Educational learning objective

1. The resident understands the mechanisms involved in calcium metabolism and is aware of the most relevant functions of the calciotropic hormones
2. The resident understands vitamin D metabolism and the main functions regarding calcium homeostasis and skeletal mineralization.
3. The resident understands the mechanisms available to coop with chronic deviations of calcium or vitamin D intake for the regulation mechanism, plasma concentrations of calcium, phosphorus and the calciotropic hormones and thus the value of its measurements in the context of dietary mistakes.
4. The resident can make a differential diagnosis of dietary diseases based on anamnesis/history, and laboratory findings (including plasma levels of calcium, phosphorous, PTH, vitD metabolites, and radiographs)

Intoxication with calcium or vitamin D

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I. Calcium metabolism

Normocalcemia is of vital importance and will be maintained at any costs (Fig. 1), especially at the costs of the skeleton being the main source and depot of body calcium. Parathyroid hormone will increase plasma calcium concentration by increasing renal calcium reabsorption and increase plasma calcium and phosphate concentration by increasing osteoclastic bone resorption; it has no direct influence on the intestinal calcium and phosphate absorption, but will increase both by increasing 1-alpha hydroxylation of 25OHvitD and thus increase the production of the most active metabolite $1,25(OH)_2$vitD (Fig.3). The latter has not only influence on increase of calcium and phosphate absorption, but also at osteoclast activity and increased renal calcium and phosphate reabsorption. An important aspect of the homeostasis of calcium is the fact that PTH lowers the tubular maximum for phosphate (T_mP): so in the above described situation with increased PTH and secondary increased $1,25(OH)_2$vitD, the increased absorbed, reabsorbed and resorbed phosphate is lost in the urine, thus preventing a hyperphosphatemia which may counteract the regulation to restore normocalcemia. In case of hypercalcemia, polyuria is the defense mechanism of the body lowering the plasma calcium concentration; for this process PTH or related molecules (PTHrP) are necessary (Hazewinkel, 2012)

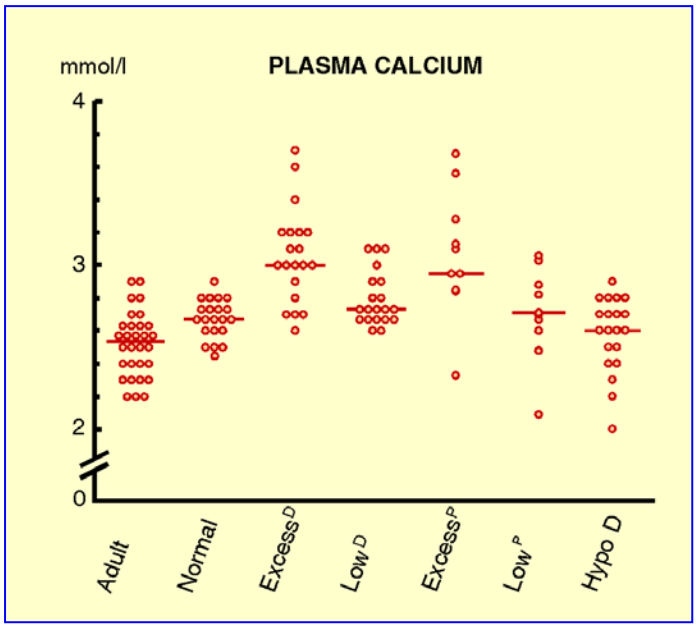

Fig. 1 Plasma calcium concentrations (range and median values in mmol/L) (D=Great Dane; P= Miniature Poodle) 1) in **adult** dogs fed a balanced dog food, 2**) normal**=young dogs raised on a diet according to NRC 1985 (with 1.1% Ca and 500 IU vitD/kg food on dmb), 3) **Excess^D**= Great Dane dogs raised on diet with 3x the Ca content (3.3% vs 1.1%, on dmb), 4**) Low^D**=Great Dane dogs raised on food with 0.55% Ca, 5) **Excess^P**=Miniature Poodles raised on food with 3.3% Ca, 6) **Low^P**= Miniature Poodles raised on food with 0.33% calcium on d.m.b., 6**) Hypo D**= Mongrel dogs raised on synthetic food without vit D added to it but otherwise according to NRC 1985, Despite a 3 x higher calcium intake, or a 50%-66% lower calcium intake, of a normal or very low vit D intake, the median plasma calcium level stays within narrow limits and is thus nót reflecting the serious problems of the dietary composition. (Hazewinkel, 1996)

Parathyroid hormone (PTH) is synthesized in the parathyroid glands; its synthesis and secretion are stimulated in case of hypocalcemia. The set point of PTH secretion can be influenced by

excessive calcium intake of excessive vitamin D intake at the post-weaning period. Calcitonin (CT) is produced in the C-cells, mainly located in the thyroid glands; it synthesis and secretion is stimulated mainly by raise in plasma calcium concentration (Hazewinkel, 2012).

Physiological situations

Increased plasma calcium concentrations can be recognized in the following situations:

1. The normocalcemia can be set at a higher level in young dogs when compared with normal adult dogs of the same breed. It is even higher in case of increased bone turn-over.
2. Factors affecting plasma calcium values may include laboratory mistakes (e.g. lipemic blood samples), dehydration (together with hemoconcentration and hyperproteinemia), hemolysis, medication (e.g. phosphate binders, acidosis) (Feldman, 2005).

Dietary calcium content

Relationship between Ca intake (VI) and fractional Ca absorption (α) in growing dogs studied at 6–27 wk of age over a wide range of VI corresponding to 0.33–3.3 g Ca/100 g diet (on dmb), in 67 Great Dane Pups and 23 Miniature Poodle pups revealed a constant passive calcium absorption of 53% of the intake. At low calcium intake active absorption contributed to a significant part of the total calcium absorption. Intestinal calcium handling did not differ between the two breeds investigated (Tryfonidou et al, 2002)(Fig.2)

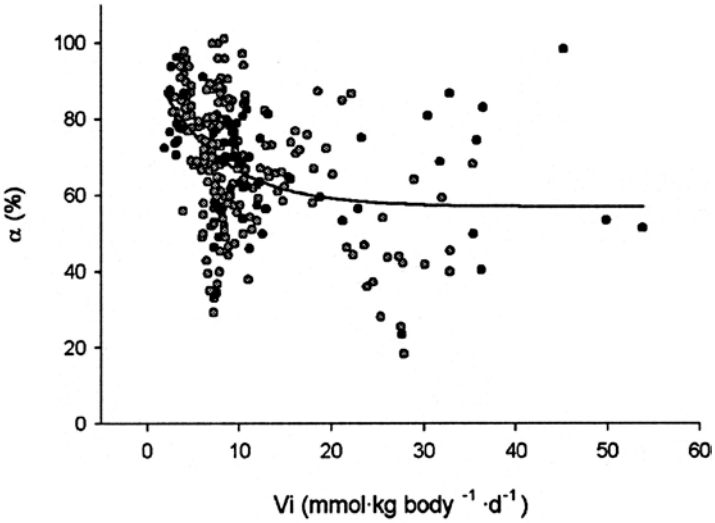

Fig. 2. Calcium absorption is a passive process of diffusion and active absorption. Passive diffusion is high in case the calcium content of the food is high and the animal is young. Another part of the calcium absorption mechanism is dependent on $1,25(OH)_2$vitD and energy and is especially playing a role in low dietary calcium content, both in young and adults. The lower the calcium content the higher the (relative!) percentage of absorption (together with a high $1,25(OH)_2$vitD plasma level), whereas the higher the calcium content (right at the x-axis) the lower the absorption percentage, but not under 50% in these Great Danes and M.poodles less than 6 months of age. These studies were performed with ^{45}Ca tracer, being less than 1% of the total calcium content of the food (Tryfonidou et al,2002).

Examples:

1. Unintentionally, young dogs may have access to food with a high Ca content, e.g. puppies with access to the bitch's diet supplemented with minerals to prevent eclampsia puerperalis, or puppies given extra artificial milk with high calcium content (Corbee et al, 2012) may cause hypertrophy of the C-cell (i.e., calcitonin producing cells in mainly the

thyroid glands). More C-cells will secrete more CT on a certain challenge of plasma calcium increase. With its mean half-life of approximately 20 minutes, hypercalcitoninism in the postprandial phase will lead to an elongated period of suppressed osteoclast activity. Enostosis (= panosteitis eosiniphylica) may develop as a consequence, leading to clinical and radiological manifestations 3-4 months later (Schoenmakers et al, 1999): Radiological indications for panosteitis were present in Great Danes (GD) but not in Miniature Poodles (MP), all raised on food with 3.3% Ca & 0.9% P. These groups GD and MP differed in their growth rate whereas the findings in calcium metabolism (absorption%, accretion and resorption) were of the same magnitude. This leads to the conclusion that growth rate has a major influence on skeletal remodelling at periods of excessive calcium intake.

