52 53 54 55 56 57 58 59 60 61 62 63 64 65

Figure 4. Measured concentrations and estimated measured concentrations for (a) flutamide, FL; (b) fenitrothion, FN; (c) linuron, LN; (d) vinclozolin, VZ. Measured data are from at least three sample points during each of two independent mixture studies conducted at Lancaster and Bergen. Estimated measured concentrations are shown as second-order regression curves (see Table S.2). The dotted black lines indicate parity between measured and nominal concentrations.

Figure 5: Concentration-response data for a mixture of four anti-androgens, tested in two different laboratories (A: Lancaster; B: Bergen). Open circles denote individual data, solid circles show the median effect, and vertical lines delineate the inter-quartile range (i.e. distance between the 25th and the 75th sample percentiles). Mixture effects were predicted according to the Concentration Addition method and are shown as an unbroken line with the approximate 95% confidence intervals as dotted lines.

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6 972 **Table 1:** Anti-androgenicity of individual compounds
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Substance (by order of IC_{50})	Concentration response function						IC10[1]		IC50[1]	
	RM[2]	$\hat{\theta}_1$	$\hat{\theta}_2$	$\hat{\theta}_3$	$\hat{\theta}_{min}$	θ_{max}	µg/L [CI]		µg/L [CI]	
vinclozolin	Logit	6.73	-7.21	-	-0.01	1	4.25E+0	[1.51E+0 - 6.45E+1][3]	8.57E+0	[6.64E+0 - 1.03E+1][3]
fenitrothion	Logit	3.57	-2.33	-	-0.33	1	2.86E+0	[1.52E+0 - 5.01E+1]	2.07E+1	[1.34E+1 - 3.50E+1]
flutamide	G.Logit I	15.93	-2.67	55241	-0.44	1	7.81E+0	[3.91E+0 - 1.33E+1]	3.63E+1	[3.08E+1 - 4.70E+1]
linuron	Weibull	3.39	-1.68	-	0.01	1	3.32E+1	[2.19E+1 - 5.07E+1]	1.72E+2	[1.21E+2 - 2.58E+2]

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32 974 [1]IC50, IC10: measured concentration provoking 50% and 10% inhibition of the effect produced by nominal 5 µg/L DHT, respectively.
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34 975 [2]The column "RM" indicates the mathematical regression function as defined by Scholze *et al.* (2001).
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36 976 $\hat{\theta}_1, \hat{\theta}_2, \hat{\theta}_3, \hat{\theta}_{min}$: estimated model parameters, given for concentrations expressed in µg/l (rounded values), θ_{max} were not estimated, but set to 1
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38 977 relating to the mean value of the DHT controls.
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40 978 [3]Values in brackets denote the upper and lower limits of the approximate 95% confidence interval.
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Table 2. Nominal and measured exposure concentrations for both mixture studies.

Components		Nominal mixture concentrations [µg/L]											
		5.71		19.04		57.12		142.8		285.6		571.21	
	Laboratory:	Lancaster	Bergen	Lancaster	Bergen	Lancaster	Bergen	Lancaster	Bergen	Lancaster	Bergen	Lancaster	Bergen
vinclozolin	nominal	0.41		1.36		4.08		10.19		20.38		40.75	
	measured[1]	0.36	0.35	0.67	0.33	1.61	1.16	4.45	2.75	7.20	6.85	16.22	12.92
	recovery [%]	87.3	85.0	49.0	23.9	39.5	28.5	43.7	26.9	35.3	33.6	39.8	31.7
	estimated[2]	0.34	0.30	0.72	0.47	1.67	1.02	3.84	2.52	7.73	5.80	16.60	15.29
fenitrothion	nominal	0.37		1.24		3.72		9.29		18.58		37.16	
	measured	1.30	0.15	1.55	0.45	2.14	1.57	4.46	3.08	6.68	13.43	16.60	23.34
	recovery [%]	349.5	41.0	125.1	36.0	57.6	42.3	48.0	33.2	35.9	72.3	44.7	62.8
	estimated	1.33	0.15	1.47	0.45	2.25	1.42	4.11	4.14	7.53	10.0	15.70	25.6
flutamide	nominal	0.72		2.40		7.21		18.01		36.03		72.06	
	measured	0.86	1.03	2.05	1.78	6.55	5.80	15.60	17.70	23.76	28.65	45.77	45.85
	recovery [%]	120.1	143.2	85.4	74.3	90.9	80.4	86.6	98.3	65.9	79.5	63.5	63.6
	estimated	0.82	0.90	2.37	2.34	6.24	8.00	13.90	13.80	25.39	26.70	46.27	53.02
linuron	nominal	4.21		14.04		42.12		105.31		210.62		421.24	
	measured	4.21	2.31	10.04	5.38	34.27	15.04	95.52	52.05	150.94	103.96	274.20	166.52
	recovery [%]	100.0	54.7	71.5	38.3	81.3	35.7	90.7	49.4	71.7	49.4	65.1	39.5
	estimated	3.87	2.13	12.10	6.08	34.05	17.18	80.31	43.29	153.28	90.19	291.86	193.66

(Footnote for Table 2)

[1]Measured values given for the individual compounds represent their geometric mean concentrations in the mixture determined during a minimum of three independent occasions of the mixture studies.

[2]Estimated concentrations were derived by a second-order regression modeling the relationship between nominal and measured values (see Table S2 for more details)

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6 992 **Table 3.** Observed and predicted spiggin induction (normalized to the means of solvent and DHT controls; see Section 2.7) for a mixture of four
7 993 anti-androgenic compounds.
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	Nominal mixture concentrations [µg/L]					
	5.71	19.04	57.12	142.8	285.6	571.21
observed (mean ± SEM)						
Lancaster	1.02 ± 0.036 (n=15)	0.76 ± 0.047 (n=15)	0.64 ± 0.062 (n=15)	0.59 ± 0.086 (n=11)	0.17 ± 0.035 (n=14)	0.04 ± 0.024 (n=15)
Bergen	0.97 ± 0.023 (n=13)	0.91 ± 0.030 (n=12)	0.69 ± 0.099 (n=13)	0.78 ± 0.035 (n=14)	0.33 ± 0.086 (n=12)	0.02 ± 0.021 (n=15)
predicted by CA (mean with 95% confidence interval)						
Lancaster	0.93 [0.88-0.96]	0.87 [0.82-0.90]	0.65 [0.59-0.70]	0.29 [0.23-0.34]	0.18 [0.13-0.24]	<0.01 [0.0-0.02]
Bergen	0.98 [0.95-.99]	0.95 [0.92-0.97]	0.77 [0.73-0.80]	0.41 [0.35-0.45]	0.10 [0.05-0.14]	<0.01 [0.0-0.02]

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Appendix

The mathematical relationship f between nominal ($c_{nominal}$) and measured concentrations ($c_{measured}$) were modeled for each compound independently by a second order regression after a $\log_{10}$-transformation of the concentration scale, i.e. for $\log_{10}(c_{measured}) = f(\log10(c_{nominal})) + error$ we set

$$f\left(\log_{10}(c_{nominal})\right) = \theta_1 + \theta_2 * \log_{10}(c_{nominal}) + \theta_3 * \left(\log_{10}(c_{nominal})\right)^2 . \qquad (A.1)$$

Here the statistical error term is assumed to be normal distributed with zero mean, and θ_1, θ_2 and θ_3 are the model parameters which have to be estimated. Occasionally we estimated a functional minimum at very low concentrations, although always below the lowest test concentration. To ensure that decreasing nominal concentrations lead always to decreasing and coherent estimates, we restricted estimates only to the strict monotonic ranges of Equation (A.1), and concentrations below this minimum were estimated by a linear function between the corresponding estimate and the zero origin. If $C_{Minimum}$ is the concentration at this global functional minimum, then function f in Equation (A.1) can be expanded to

$$f^*\left(\log_{10}(c_{nominal})\right) = \begin{cases} f\left(\log_{10}(c_{nominal})\right) & \text{for } c_{nominal} \geq C_{Minimum} \\ \dfrac{f\left(\log_{10}(C_{Minimum})\right)}{C_{Minimum}} * c_{nominal} & \text{else} \end{cases} . \qquad (A.2)$$

According to calculus, a global minimum is given only when the term $2*\theta_3$ is positive, and then $C_{Minimum}$ can be calculated as $\log_{10}(C_{Minimum}) = -\theta_2/(2*\theta_3)$.

