Combined exposure to low doses of pesticides causes decreased birth weights in rats

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Abstract (148 words)

Decreased birth weight is a common effect of many pesticides in reproductive toxicity studies, but there are no empirical data on how pesticides act in combination on this endpoint. We hypothesized that a mixture of six pesticides (cyromazine, MCPB, pirimicarb, quinoclamine, thiram, and ziram) would decrease birth weight, and that these mixture effects could be predicted by the Dose Addition model. Data for the predictions were obtained from the Draft Assessment Reports of the individual pesticides. A mixture of equi-effective doses of these pesticides was tested in two studies in Wistar rats, showing mixture effects in good agreement with the additivity predictions. Significantly lower birth weights were observed when compounds were present at individual doses below their no-observed adverse effect levels (NOAELs). These results emphasize the need for cumulative risk assessment of pesticides to avoid potentially serious impact of mixed exposure on prenatal development and pregnancy in humans.

Highlights:

- A mixture of six pesticides can cause decreased birth weight at dose levels well below the NOAELs of the individual pesticides
- Risk assessment based on single substances alone underestimates the risk for adverse effects of exposure to several pesticides with common effect
- Dose-addition model was suitable for prediction of mixture effect despite lack of mechanistic knowledge for each compound
- Cumulative risk assessment of pesticides may prevent potentially serious impact of mixed exposure on prenatal development and pregnancy in humans

Introduction

1.1 Background

Human risk assessment of chemicals is largely based on the No Observed Adverse Effect Levels (NOAELs) which are derived from experimental studies of exposure to individual chemicals in animals. However, humans are typically exposed to more than one chemical at a time. For mixtures of endocrine disrupting chemicals including pesticides, there is experimental evidence showing that substantial mixture effects on reproductive development can occur even though each of the individual chemicals is present at doses at or below their NOAELs [1-3]. For example, we have found adverse effects on male sexual development and gestation length after combined developmental exposure of rats to endocrine disrupting pesticides at dose levels below NOAELs of the individual pesticides [4-5].

Low birth weight is a marker for a non-optimal prenatal development in humans and experimental animals. Perturbations to this environment can have detrimental effects on the foetus and lead to persistent pathological consequences later in life [6], manifested in the "Barker hypothesis" on developmental origin of adult disease [7]. Low birth weight is generally considered as a predictor for increased risk of several diseases later in life, including obesity and type 2 diabetes [8]. It is suggested that this is caused by fetal programming, i.e. physiological adaptations in response to changes in the environment to prepare for postnatal life [9]. A recent Danish study on children of women who worked in green houses and thus were likely exposed to mixtures of pesticides has found lower birth weights in exposed children, but increased body fat accumulation from birth to school age [10].

Reduced birth weight is a common effect for many pesticides in experimental studies, but there is no empirical evidence on combined developmental exposure to low doses of pesticides that can decrease birth weight. The so-called component-based mixture approach for the toxicological assessment of pesticides anticipates the effects of a mixture on the basis of the toxicity of its components, and therefore allows quantitative predictions of mixture toxicities, without the need to test different mixture ratios at different mixture doses. Different concepts exist for the calculation of mixture effects on the basis of the toxicity of its components, and there is no scientifically robust data available for evaluating potential mixture effects on this endpoint and for selecting the best model for predicting the mixture effects. This study aimed to test the hypothesis that a mixture of six pesticides (cyromazine, 4-(4-chloro-2-methylphenoxy) butanoic acid (MCPB), pirimicarb, quinoclamine, thiram, and ziram) can cause decreased birth weight at doses below their individual NOAELs, and that these mixture effects can be predicted by the Dose Addition model.

1.2. Rationale for mixture study

Decreased birth weight after exposure to pesticides is often reported in regulatory studies submitted for approval of pesticides. It is a developmental toxicity effect that may be induced via different and in most cases unknown mechanisms of action. This complicates the choice of the best possible component-based mixture model, namely Independent Action (IA) and Dose Addition (DA). Both models rely on an additivity assumption, which is based on the expectation that all chemicals in the mixture exert their effects without influencing each other's action. DA is based on the idea that all components in the mixture behave as if they are simple dilutions of one another [11]. In contrast, IA is commonly thought to apply in cases where the compound exerts their effects through strictly independent, i.e. dissimilar mechanisms [12]. These competing toxicological assumptions are also reflected in their different data demands: to apply both models for predicting a mixture dose causing a 10% decrease in birth weight (ED_{10}), DA would require from all mixture compounds knowledge about their individual ED_{10}s, a data scenario that is manageable from a risk

assessment point of view. In contrast, IA would demand from all compounds effect estimates smaller than a 10% effect change, and the more compounds are present in a mixture, the lower the individual effect estimates become that are required as input values for the calculation of an IA mixture response. These experimental demands for IA are beyond what is technically achievable with the number of animals per dose group normally used in regulatory toxicity studies, and were not achievable in the current study where information about the individual compounds were derived not directly from experimental data, but rather from reports with summarising data descriptors. Consequently, we considered only DA as an option to predict the responses from a mixture of pesticides on birth weight.
The use of DA as a pragmatic approximation for the prediction of mixture effects of also non-similarly acting chemicals seems to be justified, as there is no current empirical example of a situation in which the concept of IA provides an accurate prediction that is also more conservative (i.e. cautious) than DA, supporting the use of DA as a conservative default in cumulative risk assessment [13-14]. Furthermore, an analysis of the quantitative difference between predictions based on DA or IA suggested that the differences that might be expected in practice in this study are small.