2. Excessive Ca intake (3.3% on dmb, without concomitantly an increase of other constituents like phosphorous) starting at partial weaning (i.e. 3 weeks of age till 17 weeks of age) may lead to severe hypercalcemia causing suppression, and eventually atrophy of the parathyroid glands. The chief cells were not responding to a challenge with EDTA (to produce a standardized hypocalcemia) anymore (Schoenmakers et al, 1999). Hypoparathyroidism will cause decreased hydroxylation of 25OHvitD into $1,25(OH)_2$vitD (Fig.3). Although, the plasma concentration of the latter was not decreased in this group with high calcium intake when compared with their controls (fed according to NRC 1985). This may be the result of the severe hypophosphatemia, which is caused by the increased sequestration of P in the intestinal lumen and, after its resorption, in organels and bone and a known stimulus from 1 alpha hydroxylation. The severe hypophosphatemia may be responsible for the development of rickets-like disease, despite sufficient vitamin D intake. Dogs with high Ca intakes from 6-17 weeks of age, without (HCaNP) or with (HCaHP) the same Ca:P ratio as the controls (NCaNP) revealed hampered growth but an energy intake just covering the requirements of their weight (Tabl 1). From the age of 17-27 weeks all dogs received the control food and demonstrated a restauration of their underweight (Table 1). The dogs demonstrated the ultimate defence mechanism against hypercalcemia: decreased food intake.

Table 1. (from Schoenmakers et al, 1999b)

Plasma calcium (Ca) concentration, body weight (BW), and energy intake in dogs fed diets with different Ca and phosphorus (P) concentrations from 3 to 17 wk of age and diet NCaNP from 17 to 27 wk of age[1]

Group	Age	Ca	BW	Energy intake
	wk	*mmol/L*	*kg*	*kJ/kg$^{0.75}$ BW*
NCaNP (n = 9) (1.04 g Ca, 0.82 g P)[2]	9	2.94 ± 0.021	10 ± 0.51	1317 ± 67.2
	15	2.90 ± 0.049	17.2 ± 0.69	1108 ± 91.2
	21	2.74 ± 0.055	24.2 ± 1.10	1041 ± 29.7
	25	2.78 ± 0.050	29 ± 1.3	1072 ± 36.2
HCaNP (n = 9) (3.11 g Ca, 0.87 g P)[2]	9	3.64 ± 0.056[A]	6.8 ± 0.50[A]	894 ± 71.1[A]
	15	3.51 ± 0.123[A]	10.2 ± 0.61[A]	1007 ± 38.3[A]
	21	2.68 ± 0.036	19.6 ± 0.65[A]	1410 ± 45.2[A]
	25	2.81 ± 0.039	25.2 ± 0.70[A]	1182 ± 49.2
HCaHP (n = 6) (3.10 g Ca, 2.77 g P)[2]	9	2.83 ± 0.020[B]	7.2 ± 0.64[A]	1335 ± 79.5[B]
	15	2.68 ± 0.034[B]	11.5 ± 1.17[A]	1262 ± 36.8[B]
	21	2.55 ± 0.038	19.1 ± 1.42[A]	1292 ± 74.5[A]
	25	2.71 ± 0.037	24.5 ± 1.42[A]	1257 ± 47.1[A]

[1] Values are means ± SEM.
[2] g Ca and P per 100 g dry matter.
[A] Significantly different from group fed normal Ca, normal P (NCaNP) ($P \leq 0.05$); [B] significantly different from group fed high Ca, normal P (HCaNP) ($P \leq 0.05$).

Hormonal dysregulation

Parathyroid-hormone related peptide (PTHrP) is a pro-hormone of PTH, which has structural and functional similarities with PTH. Through the homology of the amino-terminus region of PTH and PTHrP, both are able to bind to and activate the same receptor. PTHrP is formed under normal conditions in many adult and fetal tissues, where it regulates in an autocrine/paracrine fashion organogenesis. It is a causative factor of humeral hypercalcemia of malignancy. Hypercalcemia causes muscle weakness in dogs, nausea, decreased food intake and polydipsia. Consequently, chronic state of hypercalcemia will cause severe weight loss and urinary stones. In recent years it revealed that malignancy induced PTHrP causes cachexia together with decreased locomotor activity by mechanisms independent of the hypercalcemia itself. It has been demonstrated that tumor-derived PTHrP drives the expression of genes involved in thermogenesis by brown fat; neutralization with PTHrP antibodies increased body weight, muscle volume and strength (Kir et al, 2014).

Examples:

3. A tumor of a parathyroid gland will cause primary hyperparathyroidism (PHPTH) with consequently hypercalcemia due to increased osteoclast activity, increased calcium resorption and absorption, and decreased renal perfusion. The caused hypercalcemia will suppress the other sources of PTH (being the other three parathyroid glands) and will stimulate 1,25$(OH)_2$vitD production with calcium sparing effects. PTH induced lowering of the T_mP reduces plasma phosphate levels, together with hyperphosphaturia. PTH-forming tumors are seen more frequently in specific purebred dogs (e.g. Keeshond; Feldman, 2005). Neonatal primary hyperparathyroidism has been described in two German Shepherd pups and a previous pup of the same parents (being half siblings) at 2 weeks of age with hypercalcemia, hypophosphatemia, polydipsia, stunted growth, muscle weakness and increased PTH-plasma levels, and on necropsy hyperplasia of chief cell's and C-cells, osteodystrophy, mineralization's of lungs and gastric mucosa (Thompson et al 1984).

4. PTHrP may be excessively synthesized in case of in malignant lymphomas, malignant melanoma, multiple myeloma, adenocarcinomas from the apocrine glands of the anals sac region, of mammary glands and ovaries. Hypercalcemia can be the cause of the mechanism described under 3 plus possibly due to bone metastasis in selected cases. In addition RANK-ligand (a tumor necrosis factor) originating from malignancies, can be produced and acting as stimulator of osteoclastogenesis and osteoclast activity. In case of malignant lymphomas also other factors than PTHrP may cause hypercalcemia, since in those patients hypophosphatemia is often not that prominent.

Vitamin D metabolism

Vitamin D is taken in with the food, or produced in the skin; the latter is not the case in carnivores species including dogs and cats (Corbee 2014). The first hydroxylation in the liver is loosely regulated and thus reflects the amount of substrate (i.e. vitamin D), although remarkable differences are seen between different dog breeds at young age, due to differences in growth rate and thus Growth hormone (GH) levels (Fig.3). Under influence of a variety of factors, including lowered calcium or phosphorous, and especially an increase in PTH 1-alpha hydroxylase is stimulated and the biological most active vitamin D metabolite, i.e. 1,25$(OH)_2$vitD is formed in the tubuli of the kidneys (Fig. 3).

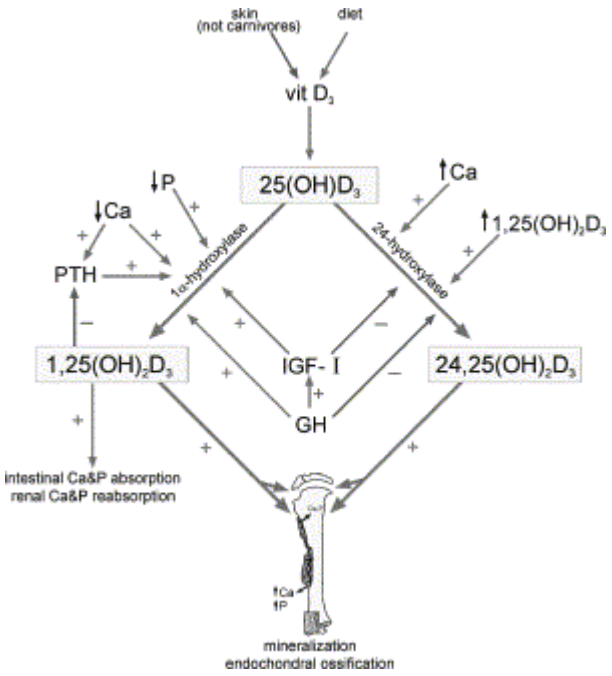

Fig. 3 Vitamin D metabolism and target organs (Hazewinkel & Tryfonidou 2002)

Contrarily, under influence of increased calcium plasma concentrations and 1,25(OH)$_2$vitD the 24-hydroxylase is stimulated and thus more 24,25(OH)$_2$vitD is formed. In young dogs in their rapid growth phase, GH and thus insulin-like growth factor I (IGF-I) concentrations will be increased, stimulating 1-alpha hydroxylase but not 24-hydroxylate. As a consequence young dogs, especially of large breeds have increased 1,25(OH)$_2$vitD and low 24,25(OH)$_2$vitD when compared with small breed dogs of the same age. 24-hydroxylase can be seen as the first defense against 25OHvitD excess: 24,25(OH)$_2$vitD has no influence on osteoclasts and decreases even 1,25(OH)$_2$vitD mediated calcium and phosphorus absorption. (Hazewinkel & Tryfonidou, 2002).