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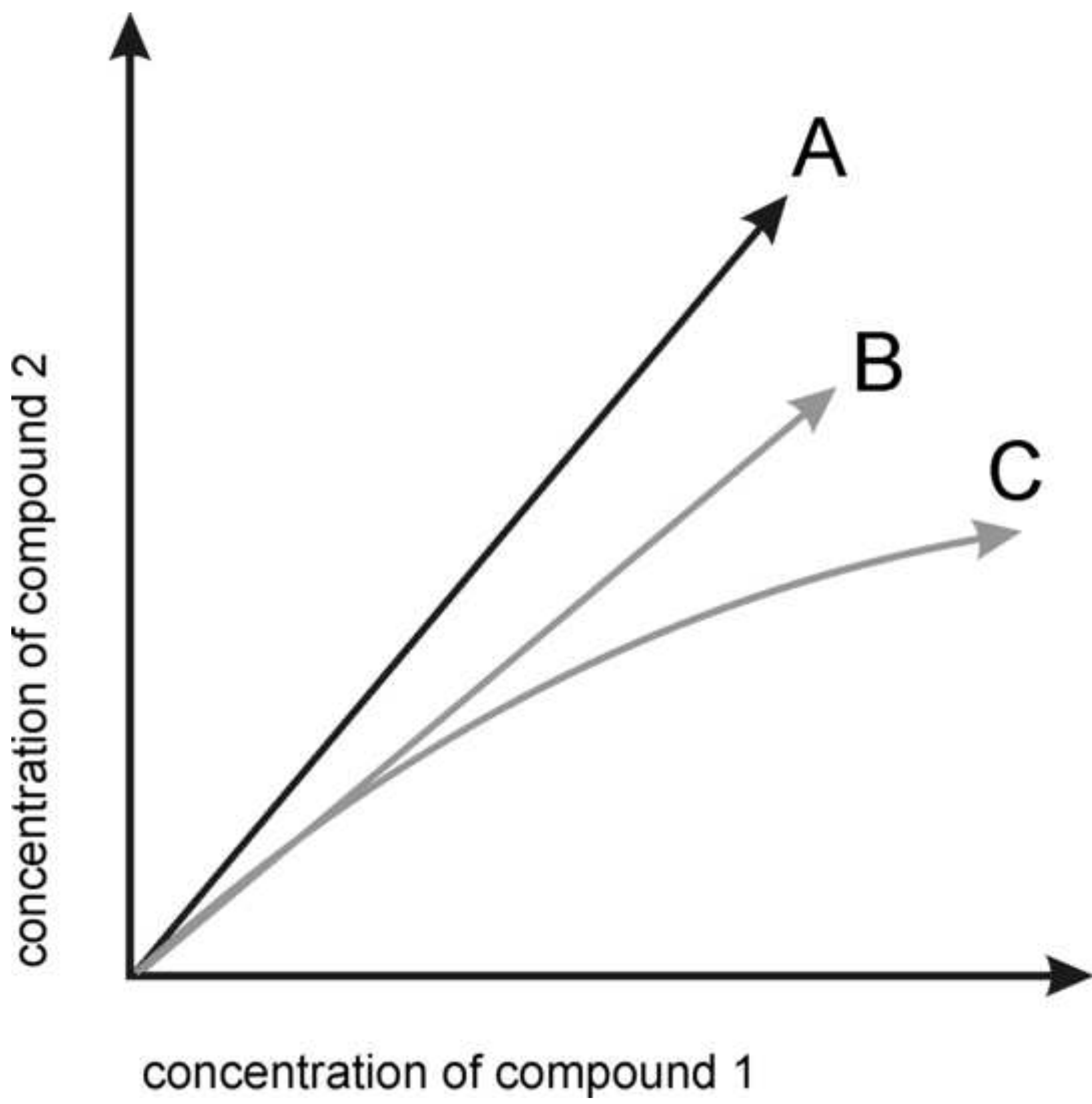

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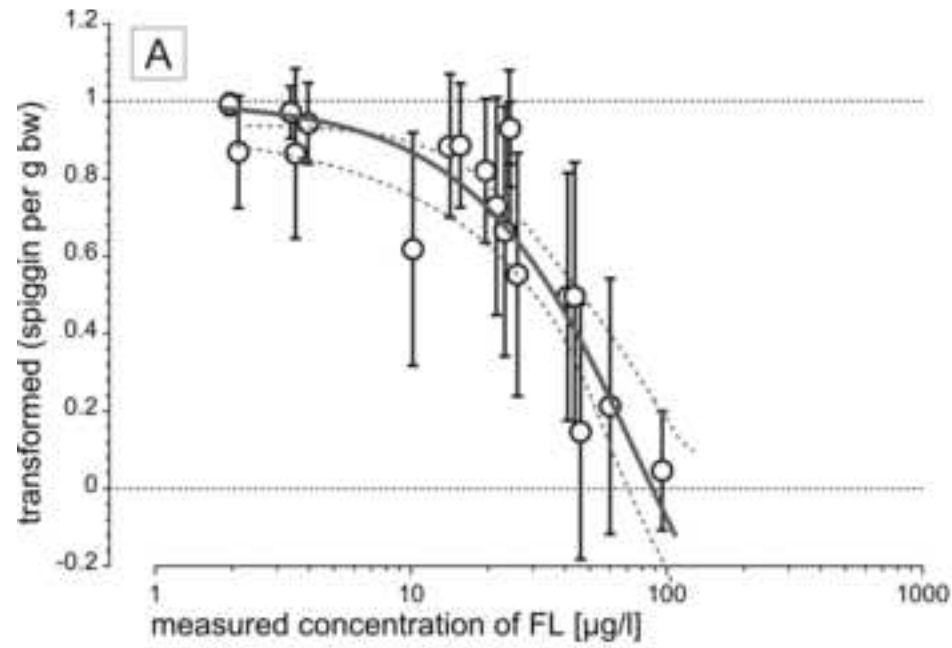

