Classification of developmentally toxic pesticides, low dose effects, mixtures – perspective of the industry

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What is meant by “Classification” in the context of this Workshop

- **CLP, GHS**
  - Classification of (agro)chemicals according to the level of concern of their reproductive and developmental toxicologic properties, independent of the dose where these were detected

- **Severity of effects of developmental toxicity**
  - Malformations, variations, retardations, minor/major anomalies, etc.
  - Fertility, fecundity, reproductive performance, maternal care etc.
  - Effects related to endocrine disruption

Mixture effects

Need for mixture risk assessments discussed for several years

- State-of-the-art reports on mixture toxicity - funded by DG Environment – were published (e.g., Kortenkamp et al. 2009).
- In the context of the regulation of pesticides in the EU (e.g., via the MRL Regulation (EC) No. 396/2005) due to the potential presence of pesticide residues in food "*… account shall be taken of … known cumulative and synergistic effects, when methods to assess such effects are available.*"
- Similar text included in Regulation (EC) No. 1107/2009, concerning the placing of PPPs on the market in the EU.

Current work ongoing to identify active ingredients with common target organs to be included in Cumulative Assessment groups (e.g., EFSA 2018; External Scientific Report 2016).

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Mixture effects

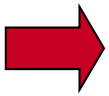
The basic assumption for conducting combined/cumulative risk assessment is dose addition for compounds with similar mode/mechanism of action.

- Often simplified by “having effects on the same target organ” (EFSA 2013)
- Evidence for synergism at low doses is rather weak (Boobis et al. 2008, ECETOC Monograph “low dose interaction”)
- Experimental evidence that dose additivity at NOAELs is a conservative assumption (Schmidt et al. 2016)

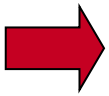
Dose additivity assumption is considered protective for human health assessments.

Mixture effects, public concerns

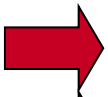
- There are effects at low doses of individual substances, which are systematically overlooked (Bisphenol A, hormesis)
- Knowledge gaps in single substance assessments (old 2-Gen studies)
- Effects are seen at mixtures of substances combined at their individual NOAELs
- More-than additive mixture toxicity



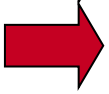
- No clear evidence, but debate is ongoing



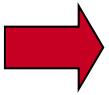
- Optimize single substance evaluation



- Only in case of common toxicity



- No human-relevant exposure situation



- No evidence (except specific combinations)

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Mixture effects

The only way to address concerns is producing reliable data and feeding them into the public discussion. This is particularly important for studies with “negative outcome”.

- Studies comparing single compound and mixed exposures at low-doses were performed at BASF Reproduction Toxicology Laboratory
- Cooperation with Metanomics
  - Metabolomics
- Cooperation with University Nice and INSERM
  - Trancriptomics
  - µRNA

metanomics
new dimensions in discovery

LRI
The Long-range Research initiative
www.cefic-lri.org

Université Nice Sophia Antipolis

Instituts thématiques
Inserm
Institut national de la santé et de la recherche médicale

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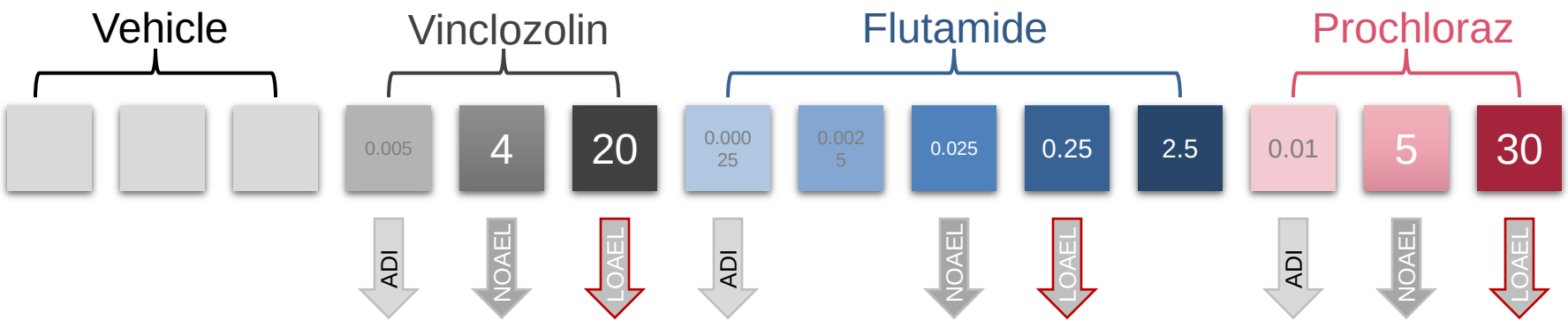
Substances and dose selection