For this mixture study, the following steps were applied to test the hypothesis that the joint effects of the pesticides on birth weight can be predicted by DA:

1) Selection of six pesticides for which clear decreases of pup birth weight has been reported at dose ranges without any signs of maternal or other toxicity. All data were obtained from regulatory studies (Draft Assessment Reports).
2) Dose-response data from each pesticide were analysed by nonlinear regression modelling.
3) For a mixture composed of pesticides in equi-effective doses, the expected mixture responses were predicted by DA, and then used for the experimental planning of the mixture studies.
4) Two mixture studies (range-finding and main study) were performed, both in a fixed-ratio design. The range-finding study was conducted to identify and avoid mixture doses in the main study that would cause marked maternal toxicity or marked effects on pregnancy parameters, such as litter size and pup survival.
5) Comparison of the predicted and observed mixture effects and evaluation of DA as tool for the cumulative risk assessment of pesticides.

2 **Materials and methods**

2.1 Selection of pesticides

Relevant data on birth weight were available from prenatal developmental toxicity studies [15] or one- or two generation studies [16-17] as described in risk assessment reports for regulatory use. This data selection was considered ideal for investigating the application of DA for regulatory purposes while limiting the number of test animals. In an evaluation based on the Draft Assessment Reports (DARs) of 224 approved pesticides in EU around 175 caused decreased fetal or birth weight [18]. Pesticides were selected for inclusion in the mixture studies only if their reported decreased birth weight was observed at dose ranges without clear signs of maternal toxicity. This was done to avoid combination toxicity on the dams or reductions in the number of offspring. It was kept in mind that decreased maternal weight gain during pregnancy can often be listed as indicative for maternal toxicity, but in reality may be due to decreased fetal weight. Other reported toxicity in dams, e.g. liver toxicity, was also avoided. Pesticides showing effects on birth weight at more than one dose with a clear dose-response relationship were prioritized to improve the

robustness of regression modelling. The six pesticides selected for the mixture testing were cyromazine, MCPB, pirimicarb, quinoclamine, thiram, and ziram.

2.2 Test compounds

The vehicle used were corn oil (product no. C8267-2.5L from Sigma/Aldrich. The test compounds were Cyromazine (97%) CAS: 66215-27-8 (product no.:551295-25G from Sigma/Aldrich), MCPB (99.8%) CAS: 94-81-5 (product no: 36145-20G from Sigma/Fluka), Pirimicarb (98.7%) CAS: 23103-98-2 (product no: 45627-15G from Sigma/Fluka), Qinoclamine (99.9%) CAS: 2797-51-5 (product no: 32719-3G from Sigma/Fluka), Thiram (99.9%) CAS: 137-26-8 (product no 45689-5G from Sigma/Fluka), Ziram (98.2%) CAS: 137-30-4 (product no: 45708-5G from Sigma/Fluka).
The same batch of substances was used in both the range-finding and the main study.

2.3 Dose-response modelling and benchmark dose estimation

Only mean values for birth weight changes have been reported, consequently only mean birth weight changes were used for data analysis. When data were available from more than one study on the same pesticide, the reported absolute weight values data were normalized by their control mean to a relative effect scale and pooled. If results were reported for both genders separately, an overall mean from both were used. Nonlinear regression analyses were performed using the best-fit approach [19], i.e. a variety of nonlinear regression functions were fitted independently to the same data set and the best-fitting model was selected using a statistical goodness-of-fit criterion. Dose-response data and regression curves are shown in Figure 1. Five percent effect doses (ED_5) were derived from these regression fits in order to establish the mixture ratio. This effect change was considered as benchmark response for birth weight, and consequently the corresponding benchmark dose (BMD_5) is identical to the estimated ED_5. Both terms are used synonymously here.

2.4. Mixture study design and predictions

To identify possible deviations from the DA prediction we favoured a balanced mixture design, with a mixture composed of pesticides in equi-effective doses, and then tested in dilutions without changing the relative mixture composition (fixed-ratio mixture design). A common effect level was operationalised as a five percent change in birth weight, and the sum of the corresponding effect doses of the individual pesticides was selected as maximal mixture dose (Mix-100%). A five percent decrease in birth weight was considered close to or below the statistical detection limits of typical regulatory study designs, and therefore we expected the Lowest Observed Adverse Effect Level (LOAEL) reported for this endpoint slightly above our estimated BMD_5. More importantly for the experimental design, it allowed us to choose a dilution of the maximal mixture dose such that each compound was present at doses below its individual NOAELs. The doses for the mixture testing were selected according to their mixture responses expected by DA, with the main focus of the range-finding study to identify the highest possible mixture dose for the main study that produces no indications for maternal toxicity and reduced pup survival. This explains the different mixture doses in the two studies (Table 1), as the outcomes from the range-finding study were mainly used to refine the dose selection. Importantly, we never tested the maximal mixture dose (Mix-100%) as we expected at these dose ranges an additive maternal toxicity. Instead, 75% of the maximal mixture dose was selected as highest dose in the range-finding study (Mix-75%), which was lowered to 37.5% in the main study (Mix-37.5%) (Table 1).
Under the assumption of dose additive combination effects, a dose-response relationship for the mixture was predicted using the best-fit dose-response regression curves of the individual pesticides. Equation 1 allows

the calculation of any effect dose of a mixture under the hypothesis of dose additivity, provided the dose-response functions of all mixture components and the mixture ratio are known

$$EDx_{mixture} = \left(\frac{p_1}{EDx_1} + \frac{p_2}{EDx_2} + \frac{p_3}{EDx_3} + \frac{p_4}{EDx_4} + \frac{p_5}{EDx_5} + \frac{p_6}{EDx_6} \right) \quad (1)$$

EDx_1, ... , EDx_6 are the effect doses of the six pesticides that on their own produce the same quantitative effect x as the mixture, and p_1, ... ,p_6 are the relative proportions of the corresponding individual doses present in the total mixture dose. It should be noted that the predicted mixture effects are also subject to a statistical uncertainty, which we could not quantify as the statistical uncertainty of the dose-response data of the individual pesticides as well as other potential sources of variation (e.g. inter-lab variability) were unknown to us.