Examples:

5. Eight fold the recommended dose of vitamin D (i.e. 4,000 IU per kg dmb) did not cause an increase in the most active vitD metabolite, 1,25(OH)$_2$vitD, but rather a decrease in plasma concentration of 1,25(OH)$_2$vitD with consequently a decreased %absorption and true calcium absorption. The noticed decrease in 1,25(OH)$_2$vitD plasma concentration can be explained being a safety measure by increasing metabolic clearance due to an increased 24-hydroxylase activity, rather than a decreased 1,25(OH)$_2$vitD production (Tryfonidou et al, 2003). The disturbances in endochondral ossification as seen more frequently in these cases than in the control dogs of that study (raised on food with 500 IU/kg), indicate that these disturbances can by a direct or indirect effect of vitamin D or its metabolites. An increase in %absorption of calcium at 22 weeks of age was seen, what was explained by the increased levels of 25(OH)vitD by its direct biological effect on calcium absorption (Table 2) or via an increase of free plasma 1,25(OH)$_2$vitD.

6. The direct relationship between high vitamin D and disturbance of endochondral ossification is supported by the findings in dogs raised on diet with hundred times the recommended amount of vitamin D as given to the controls (500 IU/ kg food on dmb). These dogs had increased level of 24,25(OH)$_2$vitD, and thus not an increased %absorption of calcium, but with severe disturbances of endochondral ossification. Both the disturbed ratio of 1,25(OH)$_2$vitD and 24,25(OH)$_2$vitD, as well as the increased CT levels found in the dogs with the 100 x vitD increased intake were hold responsible for the disturbance in

endochondral ossification. In none of the groups, either the plasma calcium or phosphorus levels were increased. In another study, Labrador retriever puppies with hampered mineralisation of joint cartilage revealed a 200% increase in plasma 25OHvitD concentration and a 300% increase in the plasma $1,25(OH)_2$vitD concentration when fed a diet with a 40 x increased vitD content compared with control dogs (of the same breed and age), fed a balanced dog food, revealed no differences in plasma calcium and phosphorous concentration, minor irregularities in endochondral ossification in their growth plates (Corbee, 2014). These Labradors ranging from 3 till 17 weeks of age, have a slower growth rate than the Great Danes of that age and thus probably a lower GH plasma level.

7. Rodenticides and psoriasis crèmes may contain massive dosages of cholecalciferol (vitD), but also commercial dog and cat foods can contain an excess of vitamin D (Morris & Earle, 1996, Wehner et al, 2013). A dosage of 1.5-8 mg/ kg b.w. is considered toxic. Since vitamin D is lipophilic and distributed predominantly in adipose tissue with an elimination half-life of approximately 2 months, the mono and bi hydroxylated metabolites (i.e. 25OHD and $1,25(OH)_2$D) circulate bound to vitamin D binding protein (DBP), but only the free form of $1,25(OH)_2$D is regarded as the metabolically active hormone.

Table 2. Relative potency of vitD metabolite in a perfused intestinal calcium transport system in chick (According to Yoshimoto & Norman, 1986)

Vitamin D (metabolites)	Relative potency
$1,25(OH)_2D_3$	10,000
1-alpha-hydroxy vitamin D_3	400
24,25-dihydroxy-vitamin D_3	137
vitamin D_3	34

Toxicity from high circulating levels of vitamin D may be due to either displacement of $1,25(OH)_2$D from DBP by excess circulating 25OHD. In addition, 25OHD can directly bind to the vitamin D receptor to activate target genes involved in calcium hemostasis (Table 2). The 25(OH)D metabolite, shows a half-life of approximately 15 days in studies of experimental hypervitaminosis in dogs, whereas $1,25(OH)_2$D has a half-life of approximately 15 h. A potency relative to $1,25(OH)_2$D, was calculated for several vitamin D metabolites. Relative potency is defined as the ratio of concentrations of $1,25(OH)_2$D /analog which were necessary to achieve perfused intestinal calcium transport system in chick. The relative potency of the D homologs tested are given in Table 2. Since $1,25(OH)_2$vitD levels are not necessarily increased, circulating PTH is not totally disappeared (Fig.3). An intake of diet with 60x the recommended amount of vitamin D to kittens and an increase of 120 x the recommended amount to puppies did not reveal disturbances in calcium metabolism (Sih et al, 2001; Tryfonidou et al 2003); apparently the adaptive mechanism of the body for calcium homeostasis is not exceeded in these cases; since skeletal abnormalities developed in the fast growing puppies exceeding the safe upper limit this cannot be without consequences, whereas long term effects on e.g. renal function and cardiovascular health have not been investigated yet. Hypervitaminosis D is characterized by hypercalcemia together with hyperphosphatemia, anorexia, muscle weakness, polyuria and eventually impaired kidney function and mineralization of kidneys, lungs, stomach and blood vessels, which may cause irreversible damage.

In conclusion

Plasma calcium concentration can be increased in young, fast growing dogs and be especially a reflexion of the bone turn over. Due to the high contribution of passive intestinal diffusion at young age, high dietary calcium content will lead to increased calcium absorption in puppies. This can influence the production of the calciotropic hormones with temporarily hypercalcitoninism (when given from 3-6 w.o.a) or even hypoparathroidism when eaten during a longer (3-17 w.o.a.) period. When the hormonal defence mechanisms do not work (yet), the animal will minimize food (and thus extra calcium) intake. Increased calcium content together with increased phosphorous content will increase skeletal mineralisation and disturb endochondral ossification even more than calcium excess alone. Eight or hundred times increased dietary vitD levels will not cause hypercalcemia or hyperphosphatemia, but mobilze the defence mechanism to metabolize $1,25(OH)_2$vitD into $1,24,25(OH)_3$vitD as was demonstrated in large breed dogs, but not demonstrated in medium size dogs or cats yet. In case of high calcium intake in case of weaned puppies or in case of PTH(rP) producing tumours, or intake of cholecalciferol rodenticides or plants containing calcitriol glycosides real hypercalcemia (> 4 mmol/L) can develop.

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Mycotoxins & Pet food safety : which threshold of warning ?

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1

- Context
  - Petfood safety is since some decades of much concern for owners
  - Among all the feed associated hazards,

    pathogens (*Salmonella* sp.)

    bioactive amines (histamine)

    pesticides, chemicals or heavy metals residues

    mycotoxin contamination

    is probably one among the most worrying for owners

2

Two main raisons :
1) mycotoxins occur frequently in raw materials …

Occurrence of 5 mycotoxins in corn grain

submitted for analysis in NC over a 9-year period (Whitlow et al., 1998)

	corn grain		
Mycotoxins	n	%	mean ± sd
Aflatoxin (>10 ppb)	231	9	170 ± 606
Deoxynivalenol (>50 ppb)	362	70	1504 ± 2550
Zearalenone (> 70 ppb)	219	11	206 ± 175
T-2 Toxine (> 50 ppb)	353	6	569 ± 690
Fumonisin (> 1 ppm)	37	60	-