	Vinclozolin	**Flutamide**	**Prochloraz**
Class of Compound	Pesticide	Pharmaceutical	Pesticide
MoA	Androgen receptor antagonist	Androgen receptor antagonist	Multiple modes of action, affects steroidogenesis
Effects at LOAEL	AGD↓, nipple/areola↑, hypospadia, demasculinization	AGD↓, nipple/areola↑ Feminization of male rat genitalia,	nipple/areola↑
NOAEL in rats	4 mg/kg bw	0.25 mg/kg bw	5 mg/kg bw
Reference value (ADI)	0.005 mg/kg bw	n.a.	0.01 mg/kg bw

Dosing



Vehicle Control

Positive Control

ADI Mix

NOAEL Mix

LOAEL Mix

Study design

Treatment

Pregnancy	Breast-feeding of their pups

GD 6 PND 21

GD 6 PND 21 PND 27 ~ 49 PND 83

Pregnancy	Suckling pups	Pre-pubescent	Post-pubescent

Measurements

General: general and reproductive health parameters,
Endocrine: anogenital distance, presence of nipples/areolas, weights of reproductive organs, full histopathology, sperm quality, hormones,
Molecular: metabolome, transcriptome, miRnome

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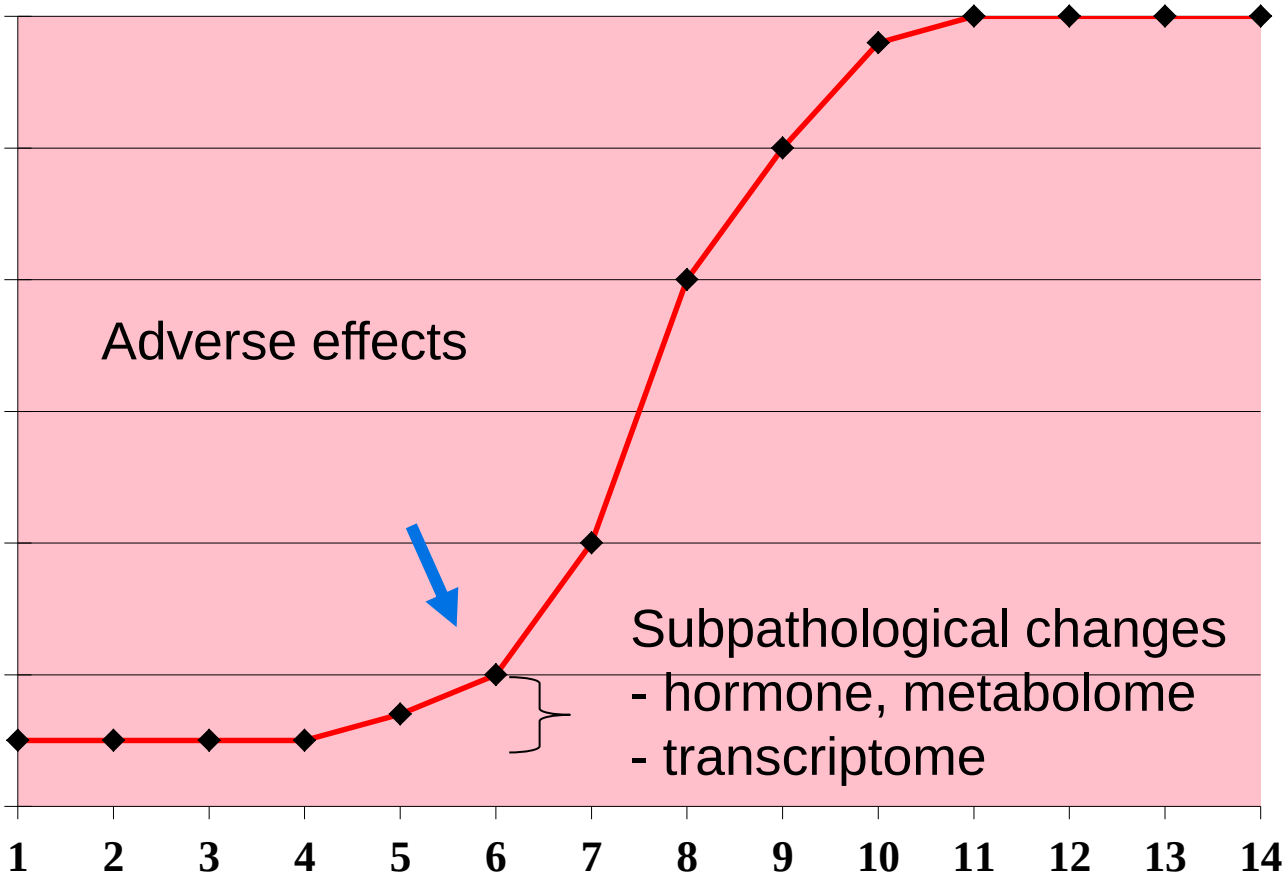
Homeostasis