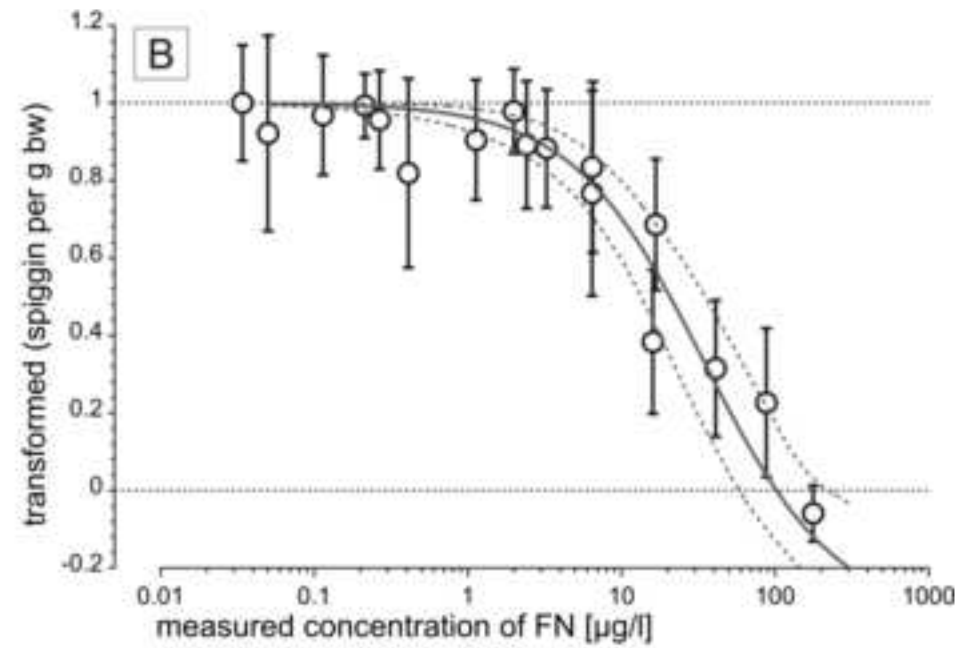

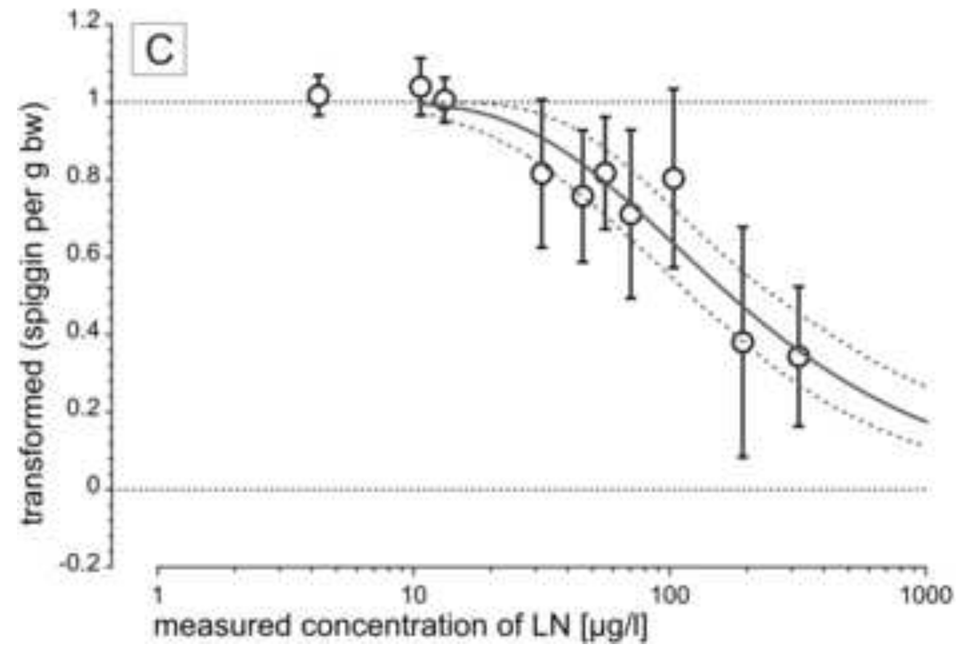

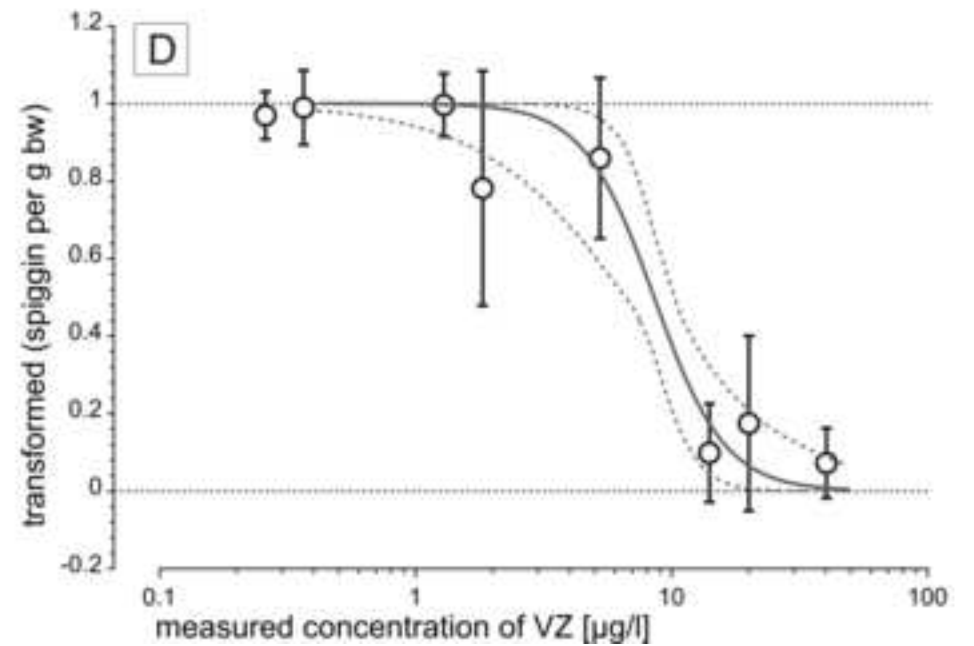

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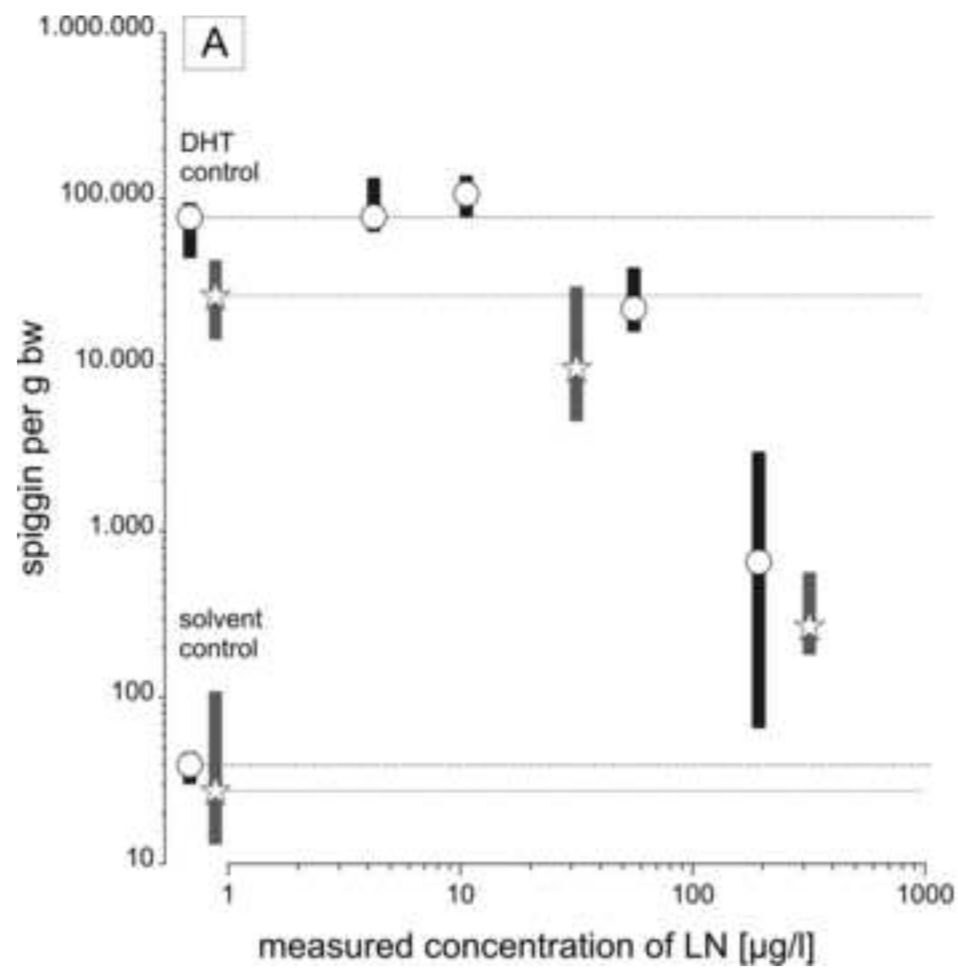