2.5 Animals and exposure

Animal experiments were carried out at the Technical University of Denmark (DTU) National Food Institute (Mørkhøj, Denmark) facilities. Ethical approval was given by the Danish Animal Experiments Inspectorate. The authorization number given is 2012-15-2934-00089 C4. The experiments were overseen by the National Food Institutes in-house Animal Welfare Committee for animal care and use.

In the *range-finding study,* four groups of 10 time-mated nulliparous, young adult animals (HanTac:WH, Taconic Europe, Ejby, Denmark) were given daily gavage doses of the vehicle (corn oil, control group) or one of three different pesticide mixture doses from gestation day (GD) 7 to pup day (PD) 16. Thus, offspring were indirectly dosed via placenta and through lactation. Mixture doses were 0, 70, 210 and 420 mg/kg/day, corresponding to 0%, 12.5%, 37.5% and 75% of the maximal mixture dose (Mix-100%). However, due to symptoms of maternal toxicity at highest mixture dose (420 mg/kg/day, Mix-75%) after 2 days of exposure (GD7-GD8), this administered dose was reduced by 1/3 to 140 mg/kg/day (Mix-25%) from GD 9 onwards. Thus the originally highest mixture exposure was changed to a median exposure, and as consequence the original median dose group Mix-37.5% became the highest mixture dose group in the range-finding study from GD 9 onwards. The doses of the individual pesticides and their combinations are shown in Table 1, with treatment groups labelled as control, Mix-12.5%, Mix-25% (or Mix-75% on GD 7-8) and Mix-37.5%.

In the *main study*, four groups of 22 time-mated nulliparous, young adult animals (HanTac:WH, Taconic Europe, Ejby, Denmark) received from gestation day (GD) 7 to pup day (PD) 16 via daily gavage either vehicle (corn oil, control group) or one of the three tested mixture doses: 28, 90 and 210 mg/kg/day (Table 1). These dose groups correspond to 5%, 16% and 37.5% of the maximal mixture dose (Mix-100%), and are labelled as Mix-5%, Mix-16% and Mix-37.5%.

On the day after arrival (GD 4) time-mated dams were pseudo-randomly distributed into four groups of animals with similar body weight (bw) distributions. Mixtures and vehicle were administered by oral gavage with a stainless steel probe 1.2 x 80 mm (Scanbur, Karlslunde, Denmark). All doses were given in vehicle (2 ml/kg) via oral gavage at the beginning of the animals' active period from 8 to 11 a.m. The solutions were prepared by a technician just before the study was performed as a stock solution and during exposure period they were stored in the animal rooms. Body weights were recorded daily during the dosing period to ensure correct dose according to body weight.

The animals were housed in pairs until GD 17 and alone thereafter under standard conditions in semi-transparent polysulfone (PSU) type III cages (PSU 80-1291HOOSU Type III, Techniplast, Buguggiate, Italy) (15x27x43 cm) with Aspen wood chip bedding (Tapvei, Gentofte, Denmark), Enviro Dri nesting material (Brogaarden, Lynge, Denmark) and Tapvei Arcade 17 (Aspen wood) shelters (Brogaarden). The cages were situated in an animal room with controlled environmental conditions (12 h light-dark cycles with light

starting at 9 p.m., light intensity 500 lux, temperature 21 ± 2°C, humidity 50% ± 5%, ventilation 8 air changes per h). A complete rodent diet for growing animals ALTROMIN 1314 (Soy- and alfalfa-free ALTROMIN GmbH, Lage, Germany) and acidified tap water (to prevent microbial growth) was provided ad libitum.
We designated the day when a vaginal plug was detectable as gestation day (GD) 1 and the expected day of delivery, GD 23 as pup day (PD) 1.

2.6 Pregnancy and postnatal development
The dams were inspected twice a day for general toxicity including changes in clinical appearance (e.g. sedation and tremor). Body weights were recorded on GD 4 and daily during the dosing period to monitor a decrease or increase in weight gain. Dam body and liver weights were recorded at sacrifice on PD 16 in the range-finding study and on PD 24 in the main study. Gestation length and number of implantation scars in the uterus was also recorded.
The weights of dams and the birth weight for individual pups were recorded after delivery in all the pregnant animals. The pups were counted, sexed, and checked for anomalies. Pups found dead were macroscopically investigated for changes when possible. The body weight of offspring was recorded on PD 6 and 14 and in the main study also on PD 24.

2.7 Statistics
For all analyses, the litter was the statistical unit. Data from continuous endpoints were examined for normal distribution and homogeneity of variance, and if relevant, transformed. When more than one pup from each litter was examined, statistical analyses were adjusted using litter as an independent, random and nested factor. Statistical significance were assessed using multiple contrast tests (Dunnett contrasts, global error rate $\alpha = 5\%$, two-sided) [20]. These tests were chosen as they are already implemented in the SAS procedure PROC GENMOD which was used for all statistical analysis (SAS Institute Inc., Cary, NC).

3 Results

3.1 Effects during gestation, i.e. before birth

Range-finding study. After two days of dosing, symptoms of toxic effects (impaired breathing) and weight loss were observed at highest mixture dose (Mix-75%). Therefore, this mixture dose were reduced by 1/3 (Mix-25%) and animals were administered by this dose from GD 9 onwards. However, one dam from this dose group did not recover from its toxic symptoms and therefore was removed from the study. One dam from Mix-37.5% was also excluded due to similar symptoms at GD 18.
Due to signs of early parturition on GD 21 (rat dams give birth on GD22-23), caesarean sections were performed on two animals, one selected from the control group and one from the median dose group (Mix-37.5%). As these two incidences occurred in a control and an exposed dam, we consider them as random findings and not related to the exposure.
The body weight gain from GD 7 to GD 21 in the animals giving birth was similar to control values in the group dosed with Mix-12.5%, but appeared to be decreased in the groups dosed with Mix-25% and Mix-37.5%, however these differences were not statistically significant (Table 2).