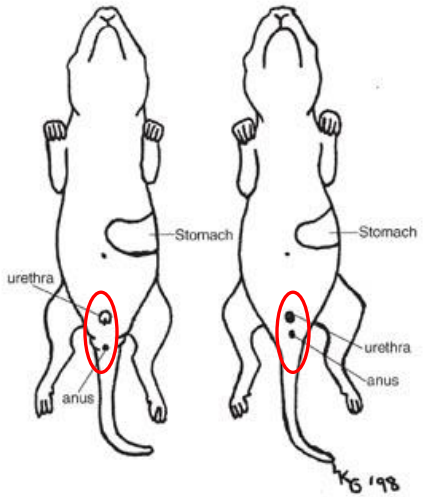
Study Design

- Dose range from ADI to Effect Level, i.e. at relevant levels for substance regulation
- Vehicle control for each study
- Positive control group exposed to 2.5 mg/kg bw/d Flutamide
- Blood and tissue samples taken to investigate sensitive markers of endocrine activity and to identify sub-pathological anti-androgenic molecular changes
- Most parameters evaluated in the same animals
- **Thus single- and mixed-exposures can be compared from molecular to pathological levels in one and the same animals**

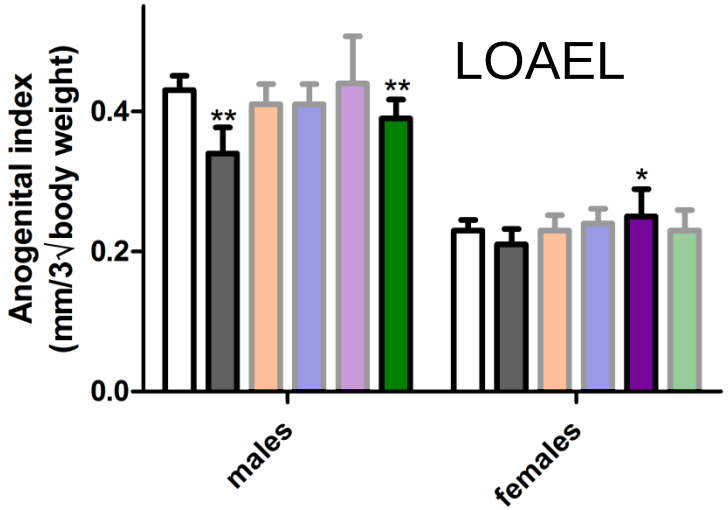
Study Results (systemic toxicity)

- No mortalities
- LOAEL Prochloraz and LOAEL Mix: slightly reduced survival of offspring
  Otherwise no signs of clinical toxicity
- LOAEL Prochloraz and LOAEL Mix: slightly decreased food consumption and body weights/weight gain during gestation/lactation in F0 females and in post-pubescent offspring
  Otherwise no effects on body weight and food consumption
- No effects on clinical chemistry
- No effects on pathology
- Specific effects in offspring:

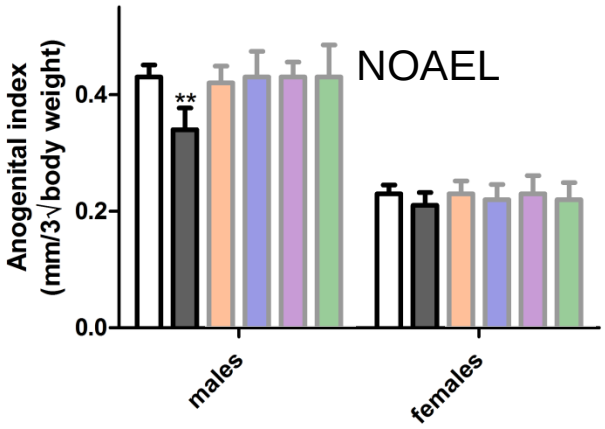
Anogenital Index

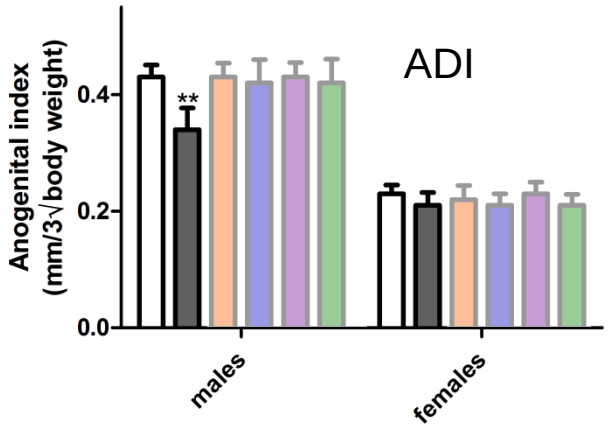


Male on the left, female on the right. Notice the distance is farther on the male between the anus and the urethra.