3

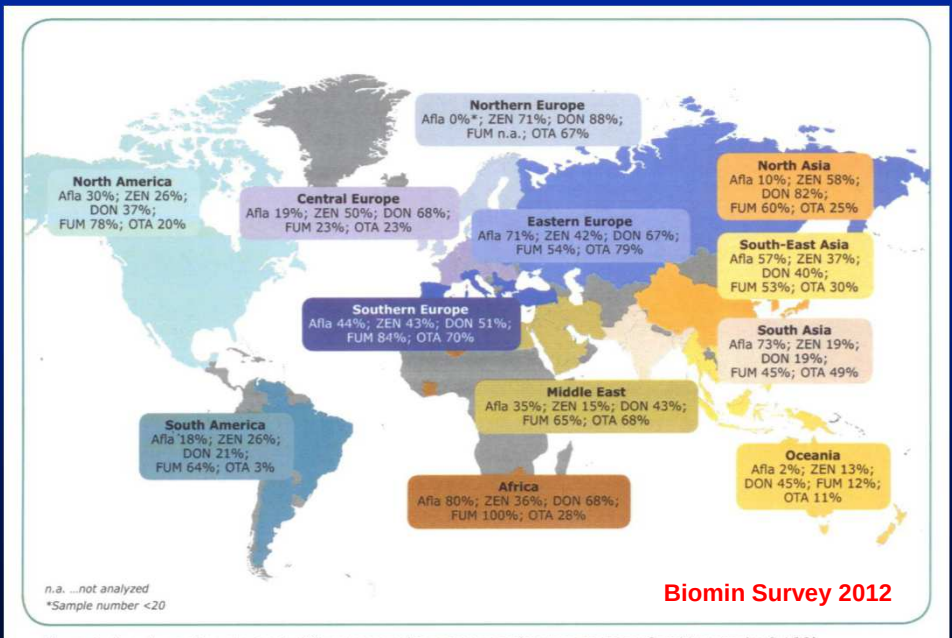


Figure 1. *Prevalence of mycotoxins in different geographic regions according to percentage of positive samples (>LOQ)*

4

… and sometimes in commercial pet foods

Results of some mycotoxins surveys in Europe

Authors	Samples surveyed	Mycotoxins detected
Scudamore	35 dry dog foods	FB1 1/35 (105 ppb)
1997 – UK	35 dry cat foods	FB1 3/35 (90-690 ppb)
Pühringer	12 dog foods (2/10)	OTA dry (0.2-13.1)
2001 – AU & PL	28 cat foods (8/20)	in 47% canned (0.2-0.8 ppb)
2003 – AU	55 cat foods (45/10)	OTA 7+7 (0.11-2.17 ppb)
Martins	20 dog foods	OTA 5/20 (2.0-3.6 ppb)
2003 – P		FB1 3/20 (12-24 ppb)
	20 cat foods	Ø
Zwierzchowski	57 dog & cat foods	ZEA in 84 %
2004 – PL		mean : 36.2 ppb ; max 299.5 ppb

2) More sensitive methods for mycotoxins detection

	Thin Layer Chromato	Gaz Chromato	High Pressure Liquid Chromato	Gaz Chromato Mass Spectro
AFB1	1 µg/kg	0.5 µg/kg	0.3 µg/kg	0.1 µg/kg
ZEA	50 µg/kg	20 µg/kg	5 µg/kg	0.5 µg/kg

So, even if mycotoxins contamination
must rationally appear as unavoidable,
it stays emotionally as unacceptable for owners

6

Agenda

- Definition and global approach
- What are the mycotoxins of interest for pets ?
- How to manage the mycotoxins risk ?

7

- Definition
  - Mycotoxins are secondary metabolites that cause pathological changes called "mycotoxicosis" in all animal species
  - Mycotoxins are low molecular weight compounds (MW : 180 to 600 da) with diverse chemical structure and biological properties
  - There are more than 600 identified mycotoxins, but less than 10 are of practical significance

8

- Molds, mycotoxins and mycotoxicosis :

  a very complex panorama !

  – Moldy feeds aren't obligatory toxic
  – A single mold can produce one or more toxins with potentially synergic effects
  – A single toxin can be produced by one or several moulds
  – A feed apparently safe can be toxic because
    - of a critical level of toxin even after the moulds have disappeared, and even after a detoxifying process
    - of the presence of masked toxins (conjugated ?)

9

- How do Mycotoxins exert their effects ?

  Three primary mechanisms :

  – Reduction of the nutritional value of the diet
  – An immuno-suppressive effect
  – Disturbance of many major biological functions

10

- 1) by reducing the nutritional value of the diet

It may append through three ways :

– 1) Decrease the nutrient content of feed
- Reduction in Vitamin E & amino-acids content (lysine)
- Hydrolysis and Oxidation of PUFA (loss of EFA)

– 2) Decrease digestibility and energy value of feed
- mycotoxin-induced irritation of the digestive tract
- interference with protein metabolism (T-2 toxin)

– 3) Decrease the feed intake
- Reduced palatability due to more acidic and rancid feeds (peroxides, ketones, etc.)
- specific impact of "refusal toxin" (vomitoxin)

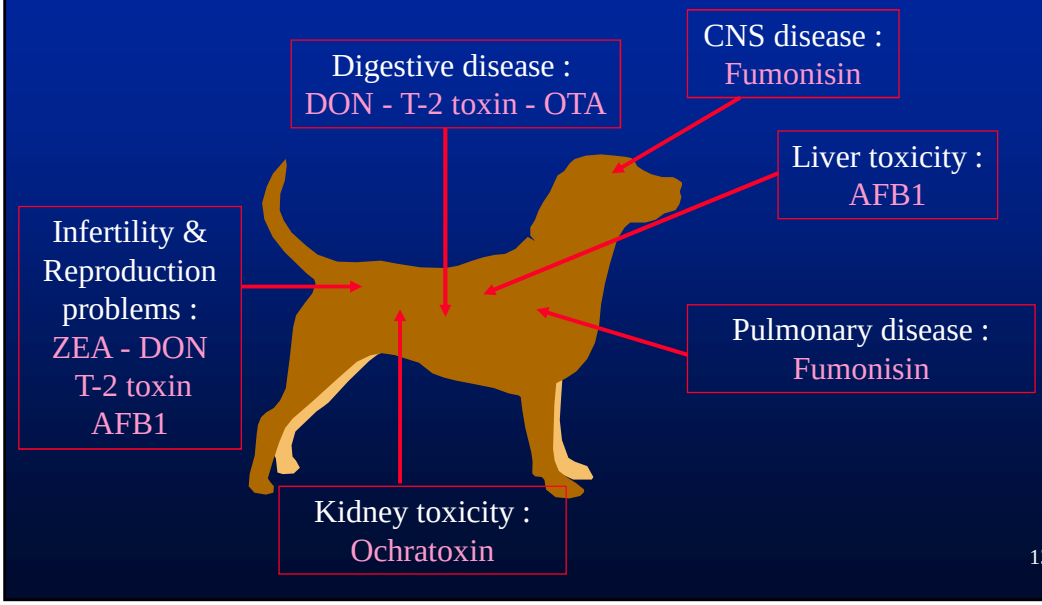
11

- 2) by an immuno-suppressive effects on humoral & cellular responses (after Galtier, 2005)

Level in feeds (mg/kg)	0,2	1	2	10	20	100
aflatoxin B1	reduced cellular response (rats)	reduced lymphocytes proliferation (pigs)				
ochratoxin A		reduced lymphocytes proliferation (chicken)	reduced phagocytosis (pigs)			
fumonisin B1				lymphocytes blastogenesis (pigs)	reduced leucocytes migration (calf)	
deoxynivalenol			reduced humoral response (mice)			
T-2 toxin				reduced humoral response (mice)		

12

- 3) by inducing disturbances of biological functions



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There is a huge variation in acute toxicity among mycotoxins

oral LD50 in mice (mg/kg)

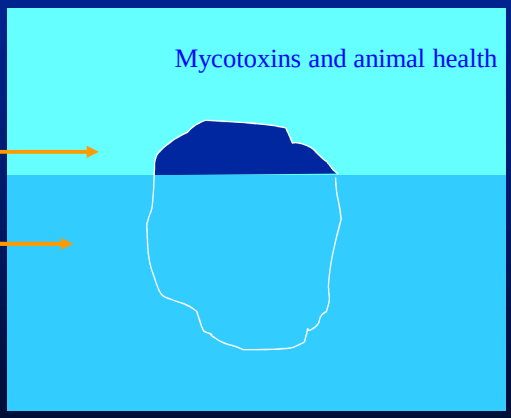
>1000	fumonisins, zearalenone	
500	pénicillic & mycophénolic acids	
200	sterigmatocystin, luteoskyrin, sporidesmin, tenuazonic acid	
100	rubratoxin B, citrinin	
60	PR toxin	
50	deoxynivalenol, nivalenol, ochratoxin A, gliotoxin	
25	patulin, verruculogen, cyclopiazonic acid, citreoviridin	
10	penitrem, *aflatoxin B1* ,	*mice, hamster*
7	diacetoxyscirpenol, T-2 toxin	*rat*
5	fusarenone X	*monkey*
2		*guinea pig*
1		*sheep, **DOG, CAT***
0.5		*pig, rabbit, duckling*

(after Galtier, 2005)

14

One challenging question about mycotoxins :

may be the acute outbreaks with clinical evidences
are not the most important for pets economy !



Are acute Mycotoxicosis
only the tip of the iceberg ?