Main study. No clinical signs of toxicity were observed in the animals before GD 21. Due to signs of early parturition on GD caesarean sections were performed on two animals, both from Mix-5% group. As this occurred only in the lowest dose group, we consider these findings not related to the exposure.
Dams giving successful birth from the dose groups Mix-16% and Mix-37.5% had significantly decreased body weight gains from GD 7 to GD 21 compared to the controls (Table 2), with both body weight gain reductions mainly detected in the first week of exposure (GD 7-14, data not shown).

3.2 Pregnancy data and maternal body and liver weights after giving birth

Range-finding study (Table 2). The maternal body weight gain from GD 7 to PD 1 appeared decreased in the groups exposed to Mix-25% and Mix-37.5%, but the difference was only statistically significant in the Mix-37.5% group ($p=0.003$). Gestational length, number of live born pups per litter and the mortality of foetuses and pups were unaffected by the exposure. On PD 16, the body weights of the dams were similar among groups, whereas the absolute and relative liver weight was significantly increased in the group exposed to the highest dose of Mix-37.5% (data not shown).

Main study (Table 2). Gestational length, number of live born pups per litter and the mortality of fetuses and pups were unaffected by the exposure. The maternal body weight gain from GD7 to PD 1 was significantly decreased in the groups exposed to Mix-16% and Mix-37.5% ($p<0.0001$). The maternal body weight gain from PD 1 to PD 24 appeared increased in the groups exposed to Mix-16% and Mix-37.5%, but the difference was only statistically significant in the Mix-37.5% group (data not shown, $p=0.009$). The sectioning of the dams was on PD 24, i.e. 8 days after the end of exposure on PD 16, and no effects on dam liver weights were seen (data not shown).

3.3 Birth weight and pup weight

Range-finding study (Table 2 and Figure 2). The average birth weight of the pups followed a decreasing dose-response trend, with 7-8% lower than control weights at highest mixture dose (Mix-37.5%) and 4% and 5% lower at lowest (Mix-12.5%) and median mixture dose (Mix-25%), respectively. However, probably due to the small sample sizes and thus low statistical power these differences could not identified as statistically significant in any of the dose groups (Table 2). Pup body weights on PDs 6 and 14 were similar to control values in the groups exposed to Mix-12.5% and Mix-25%, but appeared to be 6-7% lower in the group exposed to Mix-37.5%. However, the difference was not statistically significant.

Main study (Table 2 and Figure 2). The birth weights of the exposed pups were dose-dependently decreased compared to the birth weights of the control animals. In the Mix-37.5% group, the average birth weight was 15% lower than control weights, and decreases were 6% and 9% in the groups exposed to Mix-5% and Mix-16%, respectively. The differences were statistically significant in all of the dose groups.
Pup body weights on PD 6 were similarly decreased as the birth weights, i.e. decreases were 6%, 8% and 19% in the groups exposed to Mix-5%, Mix-16% and Mix-37.5%, respectively. However, the differences were only statistically significant at median and highest dose.
Pup body weights on PDs 14 and 24 were lower than controls at all doses, with a 6-10% reduction at lowest (Mix-5%), 7-8% reduction at median (Mix-16%) and 10-14% reduction at highest mixture dose. However, the differences were only statistically significant at highest dose.

Mixture effect on birth weight. The observed effects of the mixture on mean birth weight as well as the predicted mixture effects based on dose-addition for both the range-finding and the main study are shown in Figure 2. The observed results at all three doses in the main study appear to be slightly more marked than predicted. However, these small differences are not considered as statistically significant, as the predictions fall into the 95% confidence belt of the observed mean response. Thus, DA predicted the outcomes from both studies reasonable well.

In Table 3, the NOAEL and LOAEL for birth weight reduction from DARs are presented for each pesticide, together with our estimated BMD_5. These toxicity descriptors are compared with the individual doses of the pesticides that are present in the lowest mixture dose (Mix-5%), with each pesticide dose here being equivalent to 5% of its BMD_5 (Mix-100% would be the sum of all BMD_5s). As this mixture dose is the lowest test dose at which we could identify a statistically significant decrease in birth weight, we consider it as the LOAEL of our designed mixture ($LOAEL_{mix}$). Comparing the NOAELs or LOAELs of the pesticides with the individual doses of the $LOAEL_{mix}$ demonstrates clearly that pesticide doses well below their individual NOAELs can add up to a marked combination effect.

Table 4 shows for each pesticide the LOAELs and NOAELs reported for effects on dams, together with their doses present at lowest mixture dose (Mix-5%, $LOAEL_{mix}$). As for birth weight, these comparisons clearly show that a significant mixture response can even be expected if all individual compounds are present in doses well below their NOAELs.

4 Discussion

This project explored the hypothesis that combined developmental exposure to pesticides can cause decreased birth weight at dose levels below the NOAELs of the single pesticides. Further, we investigated if the observed mixture effects could be predicted by Dose Addition (DA).

4.1 Mixture effect on birth weight

As presented in Table 3, mixture exposure leads to effects at markedly lower doses than with exposure to the single substances. Doses of the single pesticides included in this mixture dose are approximately 20-40 times lower than the LOAELs for effect on birth weight when the pesticides are given alone, and if compared to the individual NOAELs they are approximately 5-17 times lower. Thus, mixture effects occur at dose levels at which the individual pesticides has earlier been shown to cause no effect on birth weight, i.e. clearly below the observed NOAELs for this effect (Table 3). To our knowledge, this is the first study to describe mixture effects of pesticides on birth weight in experimental animals.