Vehicle Control
Positive Control
Vinclozolin
Flutamide
Prochloraz
Mixture





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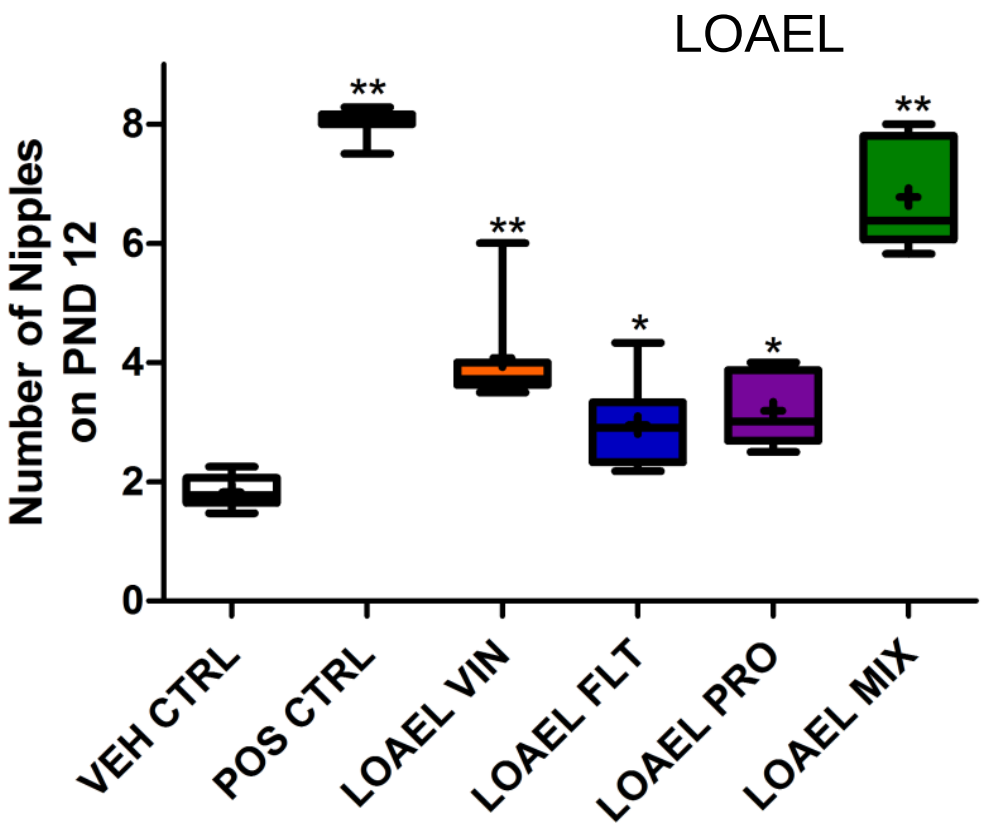
Presence of Nipples/Areolae