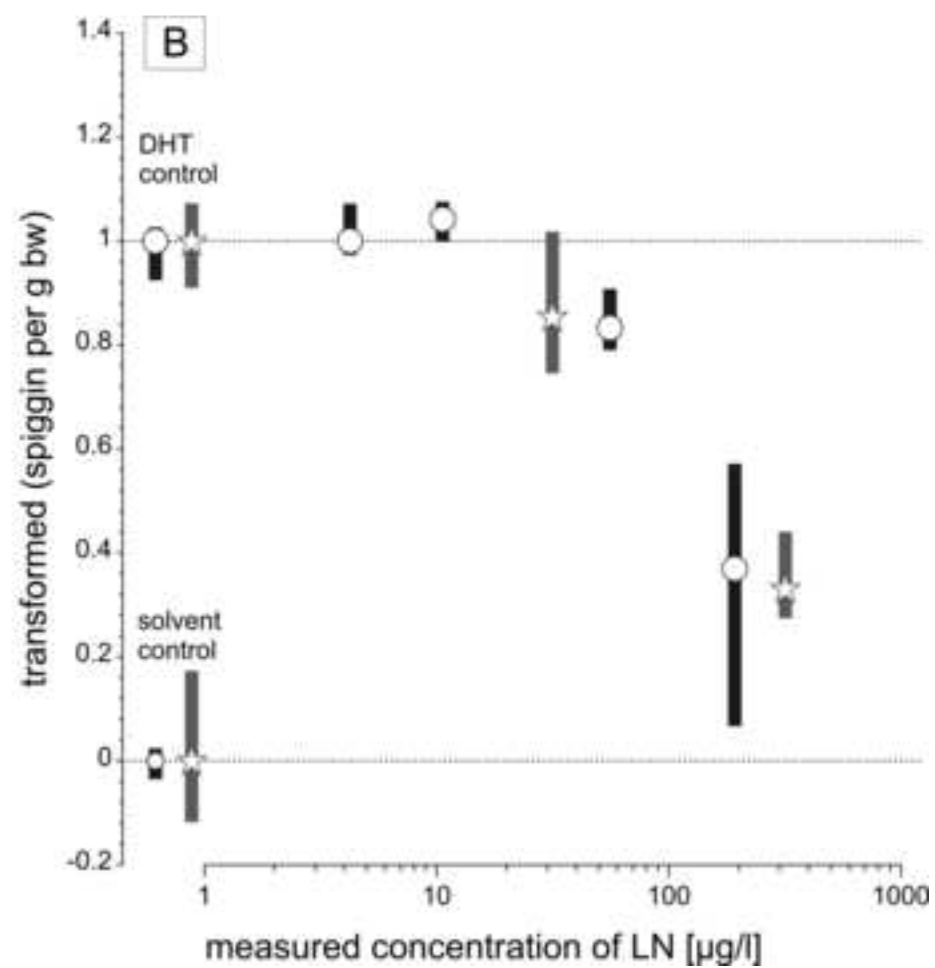

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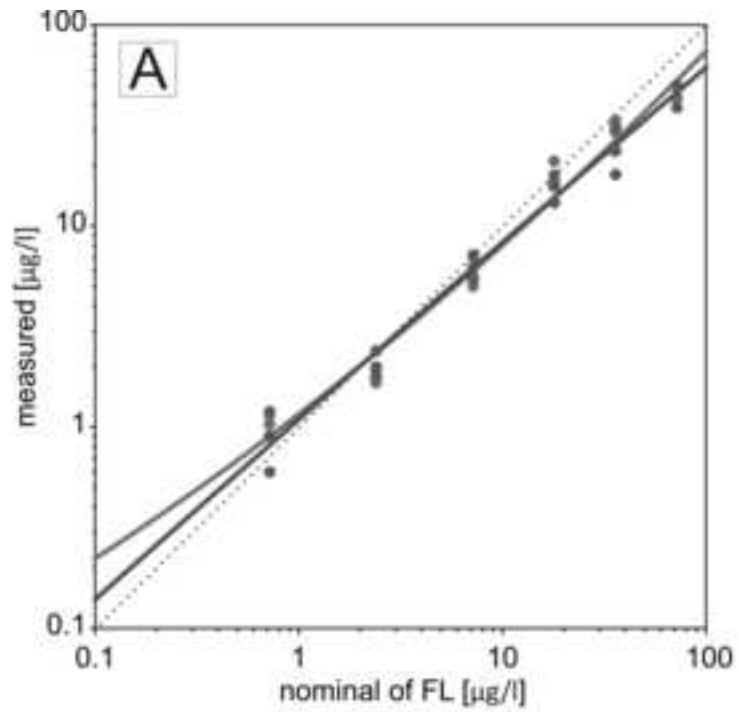