A study in humans estimated the effects of exposure to single pollutants and mixtures on birth weight in 248 mother-child pairs [21]. Several chemicals (e.g. perfluorinated compounds and methylmercury) were measured in either cord blood, maternal blood, in maternal hair or in cord plasma. Daily exposure to particulate matter was modelled and averaged over the duration of gestation. The study showed that birth weight was consistently inversely associated with exposure to pollutant mixtures. Chemicals not showing significant associations at single pollutant level contributed to stronger effects when analysed as mixtures [21]. Mixture effects on reproductive development have been seen in studies where experimental animals have been exposed to several endocrine disrupters simultaneously even though each of the individual substances were present at low, non-adverse doses [1,3-5]. We have earlier shown that a mixture of five fungicides (procymidone, mancozeb, epoxyconazole, tebuconazole and prochloraz) induced severe effects on reproductive development manifested as nipple retention and genital malformations in male rat offspring at doses where the individual pesticides appear to induce no such effects [4-5]. These mixture studies of endocrine disrupters have also included substances with dissimilar mode of action e.g. AR-antagonists and substances leading to decreased testosterone levels [1]. However, they all lead to disruption of sex hormone

functions. The present results demonstrate that a pesticide mixture can cause decreased birth weight even if each compound is present at low, non-adverse doses. This study thereby underpins the relevance of performing cumulative risk assessment for substances with similar type of effect irrespective of their mode of actions.

4.2 Prediction of mixture effect on birth weight

DA predicted the outcomes from both studies reasonably well (Figure 2). Decreased birth weight may be induced via many different and in most cases unknown mechanisms of action. This uncertainty leads to the problem on how to choose the "correct" mixture model, as both DA and IA are expected to work only if their pharmacological assumptions are fulfilled, meaning that the combined actions of pesticides happened through either similar or dissimilar sited (or modes) of action. However, even with a better toxicological understanding of the effect chains leading to the observed birth weight changes it is highly unlikely that pesticide mixtures present in humans or the environment can always be classified into similar or dissimilar mode of action groups. This classification problem lead to the pragmatic suggestion of using DA as a conservative default in cumulative risk assessment [13], supported by the conclusion that there is no current example of a experimental situation in which IA provides an accurate prediction that is also more conservative (i.e. cautious) than DA. Moreover, they analysed for selected endpoints the quantitative difference between predictions based on DA or IA, and this analysis suggested that the differences that might be expected in practice are small [13]. It remains unclear whether this holds true also for the endpoint decreased birth weight, but our results provide no indications against using DA as a conservative default assumption and therefore support the use of DA for the cumulative risk assessment of pesticides.

4.3Mixture effect on maternal body weight

Maternal toxicity in the form of reduced maternal body weight was seen at a higher mixture dose (Mix-16%) than the dose inducing decreased birth weight (Mix-5%). Table 4 clearly shows that mixture exposure can lead to lower LOAELs for effect on the dams than seen after single pesticide exposure. The doses of the single pesticides at the LOAEL of the mixture are approximately 5-11 times lower than the LOAELs for the dams when the pesticides are given alone. Also, the doses of the single pesticides at the LOAEL of the mixture are approximately 1.4-7 times lower than their reported NOAELs. This illustrates that also for effects on the dams, mixture exposure leads to lower effect levels than those seen after exposure to single substances. It also demonstrates that mixture effects can occur at dose levels where the single substances have shown no effect on dams, i.e. at or below the observed NOAELs for this endpoint.

The observed mixture effects on maternal toxicity in this study are supported by outcomes of a previous study where combined exposure to five fungicides induced severe effects in the dams (dystocia and increased gestation length) at doses where the individual pesticides induced no such effects [4-5]. In that study, the mixture ratio of the fungicides procymidone, mancozeb, epoxiconazole, tebuconazole and prochloraz was chosen according to the doses of each individual pesticide that produced no observable effects on the dams including gestation length. Application of the dose-addition model resulted in a good prediction of these observed mixture effects [4-5].

4.4 Implications for risk assessment

The experimental results in the present study imply that human risk assessments based on NOAELs for single pesticides may underestimate the risk of co-occurring pesticide exposures. Moreover, they emphasize the need for cumulative risk assessment of pesticides to avoid a potentially adverse impact of co-occurring pesticide exposure on prenatal development and pregnancy.

In the EU Regulation (EC) No 1107/2009 consideration of potential mixture effects is a requirement for human or animal risk assessment of plant protection products (PPP) and residues, as it is stated that PPPs "*shall have no immediate or delayed harmful effect on human health, including that of vulnerable groups, or animal health, directly or through drinking water (taking into account substances resulting from water treatment), food, feed or air, or consequences in the workplace or through other indirect effects, taking into account known cumulative and synergistic effects where the scientific methods accepted by the Authority to assess such effects are available; or on groundwater*" (article 4, paragraph 3(b)). These more general terms provide the basic legal background for the consideration of potential cumulative risk of PPP ingredients without addressing the necessary next steps. The EU Regulation (EC) No 396/2005 on maximum residue levels specifies in article 36, paragraph 1(c) the necessity to develop a methodology for cumulative risk assessment, as it is stated that "*studies and other measures necessary for the preparation and development of legislation and of technical guidelines on pesticide residues, aimed, in particular, at developing and using methods of assessing aggregate, cumulative and synergistic effects*". Based on this, the Panel on Plant Protection Products and their residues (PPR) developed and published in 2008 a methodology for the assessment of cumulative and synergistic risks of pesticides to human health which applies to substances with similar mode of action [22].

A State of the Science document has proposed a science-based approach for performing cumulative risk assessment also for pesticides with dissimilar modes of action [13-14]. Here, a tiered framework analysis is recommended with Dose Additivity suggested as default assessment assumption for mixtures of dissimilarly acting chemicals. At lower tiers of the analysis, all chemicals should be assessed irrespectively of their presumed modes of action. At higher tiers, when the risk estimates at lower tiers are considered unacceptable, chemicals known not to contribute to a relevant common adverse outcome can be excluded from the analysis. By way of further refining the analysis, it is necessary to apply criteria for the grouping of chemicals into common assessment groups based on common adverse endpoint.