Vehicle Control
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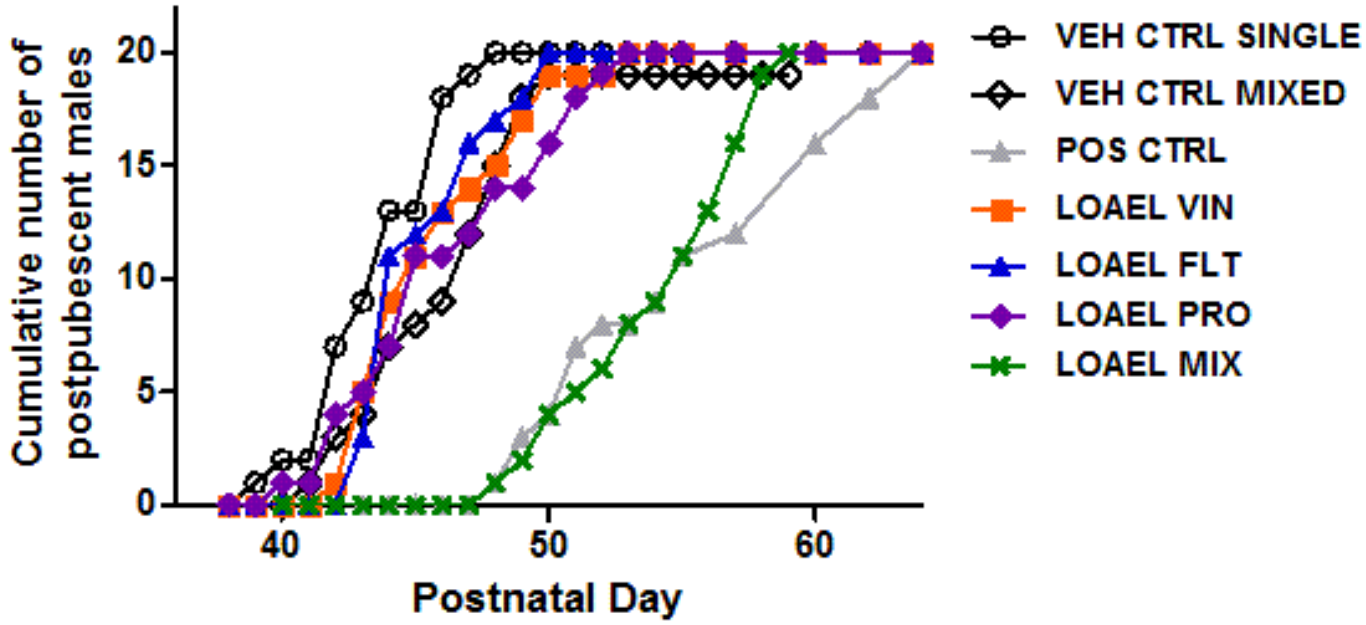
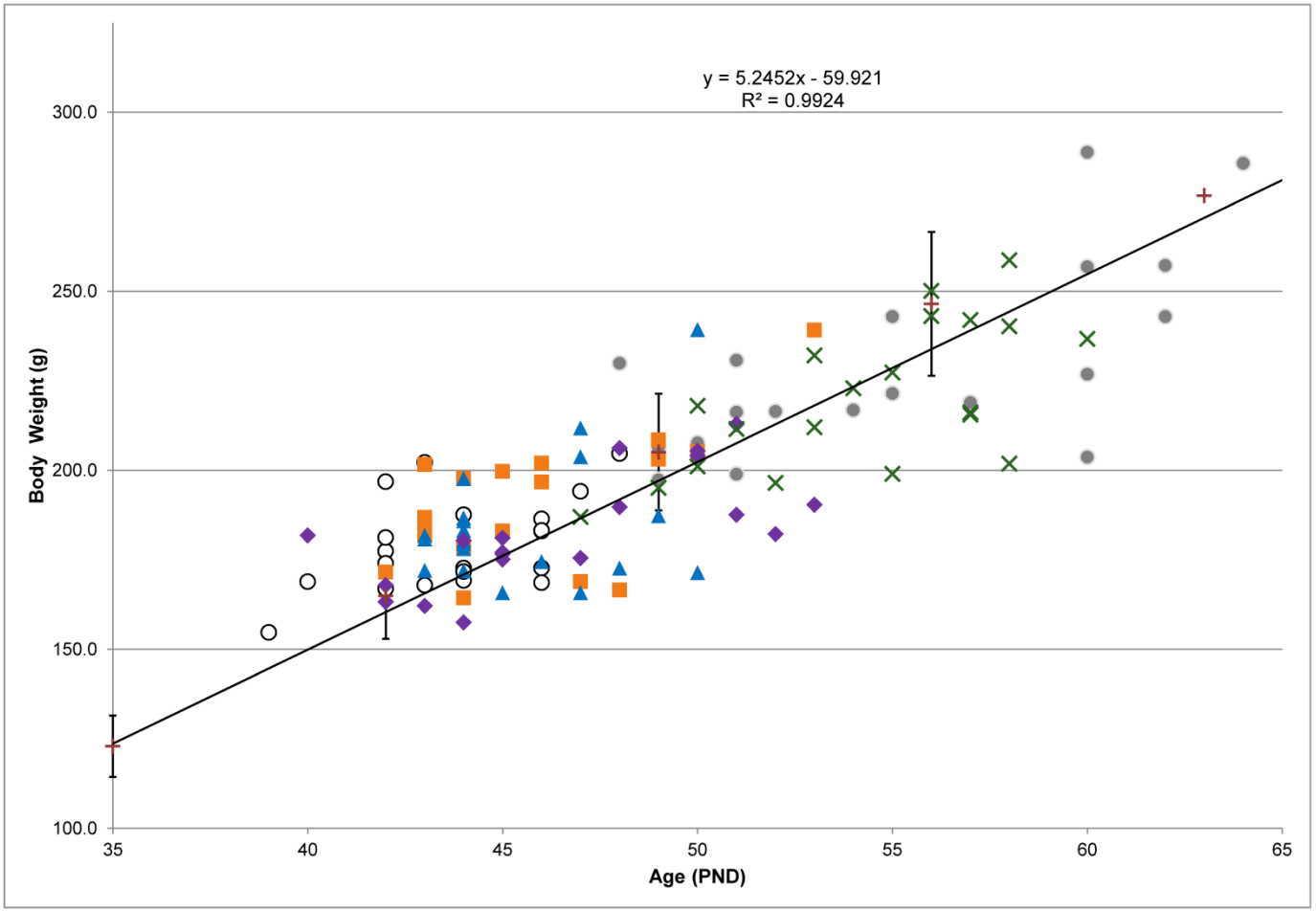
Age at Male Puberty (Preputial Separation)