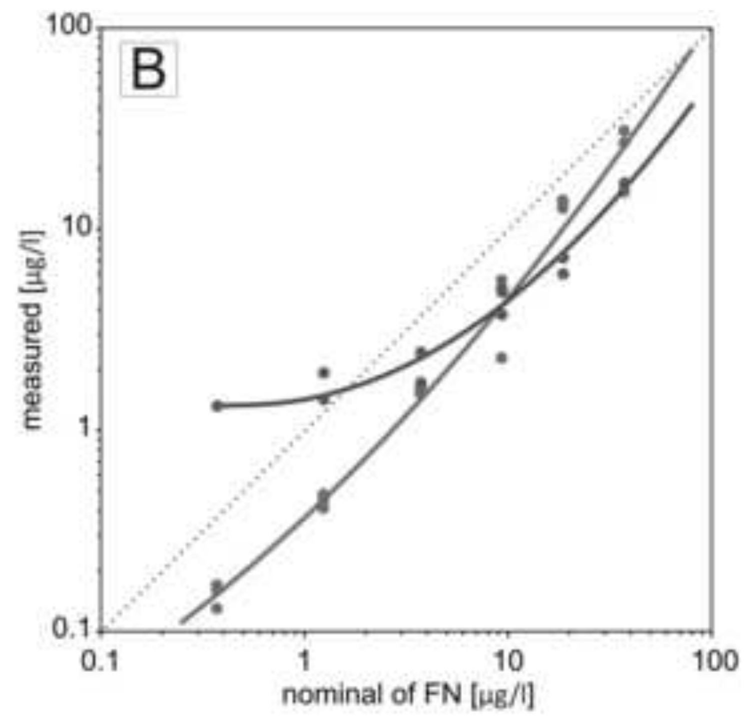

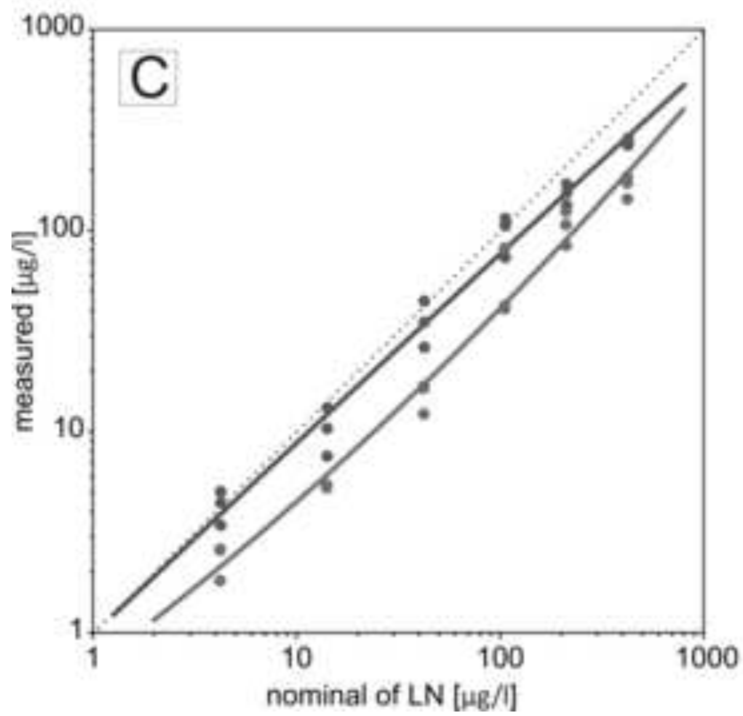

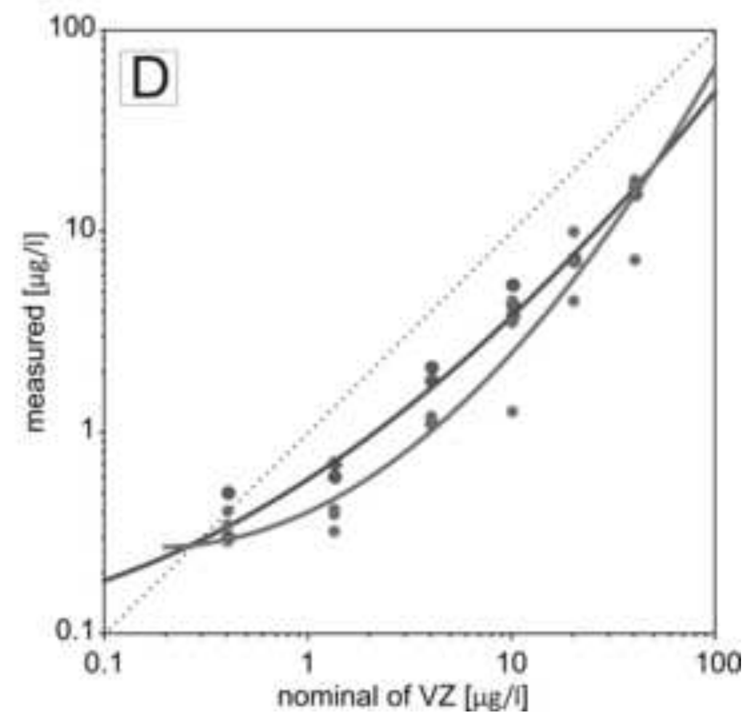

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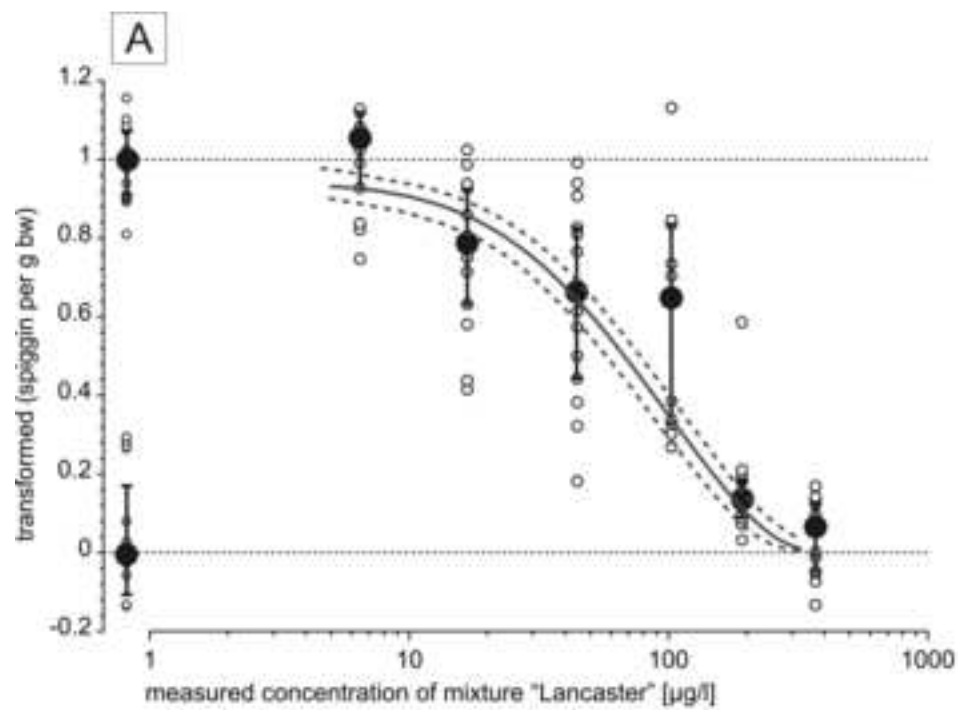

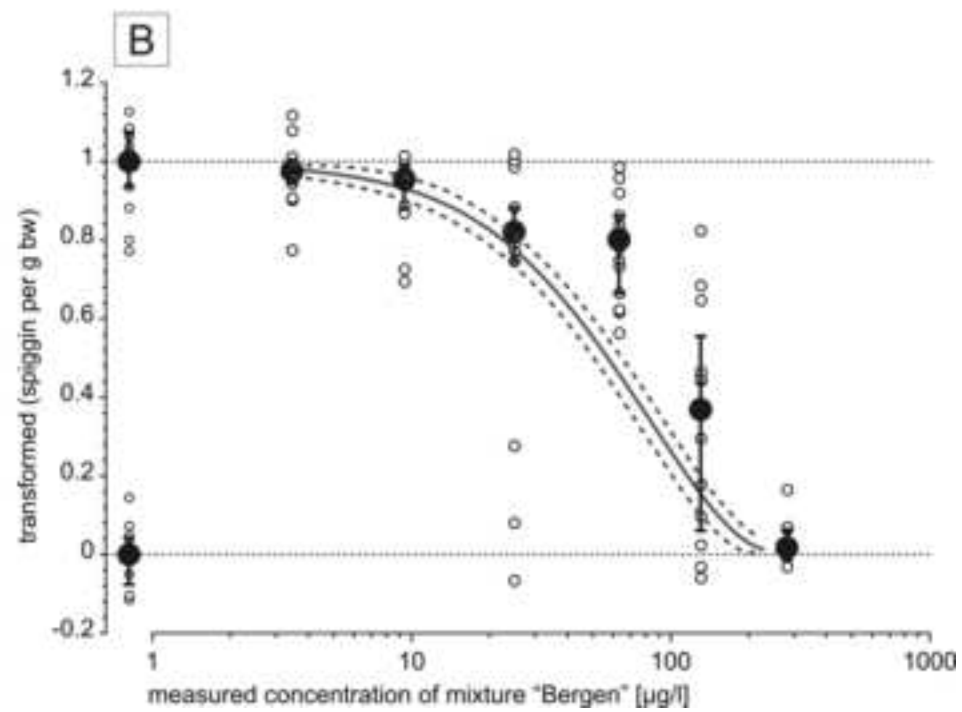

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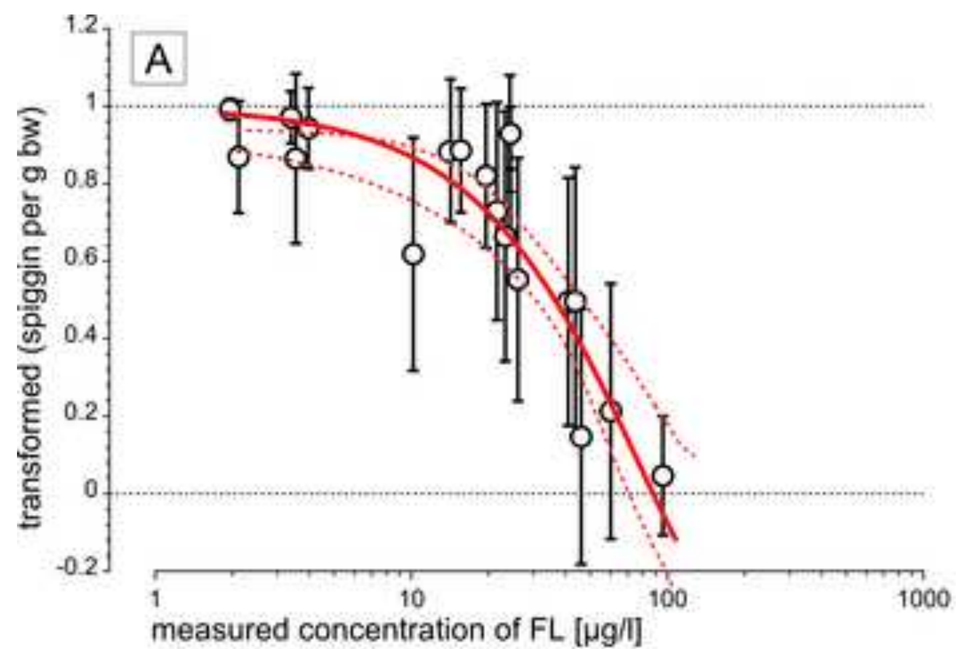