According to the above recommendations, all substances known to cause reproductive and developmental toxicity could be considered together at lower tiers. However, reproductive toxicity comprises a broad range of different effects, ranging from reductions in fertility to developmental toxicity effects. At higher tiers, such diverse effects might need to be differentiated. For developmental toxicity effects, adverse outcomes are often differentiated into structural abnormalities, functional deficiencies, death of the developing organism and altered growth [23]. Altered growth may then be further differentiated into increased or decreased growth, where decreased birth weight constitutes an important endpoint for decreased *in utero* growth.

The results of the current study support the view that grouping can be made on the basis of observations of similar types of effect, even if no knowledge on specific mode of action is available.

4.5 Conclusions

Our results clearly showed that a mixture of six pesticides can cause decreased birth weight at dose levels well below the NOAELs of the individual pesticides for this endpoint. The same was seen for reductions in maternal body weights. Although the toxicity data of the individual pesticides were not produced in our laboratory, but collected from DAR reports, which defined a non-optimal data situation for a comparative assessment between observed and predicted mixture effects, the dose-addition model predicted the observed increases in birth weight adequately. This supports the robustness of this prediction tool not only in terms of data demands and their uncertainty, but also indicates that knowledge about the underlying toxic mechanism of each compound is not necessarily an essential prerequisite for this tool. These characteristics strengthen the suitability of the dose addition model for the cumulative risk assessment of pesticides.

The significance of these findings for human risk assessment must be emphasised, because they clearly indicate that risk assessment based on single substances alone may underestimate the risk for adverse effects when humans are co-exposed to several pesticides with common effect outcomes regardless of their toxicological mechanisms.
Thus, there is a need for cumulative risk assessment of pesticides to avoid potentially serious influences of mixed pesticide exposure on prenatal development and pregnancy. Last but not least, it is important to note that several other classes of industrial chemicals may also cause decreased birth weight. As humans are exposed to numerous chemicals simultaneously, cumulative risk assessment should ideally include all chemicals, e.g. pesticides, industrial chemicals, and environmental contaminants from food, dust, cosmetics and other sources.

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Figure Legends

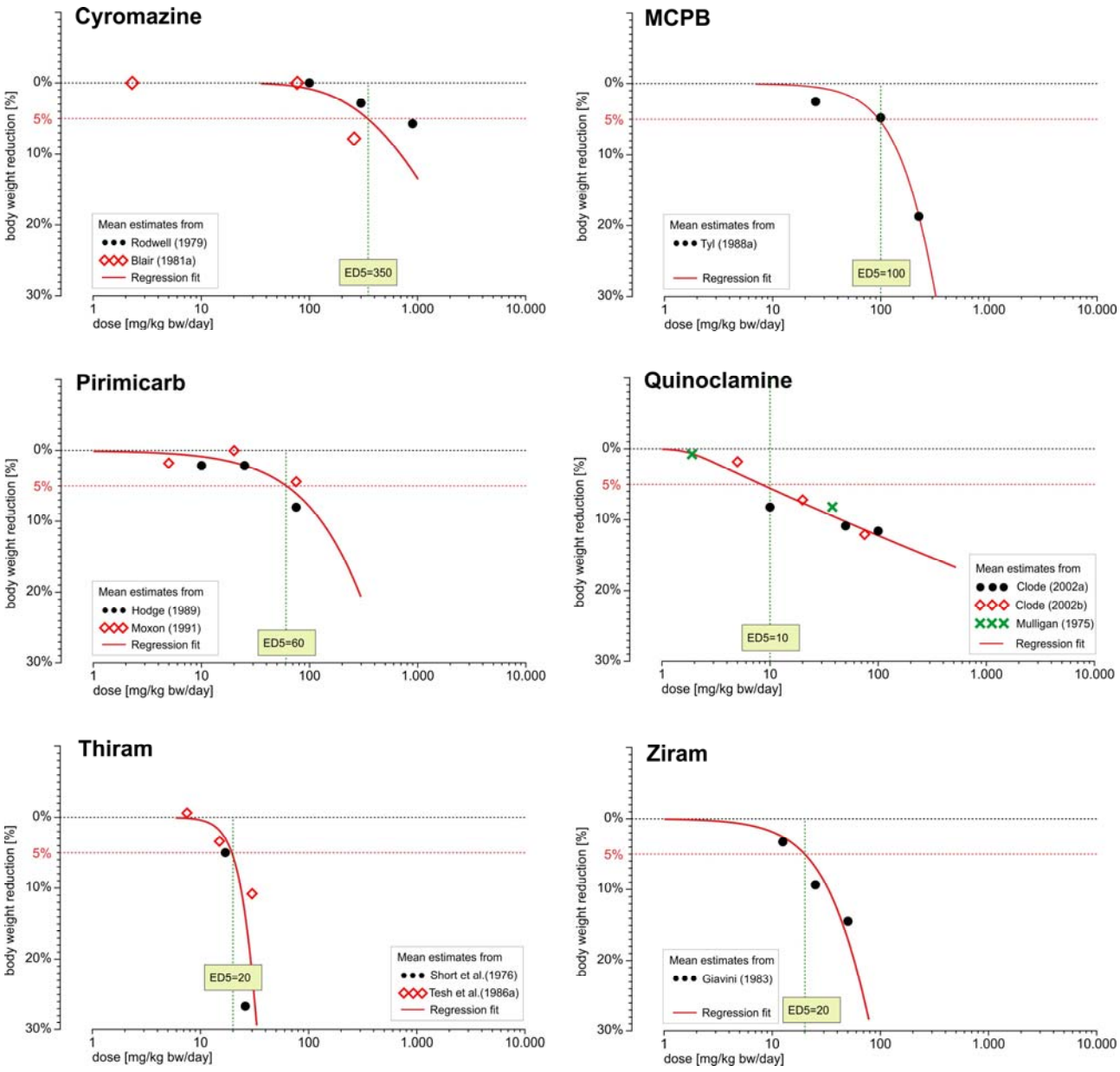

Figure 1. Dose-response data and regression curve estimates for six pesticides. Symbols are mean estimates and refer to their study origins. Vertical lines correspond to the control and 5% reduction level, the horizontal line indicates the dose that will most likely produce a 5% reduction in birth weight (designated BMD_5 or ED_5). The data are derived from Draft Assessment Reports, see text for details