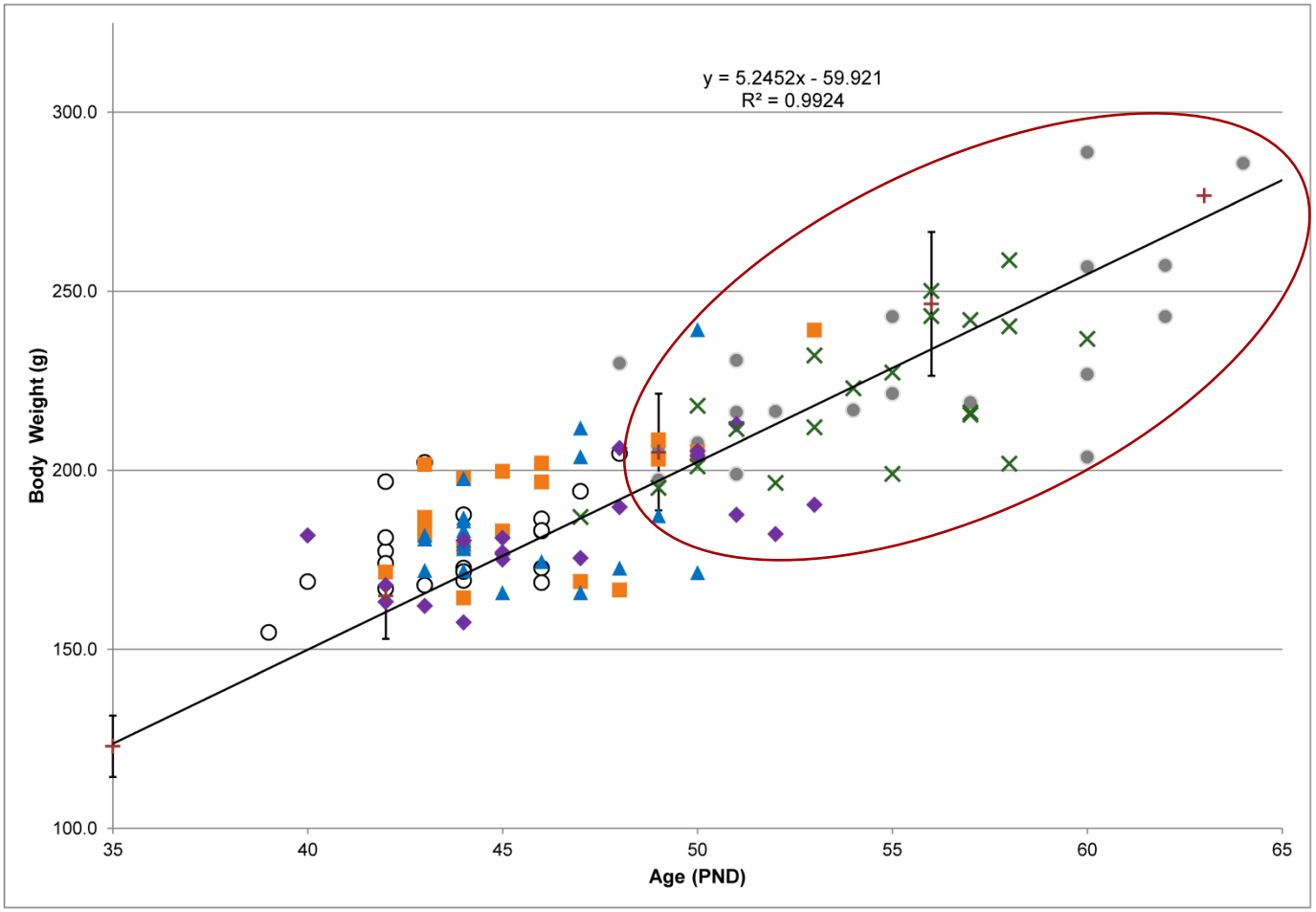


Male Sexual Maturation and Body Weights



○ VEH CTRL
● POS CTRL
■ LOAEL VIN
▲ LOAEL FLT
♦ LOAEL PRO
X LOAEL MIX
+ MEAN ± SD OF CTRLS