Chronic Mycotoxicosis

lesser animal productivity (growth, fertility)
decreased efficiency of vaccination & therapy
increased susceptibility to diseases

(after Galtier, 2005)

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I - Which mycotoxins are of interest for pets ?

- Two main circumstances are involved in pet mycotoxicosis :
  - 1) Dry feeds including a significant part of contaminated
    - cereals (corn, wheat) and/or their byproducts
      particularly bran where toxins (AFB & Zea) are at least 3-fold more concentrated compared to the raw kernel
    - seeds (soybean, cotton, sunflower) and/or their derived meals

    - The more frequent *moulds* / toxins association observed are :
      - *Penicillium* sp / Aflatoxin B1 and Ochratoxin A
      - *Fusarium* sp / Trichothecenes : Deoxynivalenol and T-2 toxin, Zearalenone and Fumonisins

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– 2) Eating spoiled food and moldy garbage

- Moldy starch based foods (bread, cakes, pastas) contaminated by *Penicillium* producing toxins (Aflatoxin B)
- Moldy Dairy product contaminated by *Penicillium* producing tremorgenic toxins (Penitrem A; PR toxin)

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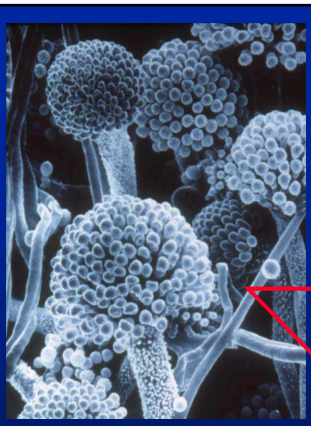
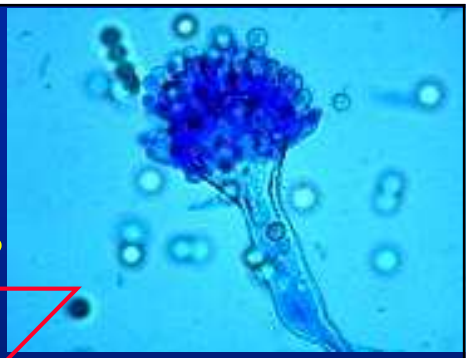
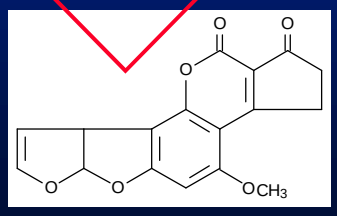


Aflatoxicosis



Aspergillus parasiticus

Aspergillus flavus. (CABI)



Aflatoxin B1

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Many outbreaks described in Dogs, None in Cats !

- Many outbreaks of food borne hepatitis described in dogs since 50 years (first description of hepatitis X by Seibold in 1952, experimentally proved in 1960 by Newberne)
- From 1995 to 2005, 11 outbreaks in USA (one in 1998 has killed 48 dogs) (Stenske, 2006)
- In 2005 Purina Venezuela recall 8700 T dry petfood because of AFB1 contamination – more than 400 dogs died ?
- Last outbreak : recall on Dec 2005 of dry petfood produced in SC by Diamond Pet Food with moldy corn (4 bins from 90 to 1850 ppb AFB1 !) – more than 150 dogs died ?

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Toxicological profile of aflatoxin B1 : hepatotoxic

	Dog	Cat
LD 50	0.5-1.5 mg/kgBw	0.3-0.6 mg/kgBw
Acute toxicity	>6000 ppb	??
(vomiting, depression, polydipsia, polyuria, hepatitis, death within days)		
Sub-acute toxicity	300-500 ppb	??
(anorexia, lethargy, jaundice, dissem intrav coagul°, death within weeks)		
Chronic toxicity	60-300 ppb	??
(*liver dysfunction, hypoproteinemia, death within months)*		
NOAEL dose ??	< 50 ppb ??	

ppb = µg/kg feed NOAEL : no observed adverse effect level

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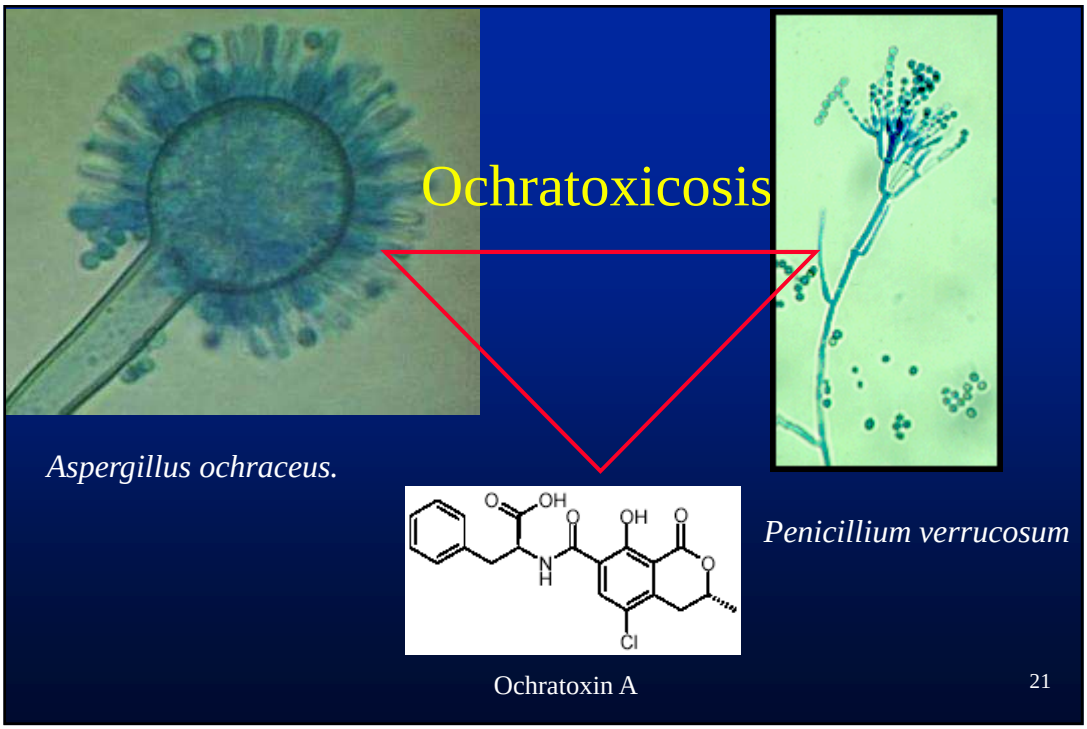


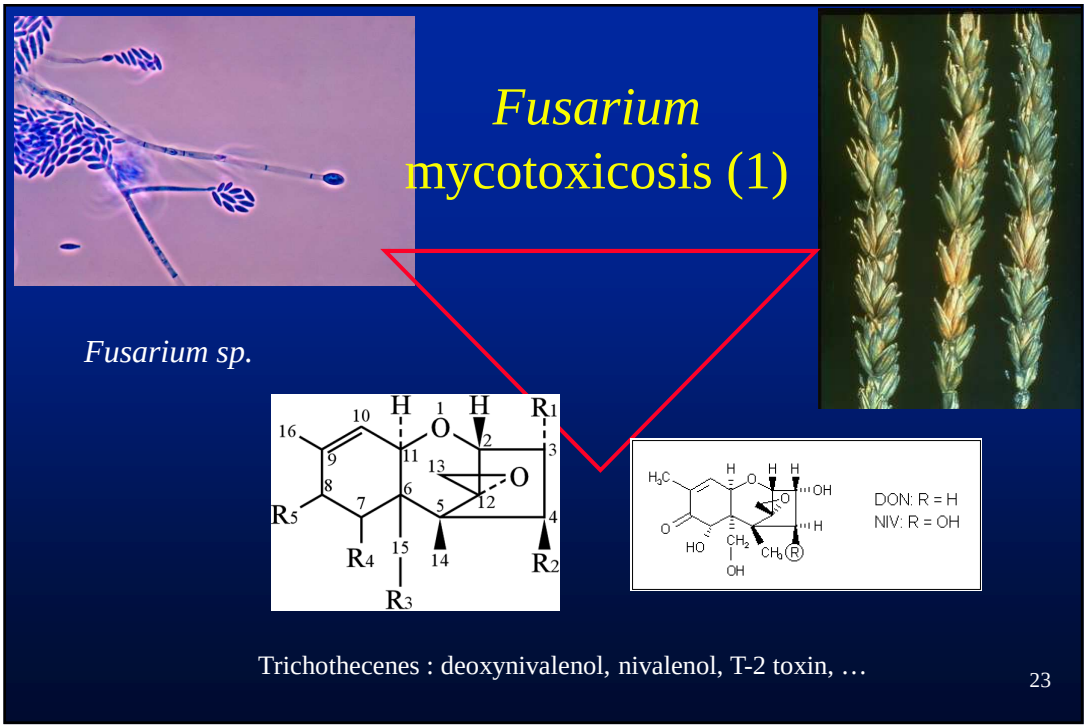
Toxicological profile of ochratoxin A : nephrotoxic

	Dog	Cat
LD 50	?? 20-30 mg/kgBw	??
Acute toxicity	>3000 µg/kgBw	??
(vomiting, severe kidney damage, anorexia, weight loss, death within days)		
Sub-acute toxicity	300 µg/kgBw	??
(anorexia, PUPD, dehydration, vomiting, prostration, death within weeks)		
Chronic toxicity	?? No data ??	??
in Pig : 800 ppb (16 µg/kgBw) mild nephropathy within a year		
NOAEL dose (3 weeks)	< 100 µg/kgBw	??