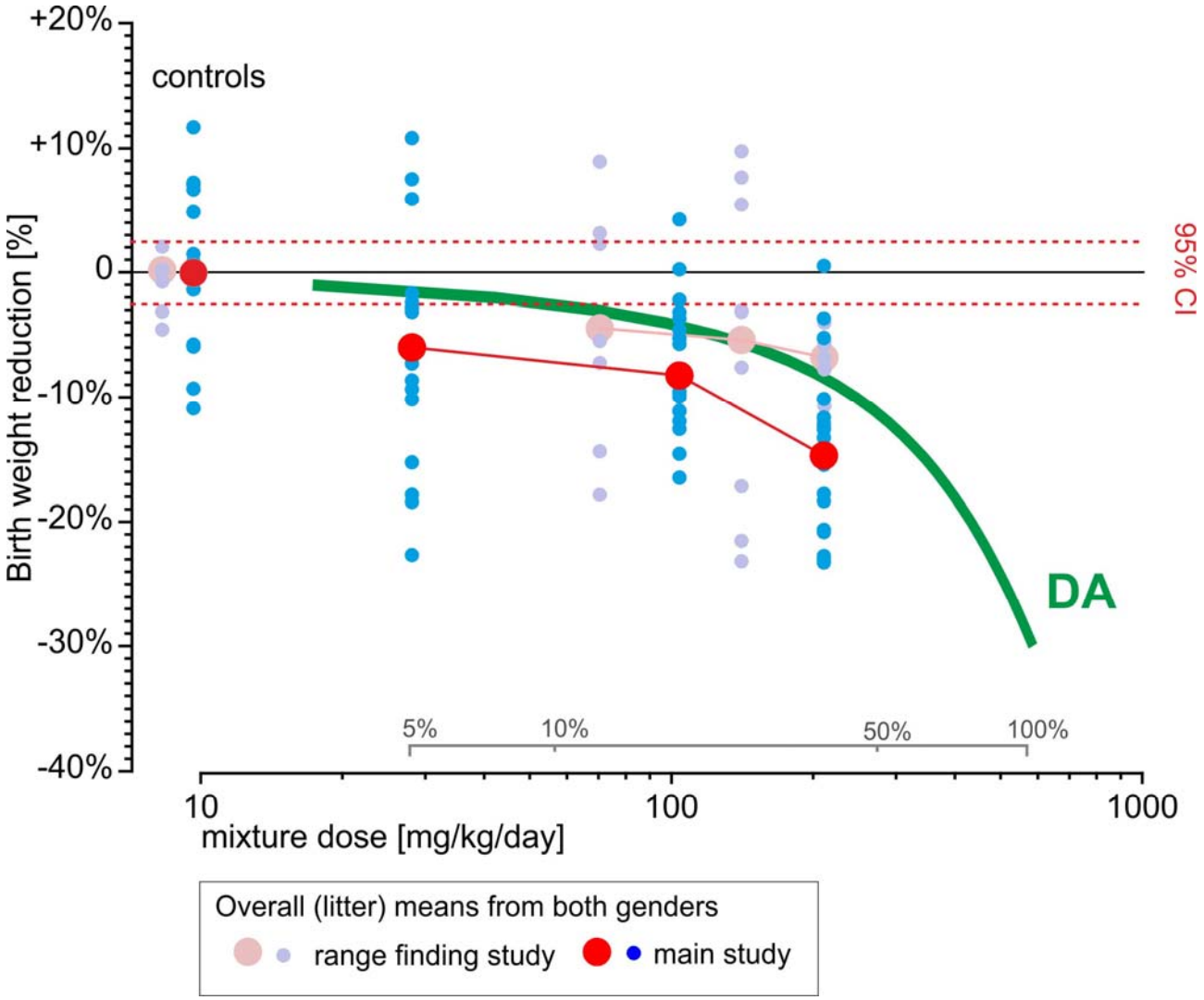

Figure 2. Birth weight decrease in pups following gestational exposure to a mixture of six pesticides. Results are from the range-finding study (see notes about dosing in text) and the main study, with predicted mixture effects shown as dose-response curve (thick green curve) and observed data as litter means (small dots) and group means. The 95% confidence belt around the control mean is included as horizontal dotted line. Also added is a scale indicating the dilutions of the mixture doses (grey x scale), with 100% referring to the sum of the ED_5s (BMD_5s) of the six individual pesticides.

Table 1 Composition of pesticide mixture tested in the range-finding and main study (doses in mg/kg bw/day)

	Range-finding study					**Main study**			
Pesticide	**Control**	**Mix-12.5%**	**Mix-25%****	**Mix-37.5%**	**Mix-75%***	**Control**	**Mix-5%**	**Mix-16%**	**Mix-37.5%**
Cyromazine	0	43.75	87.5	131.25	262.5	0	17.5	64,8	131.25
MCPB	0	12.50	25.0	37.50	75.0	0	5.0	18.5	37.50
Pirimicarb	0	7.50	15.0	22.50	45.0	0	3.0	11.1	22.50
Quinoclamine	0	1.25	2.5	3.75	7.5	0	0.5	1.9	3.75
Thiram	0	2.50	5.0	7.50	15.0	0	1.0	3.7	7.50
Ziram	0	2.50	5.0	7.50	15.0	0	1.0	3.7	7.50
Pesticide mixture	**0**	**70**	**140**	**210**	**420**	**0**	**28**	**90**	**210**

* Only used on GD 7-8. Hereafter changed to Mix-25% due to maternal toxicity

** From GD 9 to PD 16

Table 2 Pregnancy and litter data from gestationally and perinatally exposed rat dams (data are expressed as group means ± STD)