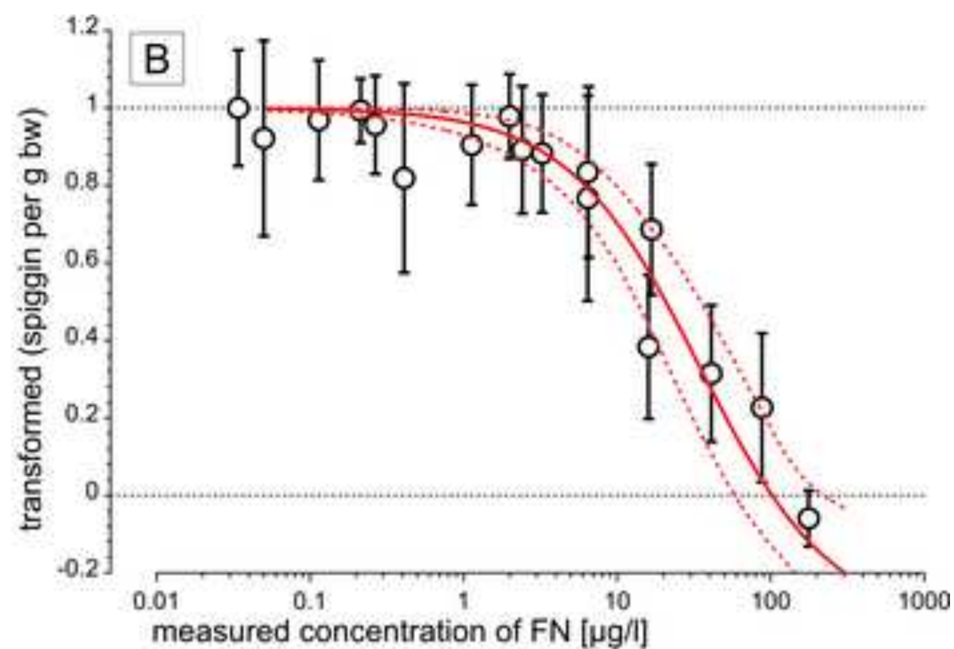

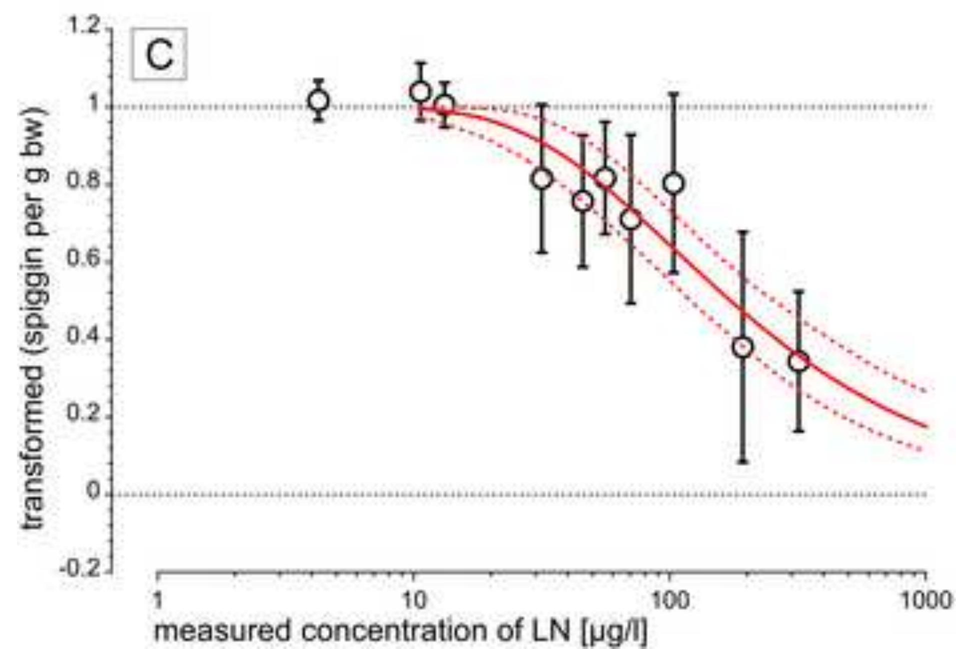

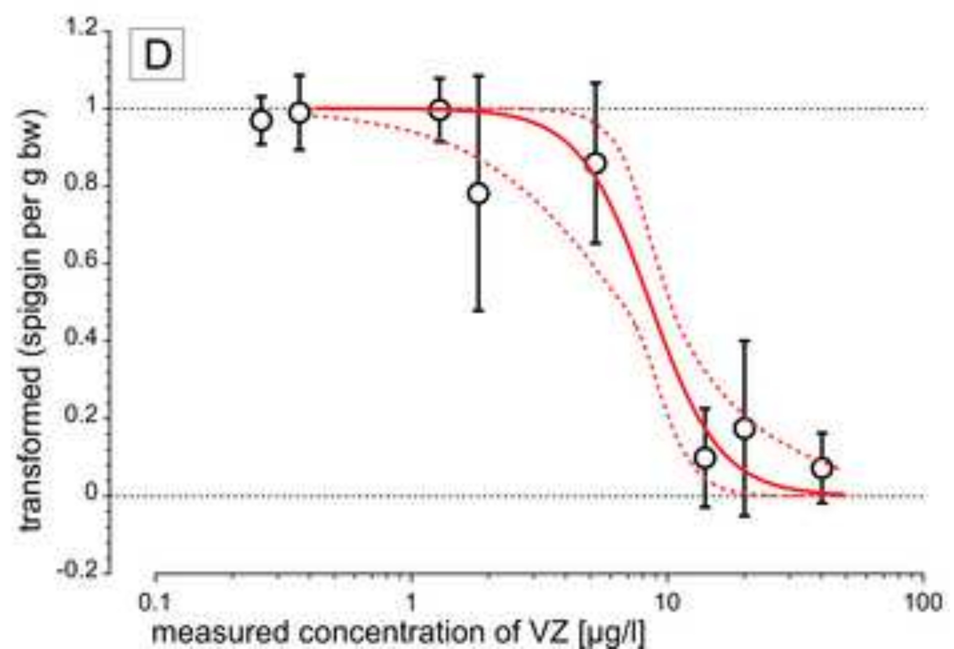

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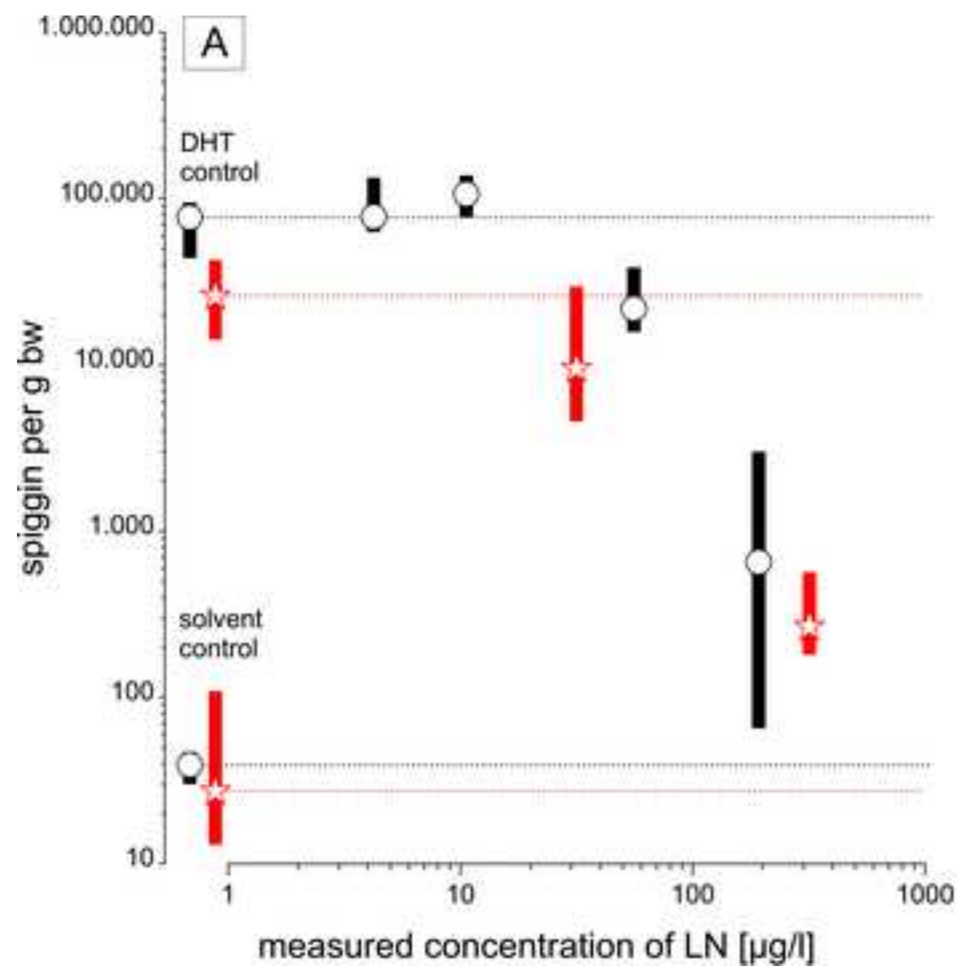