(Szczech, 1973)

ppb = µg/kg feed

[only experimental data] 22

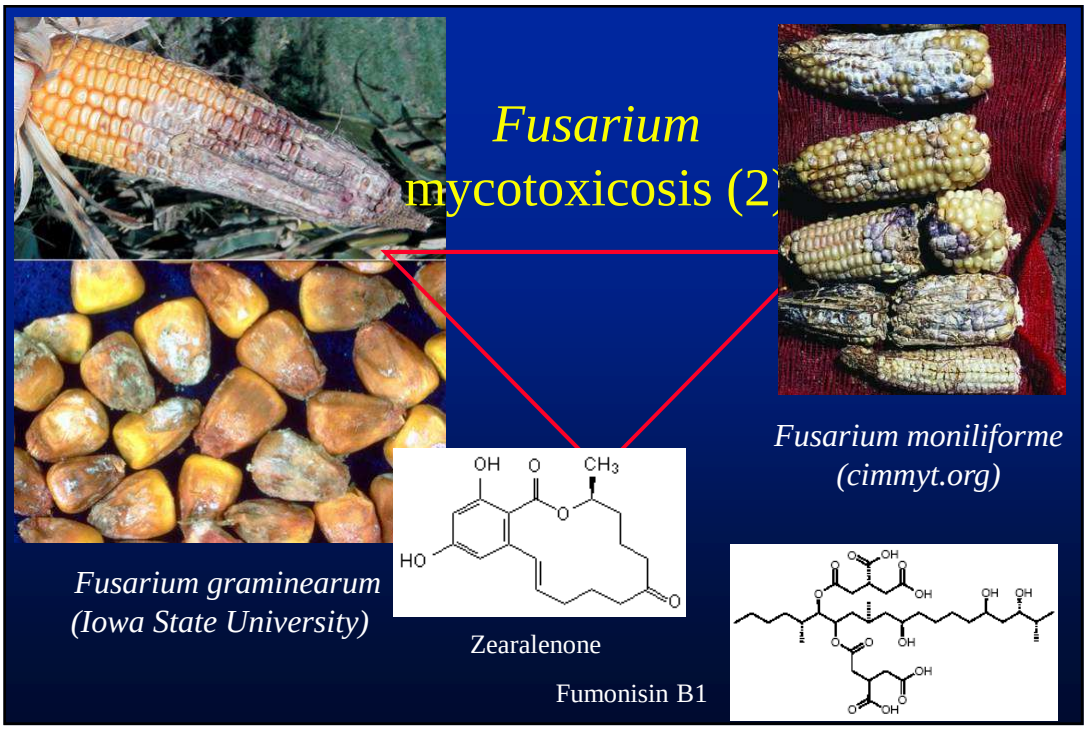


Toxicological profile of trichothecenes : hematotoxic

	Dog	Cat
LD 50	?? T-2 toxin >>> DON ??	
Acute toxicity (T-2 toxin) (Lutsky & Mor, 1981)	??	80 µg/kgBw *(bone marrow aplasia, death within days)*
Sub-acute toxicity	??	???
Chronic toxicity (DON) (Hughes, 1999)	4,5 ppm *(vomiting, feed refusal, anorexia, weight loss)*	7,7 ppm
NOAEL dose (DON) 2w trial	< 60 µg/kgBw *(3 ppm)*	< 110 µg/kgBw *(6 ppm)*

ppm = mg/kg feed

[only experimental data] 24



Toxicological profile of zearalenone : estrogenic

	Dog	Cat
LD 50	very high > 4000 mg/kgBw ??	
Acute & sub acute toxicity	??	??
Chronic toxicity	200 µg/kgBW	??
(changes [hyper-estrogenism] in canine reproductive system within weeks)		
in Gilts : 4-8 ppm (130 µg/kgBw*) hyper-estrogenism within weeks*		
	25-50 µg/kgBW	??
immuno-suppressive – degeneration, atrophy, edema uterus wall within 100d		
NOAEL dose in Gilt (reprod)	10 µg/kgBw	??

ppm = mg/kg feed

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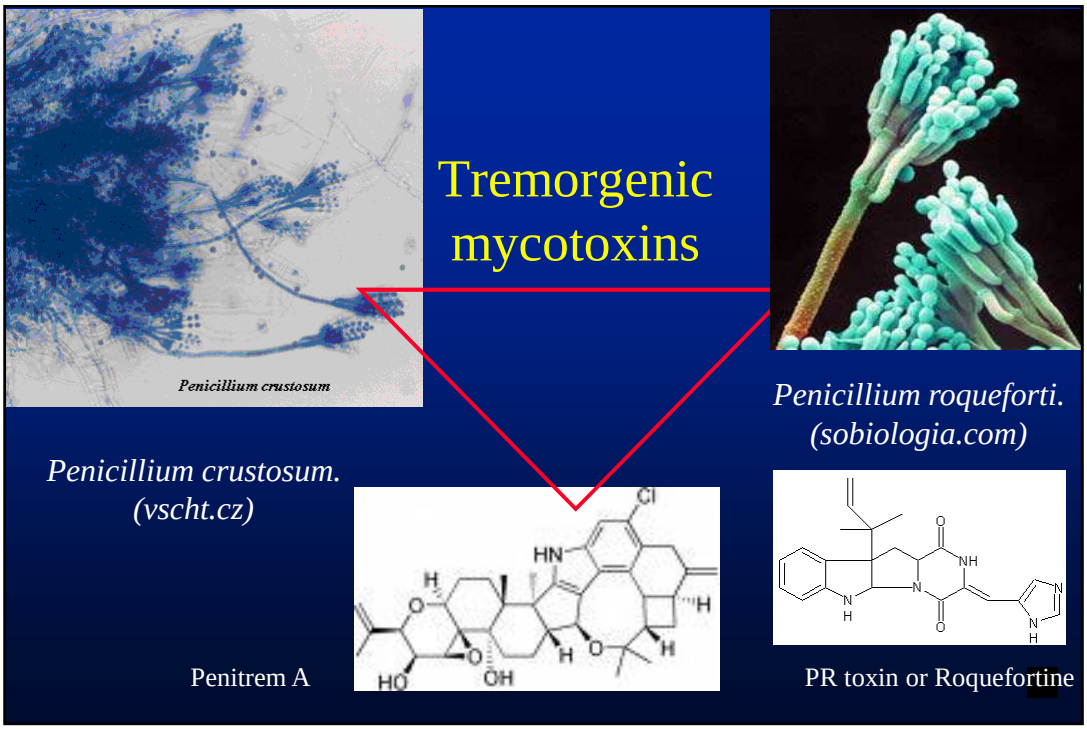

Many cases of penitrem A toxicosis described in Dogs, None in Cats !

– 8 reports of tremorgenic mycotoxicoses have been described in dogs (n=13) since 30 years (first description by Arp & Richard in 1979)

– Never associated with commercial pet food

– Only moldy food & garbage
- Dairy products kept too long time in fridge (3 cases)
- Moldy nuts (2), bread (1), rice (2), etc.
- Garbage (5 cases)

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Toxicological profile of penitrem A : tremorgenic

	Dog	Cat
LD 50	?? *[mice : 1.1 mg/kgBw]* ??	
Acute toxicity	>0.5 mg/kgBW (IP)	??
as an inhibitor of neurotransmitters (glycine, γ–aminobutyric acid)		
(salivation, vomiting, muscle tremor, convulsions, death within hours)		
Sub-acute toxicity	0.1-0.2 mg/kgBW *(vomiting, muscle tremor, ataxia)*	??
Chronic toxicity	??	??
NOAEL dose ??	??	??