Male Sexual Maturation and Body Weights



○ VEH CTRL
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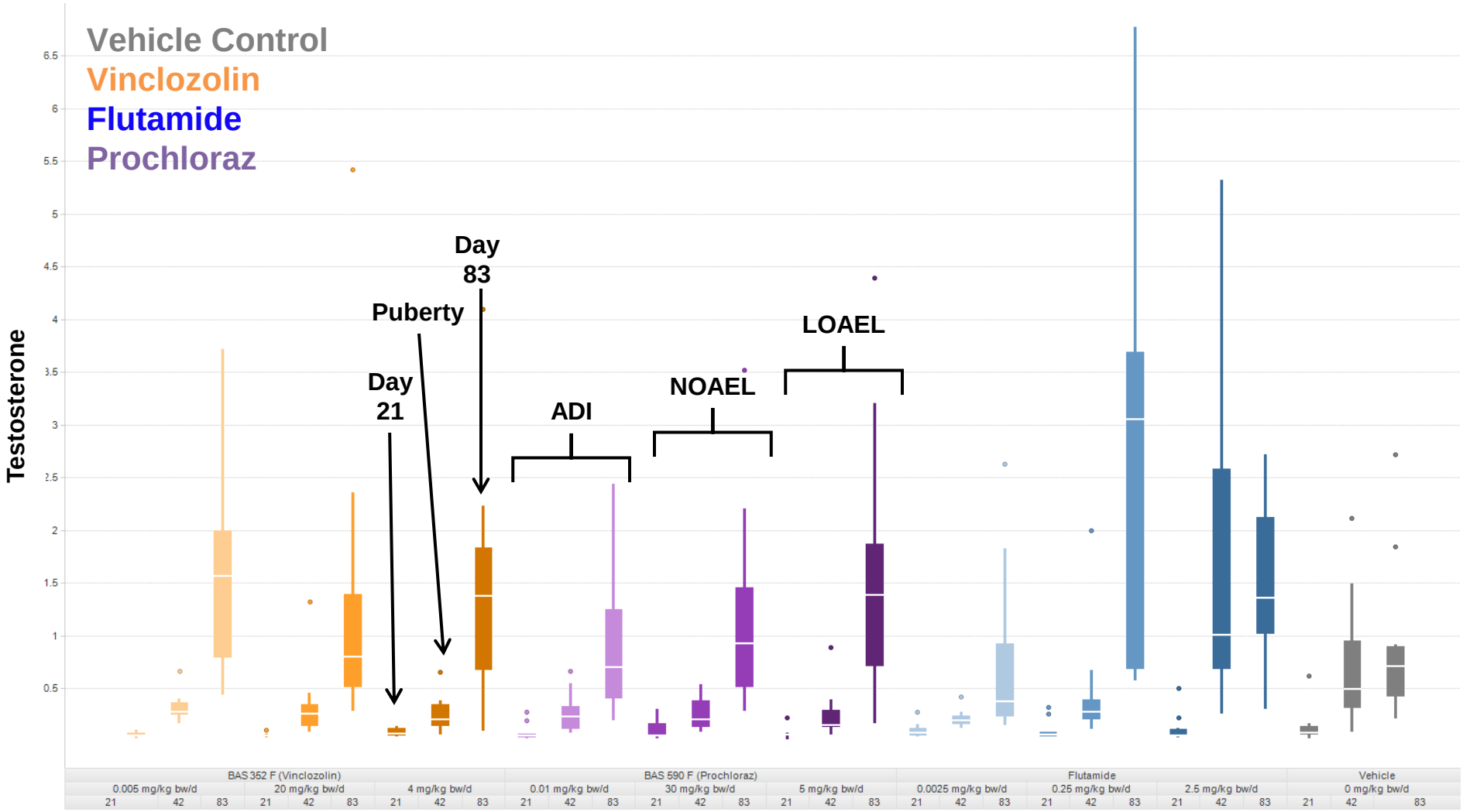
Steroid Hormone Levels