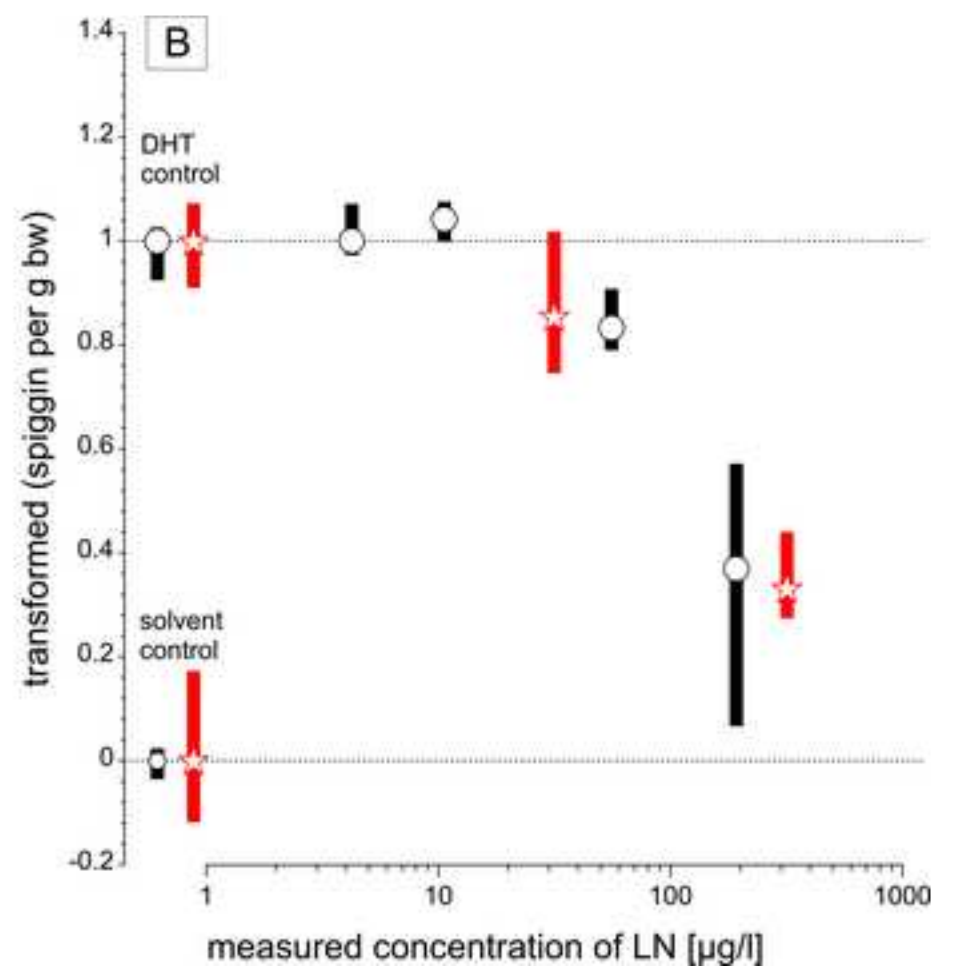

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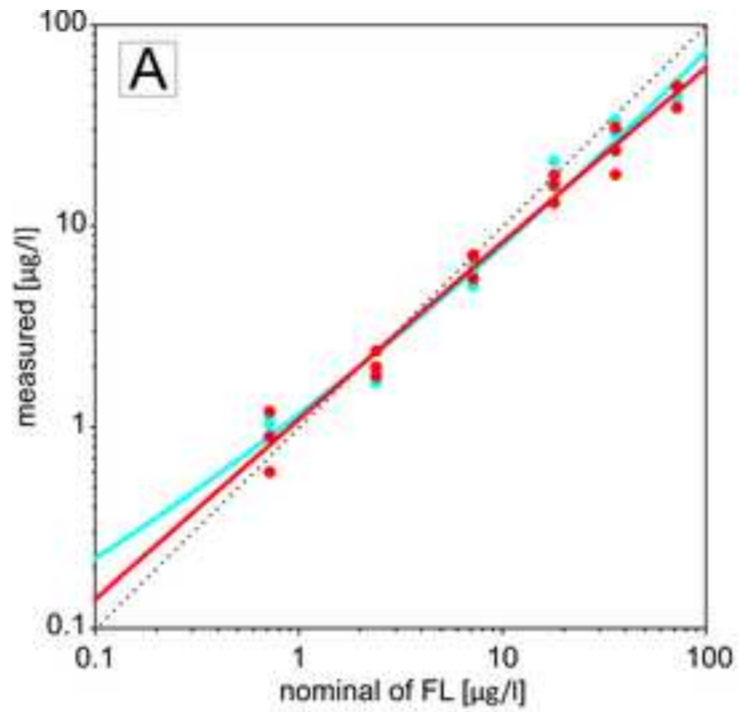