	Range-finding study				**Main study**			
	Control	**Mix-12.5%**	**Mix-25%[a]**	**Mix-37.5%**	**Control**	**Mix-5%**	**Mix-16%**	**Mix-37.5%**
Time-mated females (no.)	10	10	10	10	22	22	22	22
Toxicity symptoms after GD 9 (no.)	0	0	1	1	0	0	0	0
Non-pregnant (no.)	1	2	0	2 (3[b])	0	0	3	2
Pregnant, but caesarean section (no.)	1	0	0	1	0	2	0	0
Pregnant, but no pups on PD1 (no.)	0	1	0	0	2	0	0	1
Pregnant, but not continued due to small litter size, i.e. below 4 (no.)	0	0	0	0	1	1	2	0
Pregnant, but early total litter loss after PD 1 (no.)	0	0	0	0	1	0	0	0
Females giving birth/litters (no.)	8 (7[c])	7	9	6	21	20	19	20
Maternal bw GD7 (g)	226.0±3.7	227.2±10.0	225.8±7.5	226.6±8.3	225.7±9.9	228.8±10.4	225.6±11.1	225.7±9.4
Maternal bw gain GD7-GD21 (g)	71.6 ± 15.5	78.0 ± 11.9	62.0 ± 12.4	58.0±12.0	73.8 ± 17.2	72.0 ± 18.7	57.0 ± 17.4*	43.1±16.0*
Maternal bw gain GD7- PD1 (g)	12.1±10.6	10.0±6.2	2.1±6.6	-4.7±10.3*	18.8±7.8	12.3±9.7	4.19±10.1*	-3.5±8.1*
Gestational length (d)	23.1 ± 0.4	22.9 ± 0.4	22.9 ± 0.4	23.0 ± 0.0	23.1 ± 0.3	23.0 ± 0.5	23.1 ± 0.2	23.1 ± 0.4
Litter size, live pups, PD 1 (no.)	9.4±4.0	11.7±2.3	9.9±3.8	11.3±1.4	8.7±3.1	10.4±4.3	8.8±3.8	8.6±3.5
Pup mortality, stillborn and dead after birth (%)	1.3 ± 3.4	1.1 ± 2.9	3.6 ± 7.9	0.0 ± 0.0	1.3 ± 5.7	0.0 ± 0.0	5.3 ± 22.9 (0.0± 0.0[d])	1.5 ± 3.6

Birth weight (g)	6.2 ± 0.3	5.9 ± 0.6	5.8 ± 0.8	5.7 ± 0.1	6.5 ± 0.4	6.1 ± 0.6*	6.0 ± 0.6*	5.6 ± 0.4*
Body weight, PD 6 (g)	12.1 ± 1.6	11.8 ± 1.5	12.0 ± 2.3	11.3±0.7	13.5 ± 1.4	12.8± 1.8	12.5± 1.5*	11.0±1.4*
Body weight, PD 14 (g)	25.2 ± 4.9	24.6 ± 3.9	26.4 ± 6.3	23.7 ±1.7	30.0±4.4	28.3± 5.3	27.8± 4.7	26.0±4.6*
Body weight, PD 24 (g)	ND	ND	ND	ND	57.5± 7.8	52.1± 9.2	52.8± 6.9	51.9± 7.6*

* $p < 0.05$. ND: not determined as range-finding study was terminated at PD 16.

a) Exposed to Mix-75% from GD 7-8 and Mix-25% from GD 9-PD16
b) The dosing of one of the non-pregnant females was already stopped due to toxicity
c) One litter with only 1 male pup was terminated on PD 6 due to very low pup weight. It is common that litters with only 1-2 pups leads to insufficient maternal care, most likely due to insufficient stimulation of milk production in the dam
d) Excluding one litter with only 2 pups which died shortly after PD 1. It is common that litters with only 1-2 pups leads to insufficient maternal care, most likely due to insufficient stimulation of milk production

Table 3 LOAEL, NOAEL and BMD_5 (ED_5) for decreased birth weights after developmental exposure to pesticides* (suffix P), compared to the individual pesticide doses present at the lowest mixture dose (Mix-5%) that produced a significant effect on birth weight (i.e. $LOAEL_{mix}$). All doses are mg/kg bw/day.

Endpoint: Birth weight	**Individual pesticides***			**Mixture**	**Comparisons**	
	$LOAEL_P$	**$NOAEL_P$**	**BMD_{5P}**	**$LOAEL_{mix}$ (mix-5%)**	**$LOAEL_P$ /$LOAEL_{mix}$**	**$NOAEL_P$/ $LOAEL_{mix}$**
Cyromazine	600	300	350	17.5	34	17.1
MCPB	100	25	100	5	20	5.0
Pirimicarb	75	25	60	3	25	8.3
Quinoclamine	20	5	10	0.5	40	10.0
Thiram	30	16	20	1	30	16.0
Ziram	25	12.5	20	1	25	12.5

* Data derived from DAR, see text for details.

Table 4 Mixture effect on dams. LOAELs and NOAELs for the single pesticides given alone (suffix P) compared with the individual pesticide doses present at the lowest mixture dose (Mix-5%) that produced a significant effect on the dams (i.e. LOAELmix). The doses are mg/kg bw/day

Endpoint: maternal body weight gain	**Individual pesticides***		**Mixture**	**Comparisons**	
Pesticide	**$LOAEL_P$**	**$NOAEL_P$**	**$LOAEL_{mix}$ (mix-16%)**	**$LOAEL_P$/ $LOAEL_{mix}$**	**$NOAEL_P$/ LOAELmix**
Cyromazine	300	100	64.8	5	1.5
MCPB	100	25	18.5	5	1.4
Pirimicarb	75	25	11.1	7	2.3
Quinoclamine	20	5	1.9	11	2.6
Thiram	17	-	3.7	5	>5
Ziram	25	-	3.7	7	>7

* Data derived from DAR, see text for details.