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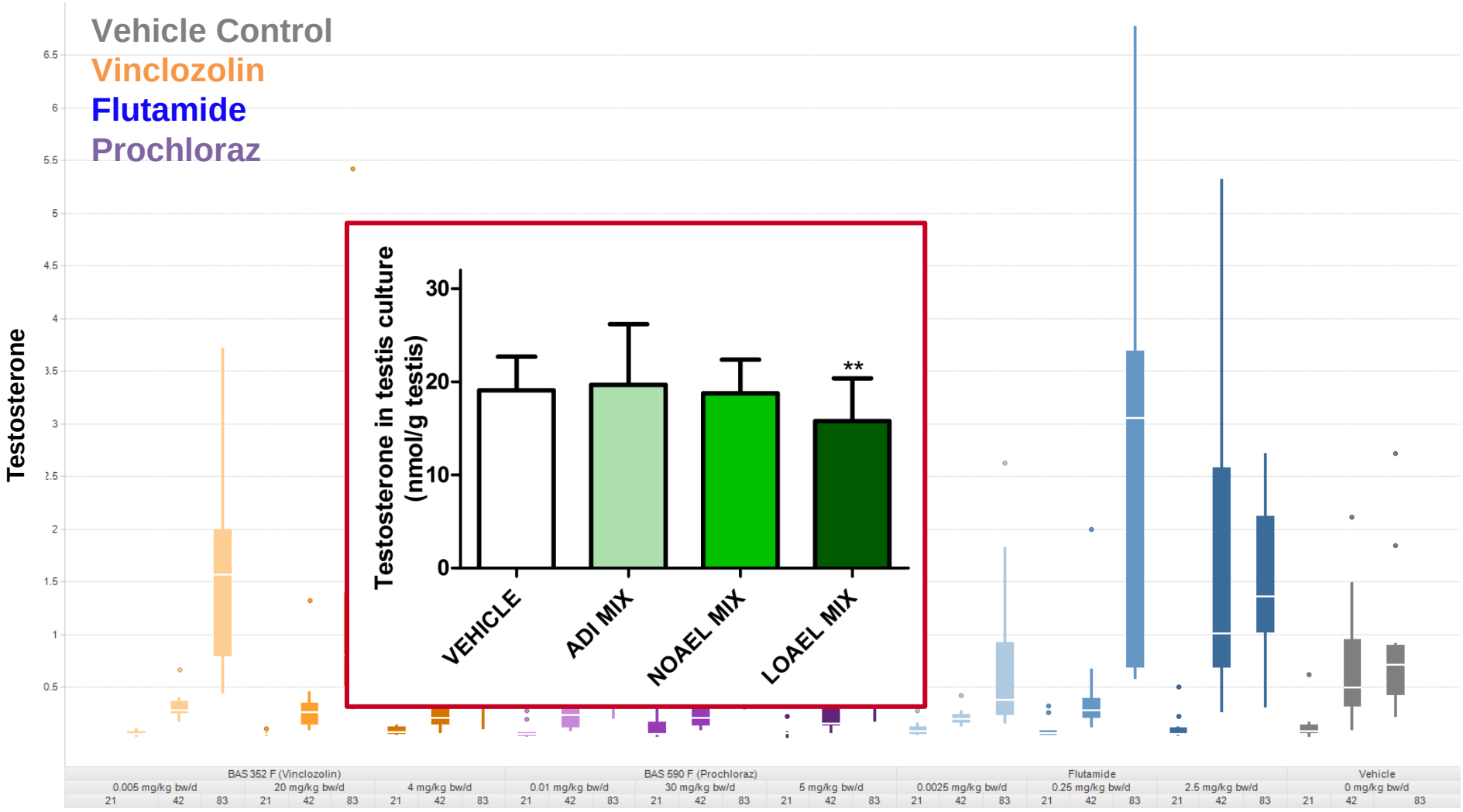
Steroid Hormone Levels



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Study Results

- Significant ‘cumulative’ effects in the maturing LOAEL mixture F1 animals
  - Decreased anogenital distances/indices
  - Increased nipple retention
  - Delayed male sexual maturation
  - Reduced sex organ weights
- No effects seen in ADI or NOAEL mixtures

Conclusions

Among the effects seen at LOAEL mixture

- Nipple/areola counts appeared to be the most sensitive measure of effect, closely followed by age at sexual maturation,
- then anogenital distance/anogenital index and
- then male sex organ weights, esp. ventral prostate weight, and finally
- gross and histopathological findings.

Conclusions

- Combined exposure at LOAEL level resulted in more than additive responses for decreased male anogenital index (but not for anogenital distance), and delayed preputial separation in comparison to the expected effects of the individual compounds.
- The quantification of circulating hormone levels showed little consistency when comparing possibly treatment-related changes with those from same dose group at other developmental stages or when mixed- and single-substance exposure data were compared.
- In contrast, testosterone changes in testes of male fetuses at GD 20 seems to represent an appropriate biomarker for potential anti-androgenic effects in a sensitive tissue during a critical developmental window.

Conclusions

While these endpoints for anti-androgenicity had varying sensitivities, when taken together the data reveal two important observations:

- The dose–response curve clearly indicates a monotonic process
- No evidence for an interaction of the compounds at the individual NOAEL or lower doses

Thus: For the antiandrogens tested in this project the NOAELs and Reference Values were protective even when considering potential mixture effects

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