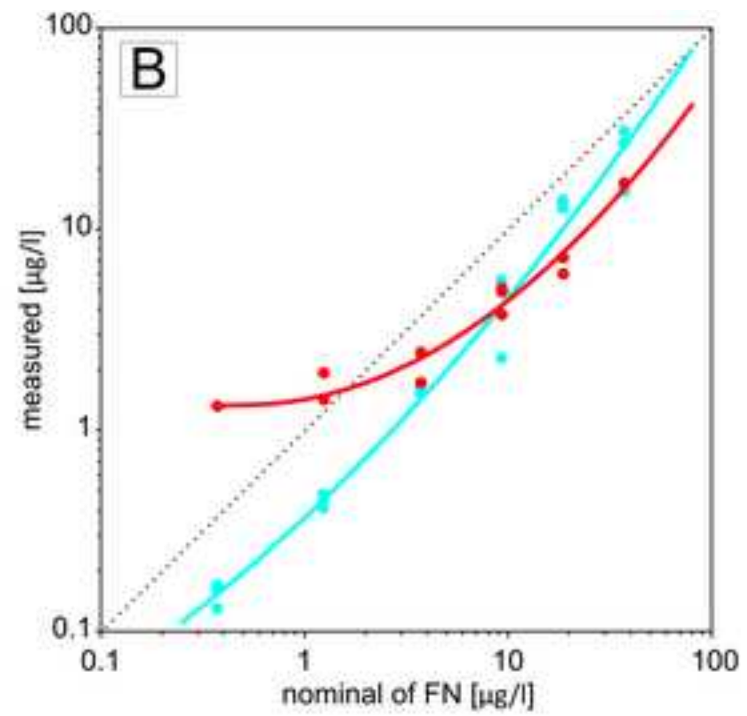

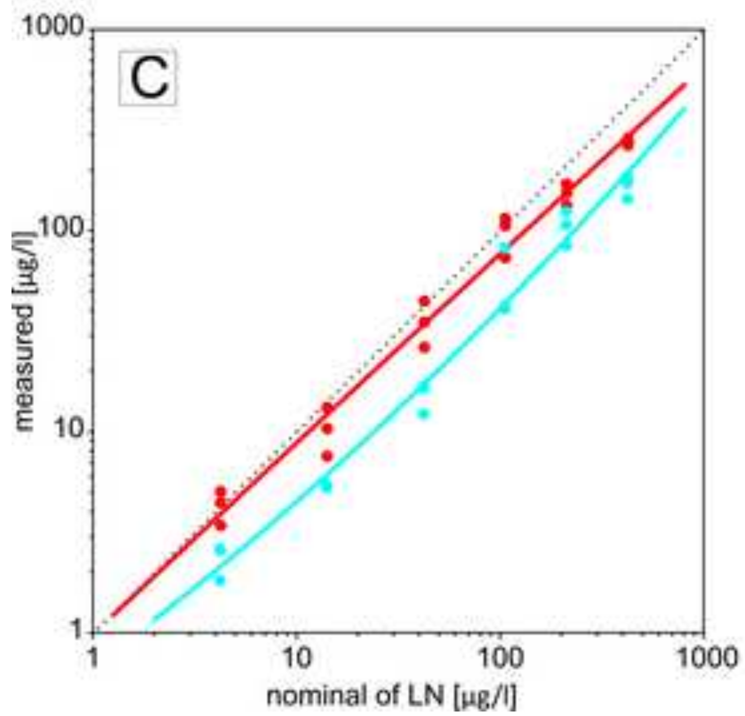

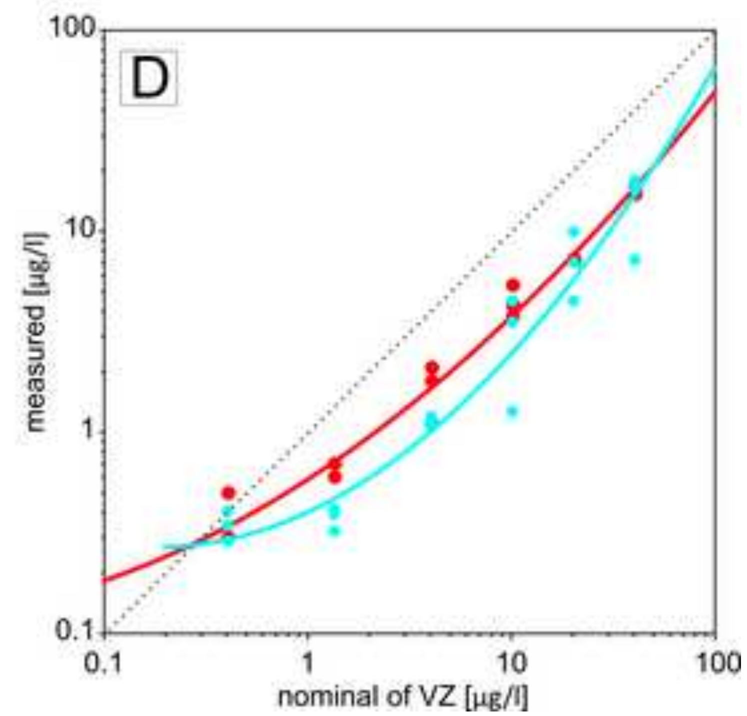

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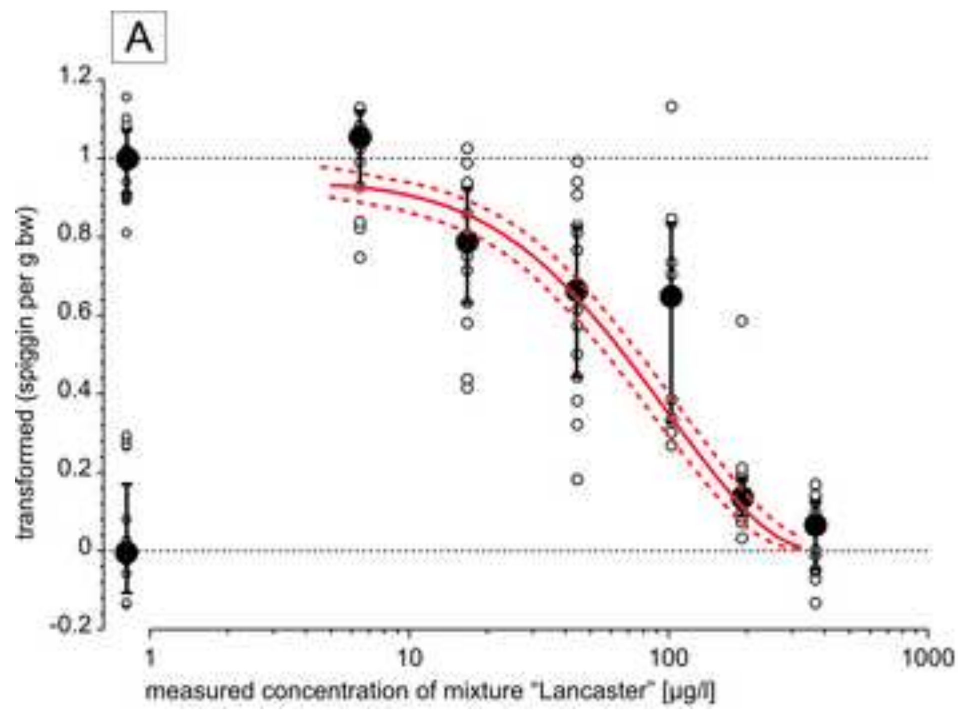

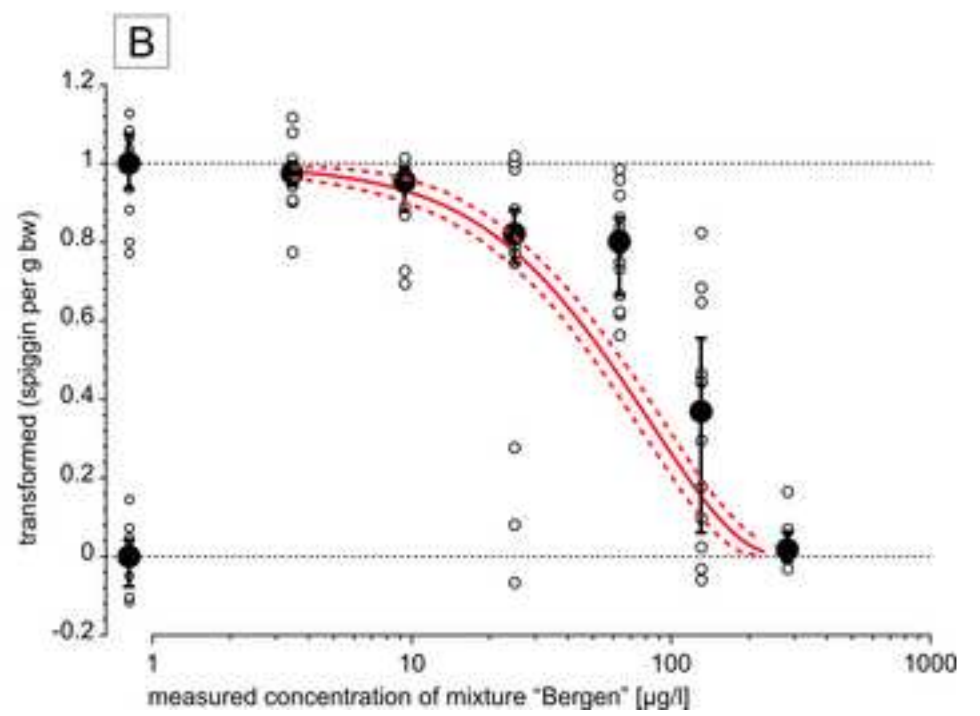

Supplementary Material

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*Highlights (for review)

Highlights

- Spiggin synthesis was stimulated in female sticklebacks by exposure to androgen
- The inhibition of spiggin production by four anti-androgens (AAs) was assessed
- An equipotent mixture of the AAs was formulated using the single agent data
- Concentration addition was used to predict the response of fish to the mixture
- Good agreement between the actual and the predicted outcomes was obtained

Supplementary material

Table S1. Study design of the four component mixture study. FL - flutamide; FN – fenitrothion; LN – linuron; VZ – vinclozolin; DHT - dihydrotestosterone

			Nominal concentrations (µg/L)					
	Treatment	**Tank**	**FL**	**FN**	**LN**	**VZ**	**Mixture**	**DHT**
Controls	Water	A	-	-	-	-	-	-
	Methanol	B	-	-	-	-	-	-
	DHT	C	-	-	-	-	-	5
Single chemical (concentration = IC50)	FL	D	72.06	-	-	-	-	5
	FN	E	-	37.20	-		-	5
	LN	F	-	-	421.24	-		5
	VZ	G	-	-	-	40.75	-	5
Single chemical (concentration = IC50/10)	FL	H	7.20	-	-	-	-	5
	FN	I	-	3.70	-	-	-	5
	LN	J	-	-	42.12	-	-	5
	VZ	K				4.08	-	5
Mixture (dilutions of stock containing all chemicals at respective IC50)	Mix (1.0)	L	72.06	37.16	421.24	40.75	571.21	5
	Mix (0.5)	M	36.03	18.58	210.62	20.38	285.60	5
	Mix (0.25)	N	18.01	9.29	105.31	10.19	142.80	5
	Mix (0.1)	O	7.21	3.72	42.12	4.08	57.12	5
	Mix (0.33)	P	2.40	1.24	14.04	1.36	19.04	5
	Mix (0.01)	Q	0.72	0.37	4.21	0.41	5.71	5

9 **Table S2.** Mathematical relationship between measured and nominal concentration of

10 components within test mixtures

		Concentration response function[1]		
Components	Laboratory	$\hat{\theta}_1$	$\hat{\theta}_2$	$\hat{\theta}_3$
vinclozolin	Lancaster	-0.233*	0.657*	0.153*
	Bergen	-0.395*	0.468*	0.318*
fenitrothion	Lancaster	0.146*	0.185*	0.308*
	Bergen	-0.436*	0.948*	0.145
flutamide	Lancaster	0.038	0.888*	-0.006
	Bergen	0.065	0.778*	0.062
linuron	Lancaster	-0.008	0.957*	-0.006
	Bergen	-0.165?	0.744*	0.072

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12 [1] second-order regression, i.e. $\log_{10}(c_{measured}) = \theta_1 + \theta_2 * \log_{10}(c_{nominal}) + \theta_3 * \log_{10}(c_{nominal})^2$, given

13 for concentrations expressed in µg/l.

14 $\hat{\theta}_1, \hat{\theta}_2, \hat{\theta}_3$ estimated model parameters (rounded values)

15 * indicates statistical significance

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