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- This overview on mycotoxin toxicity in pet animals clearly underlines
  - the large number of potential mycotoxins
  - the lack of research done specifically with pets and particularly with cats
  - the huge differences between animal species
  - the importance, among many moderating factors, of duration of exposure
  - few information if any on interaction of mycotoxin between them and with feed matrix in natural outbreaks
- With, as a consequence, difficulties to manage mycotoxin risk and to establish levels of safety

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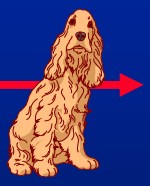
How to manage the mycotoxins risk ?



Feedstuffs production level :	Pet food production level :	Owner level :
Good agricultural practices • pre-harvest control (resistant varieties; balanced fertilization; irrigation; crop rotation) • post harvest control/storage (moisture control; drying; mould growth inhibitors -organic acids-; safe transportation)	grain processing quality of sampling/analysis quality of storage (prevent condensation & re-wetting) removing contamination preventing strategies *in vivo*	good storage of commercial pet food & HMD

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- The petfood manufacturer point of view :
  a technical approach of quality management
  - feed processing & removing contamination
  - systematic sampling/analysis
  - quality of storage
  - preventing strategies *in vivo*

- The regulatory authority point of view : do we need specific mycotoxins contamination thresholds for pets ?

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Pet food manufacturer and quality management (1)

- Feed processing : 3 main processing techniques to reduce mycotoxin content of feeds :
  - Sieving cracked, damaged and improperly developed kernels (↘ 60-80% ZEA & DON)
  - Washing because of mycotoxins are primarily found on the outer surface of grains (↘ 70-90 % ZEA & DON)
  - Abrasive pearling of barley (↘ 66 % DON) and dehulling of corn (↘ 90 % AFB)

  Warning : mycotoxin are logically more concentrated in bran (at least 3-fold for AFB, ZEA & Fumonisins)
- Mycotoxin removal can be done by ozonation and ammonia treatment (AFB)

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Pet food manufacturer and quality management (2)

- Systematic sampling/analysis
  - Sampling operation is the biggest source of error due to uneven distribution of mycotoxin (focus on "hot spot")

  [AFB in corn : n = 72; moy = 15 ppb (0-332 ppb) Johnson, 1969]
  - Individual mycotoxin screening has to be done as soon as possible with a simple, cheap, rapid test (ELISA)
  - If necessary later confirmation with more accurate (& expansive!) methods (TLC, LC, HPLC, GC-MS)
  - Anyway, the mycotoxin screening has to be done preferably before process to avoid toxin-matrix interactions making them less or undetectable to the usual analytical procedures

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Pet food manufacturer and quality management (3)

- Quality of storage :
  - Temperature, moisture (below 15 % for grains) and water activity (below 0.7) must be checked
  - Adding mould inhibitors before loading fresh grain but it doesn't influence the mycotoxins produced earlier !
  - Watch to "wet spot" resulting from moisture transfer consecutive
    - to mixing grains with different temperatures
    - or to "cold wall" effect after sunlight/shade variation with condensation and re-wetting

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Pet food manufacturer and quality management (4)

- Preventing strategies *in vivo* : 3 main processing techniques to reduce mycotoxin content of feeds :
  - Nutrient supplementation
  - Sequestering agents
  - Microbial deactivation

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- Nutrient supplementation : many have been proposed but seem of limited value
  - Neutral AA (Val; Leu; Ile; Tyr & Phe) to compete with Try for active transporter carriers across blood-brain barrier (prevention of neurological effects of T-2 toxin & DON)
  - Antioxidants (Se, vit A, C & E) to limit damage to cell membranes by lipid peroxidation and reduce DNA adduct formation (OTA; ZEA)
  - PUFA (EPA; DHA) to attenuate pro-inflammatory response and renal damage (DON)

Warning : the feasibility of using these dietary supplement to treat mycotoxicosis in pets remains to be confirmed

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- Mycotoxin sequestering agents : old and new facts
  - Clays : Bentonites, Sepiolites and HSCAS (hydrated sodium calcium aluminosilicate, zeolites) used since a long time
    - can reduce intestinal mycotoxin absorption of polar toxin (AFB >> OTA, ZEA >>DON & T-2 toxin)
    - require high inclusion rate (0.5 to 1% clays; 0.1 to 0.5% HSCAS)
    - may also bind useful nutrients (trace elements)
    - Bentonite (UE approved tech add R (UE) N° 1060/2013 Rumt Poultry Swine for AFB)
  - Glucomannan-containing polymer from yeast cell wall :
    - high absorptive capacity for binding a larger combination of different mycotoxins
    - binding capacity function of β-glucan concentration
    - require a much lower inclusion rate (0.05 to 0.2%)
    - efficacy *in vitro* has to be confirm *in vivo* in all species

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Mycotoxins binding capacity of glucomannan *in vitro*

(after Devegowda, 2000)

Mycotoxins	binding capacity (%)
aflatoxins	95,0
fumonisins	67,0
zearalenone	77,0
T-2 toxin	33,4
deoxynivalenol	12,6
ochratoxin A	12,5
nivalenol	8,2

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- Microbial deactivation : a very promising topic
  - a new concept inherited from the ability of rumen microbiota to deactivate almost partially most of the mycotoxins
  - the microorganisms act in the intestinal tract of animals prior to mycotoxin absorption

R (CE) N°386/2009 has opened a new functional group of technological additives : 1) m) mycotoxins adsorbants or denaturants

Several strains are studied – up to now only one received UE agreement :

- *Eubacterium* / *Coriobacteriaceae* family

  ⟹ Biomin BBSH 797 (UE approved R (UE) N°1016/2013)

  efficient on TTC (DON by enzymatic reduction of epoxide group)
- *Trichosporum* (Schatzmayr, 2006)

  A yeast supposed to be active on OTA & ZEA

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The regulatory authority point of view :
do we need specific mycotoxins contamination thresholds for pets ?

- Pet food is regulated by a maximum mycotoxin contamination as for all feedstuffs but there is NO pet specific legislation
- At present, only AFB1 is considered by EU regulation
  [Council Dir 2002/32/CE modified by Dir 2003/100/CE]
- For the others mycotoxins there are only issued guidance values [Commission Recommendation 2006/576 & 2013/637]

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- Most of these guidelines do NOT specifically focus on Pets
- UE regulations of mycotoxin contamination reflects more analytical detection limits and regional prevalence than the search of a safe limit for mycotoxin exposure in pet animals

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Do we need specific aflatoxin B1 contamination thresholds for pets ?

- Present regulation (Dir 2002/32/CE modif by Dir 2003/100/CE) :
  - Commercial feeds for Farm animals : 20 µg/kg
  - Other animals (implicitly Pets) : 10 µg/kg
- Is-it adequately safe for pets ?
  - NOAEL dose : < 50 µg/kg
  - Safety margin : 5/1
  - Safe level proposed : 10 µg/kg So YES it is

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Do we need specific ochratoxin A contamination thresholds for pets ?

- NO regulation Only advisory guideline (Recomm 2006/576) :
  - Commercial feeds for Pigs : 50 µg/kg
  - Even implicitly, none for Pets : ???
- How can we propose an adequately safe level for pets ?
  - NOAEL dose 3 w trial (Szczech, 1973) : 100 µg/kgBw
  - LOAEL with pigs (one year) 16 µg/kgBw
  - A 15 kgBW dog eating 300g dry food : 16x15/0.3 ≅ 800 µg/kg
  - Safety margin : 10/1
  - Safe level proposed : 80 µg/kg

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Do we need specific trichothecenes (*DON*) contamination thresholds for pets ?

- NO regulation Only advisory guideline (Recomm 2006/576) :
  - Commercial feeds for Pigs : 0.9 mg/kg
  - Other animals (implicitly Pets) : 5 mg/kg
- How can we propose an adequately safe level for pets ?
  - NOAEL with dogs/cats (2 weeks) 60/110 µg/kgBw
  - A 15 kgBW dog eating 300g dry food : 60x15/0.3 ≅ 3 mg/kg
  - A 4 kgBW cat eating 70g dry food : 110x4/0.07 = 6 mg/kg
  - Safety margin : 5/1
  - Safe level proposed (dog/cat) : 0.6 / 1.2 mg/kg

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Do we need specific trichothecenes (*T-2 toxin*) contamination thresholds for pets ?

- NO regulation only a recent advisory guideline (Rec 2013/637) :
  - Commercial feed for cats : 50 µg/kg
- Is-it adequately safe level for pets ?
  - Cat seems to be highly sensitive
    acute toxicity : 80 µg/kgBw
  - A 4 kgBW cat eating 70g dry food : 80x4/0.07 = 4570 µg/kg
  - Safety margin : 100/1
  - Safe level proposed (cat) : 45 µg/kg
    so UE proposal seems to be OK

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Do we need specific zearalenone contamination thresholds for pets ?

- NO regulation Only advisory guideline (Recomm 2006/576) :
  - Commercial feeds for Gilts/Sows : 100/250 µg/kg
  - Even implicitly, none for Pets : ???
- How can we propose an adequately safe level for pets ?
  - NOAEL with gilts (reprod) 10 µg/kgBw
  - LOAEL in Bitches (immun/reprod) : 20/25 µg/kgBw
  - A 4 kgBW breeding Queen eating 100g dry food
    A 15 kgBW breeding Bitch eating 400g dry food :
    10x4/0.1 = 400 µg/kg & 10x15/0.4 = 375 µg/kg
  - Safety margin : 5/1
  - Safe level proposed : 80 µg/kg

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Do we need specific fumonisins B contamination thresholds for pets ?

- NO regulation Only advisory guideline (Recomm 2006/576) :
  - Commercial feeds for Horse & Pets : 5 mg/kg
- Is-it adequately safe for pets ?
  - LOAEL (Horses, the most sensitive species) : 0.2 mg/kgBW
  - NO data available for pets
  - A 15 kgBW dog eating 300g dry food : 0.2x15/0.3 ≅ 10 mg/kg
  - Safety margin : 5/1
  - Safe level proposed : 2 mg/kg

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Take Home Message

- Zero risk doesn't exist ! Pets (and owners) have to live with food borne risk, and primarily mycotoxins
- Specific maximum contamination levels for pets have to be fixed to make owners more confident with pet food
- We need also to answer to three hot questions :
  - 1) what are the long term effects of small doses of mycotoxins and particularly for immunity, disease resistance, fertility ? Specific trials have probably to be conducted on pets !
  - 2) what about synergistic interactions among mycotoxins in pets ?
  - 3) are we sure to analyze the true quantity of mycotoxins presents in feed ? What about masked and matrix-binded mycotoxins escaping laboratory detection but still toxics ?

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Stiftung Tierärztliche Hochschule Hannover
University of Veterinary Medicine Hannover, Foundation
Institute for Animal Nutritition (Head: Prof. Dr. J. Kamphues)

RESIDENCY CLASS UTRECHT
Workshop on toxic plants
September 10th

ESVCN Congress 2014
Utrecht University

Cause and Effect: Plant Poisoning in Large Herbivores (Horse, Goat)

Sabine Aboling

What we will do during the workshop on toxic plants

Indoor

- Thinking about four questions
- Following four case reports
- Trying to answer question No. 4
- **Highlight 1**: Survival strategies of large herbivores

  → How to avoid plant poisoning?

Outdoor

- **Highlight 2**: Survival strategies of plant species (Hortus Botanicus)

  → How to defend oneself?

Daily situation

Dear Vet,

my young goat is browsing around, and I was told that foxglove and cherry are toxic for goats and that it (the goat) is going to be poisoned.

However, it is impossible to remove at least the trees (see picture). Shall I put my goat into the stable?

Sincerely

K. H.

„Toxic plants“: Getting into the topic by arising questions

1. What is a toxic plant?

   ……

2. What can be done best in order to shield animals from poisoning?

   ……

3. What happens if animals come into contact with toxic plants?

   ……

4. In which context does a plant have a toxic effect on animals?

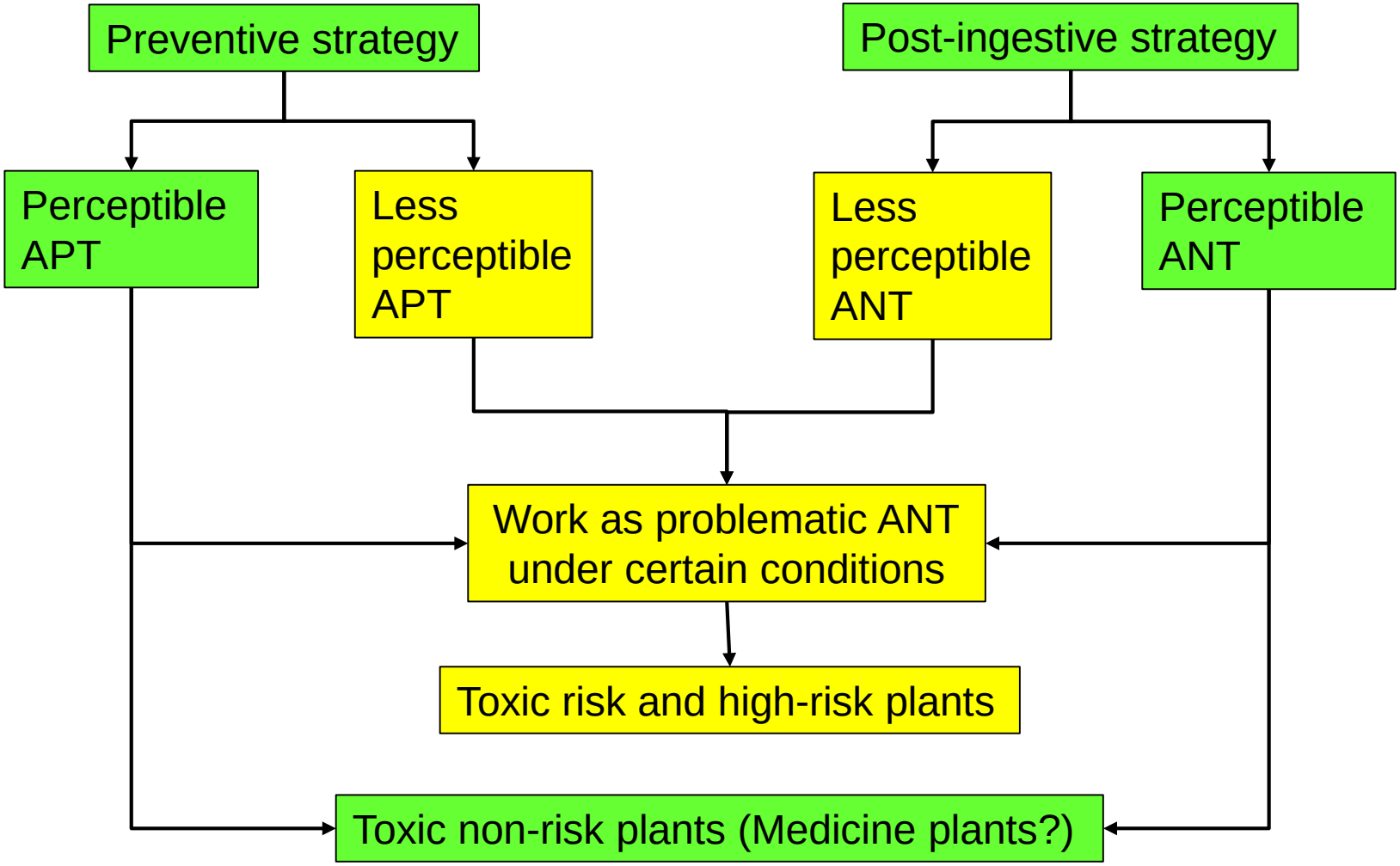
Plant-herbivore-interaction under natural conditions

1. **Preventive strategy** →
   Reduce or prevent eating by mechanical **anti-pastoral traits (APT)** as thorns, hairs and chemical compounds as lignin, silica, bitterns that are supposed to be perceptible by herbivores in advance.

2. **Post-ingestive strategy** →
   Reduce or prevent eating by **anti-nutritional traits (ANT)** as alkaloids, phenols, glycosides, that are supposed to be perceptible by herbivores after they have eaten the plant.

3. **Trading-off strategy** →
   Lacking or possessing only minor ANT and APT. Thus, these plants are rather palatable. Moreover, they regenerate fast, allowing herbivores to feed extensively on them.

When APT and ANT become problematically



Checklist of 9 possible causes (context) of plant poisoning along with prominent examples

Context of plant poisoning	Fact and effect	Plant	Animal
Failing of preventive or post-ingestive strategies	Animal consumes unlimited amount of ANT or APT	*Taxus*	Goat
Exceptional situation	• Curiosity leads to eating (much) • Not adapted animal • No perception of APT/ANT	*Taxus*	Goat
Humid climate (Pastures)	Fungi can be present	*Claviceps Pastinaca*	Horse
Early or late in the year (Pastures)	Fruits and fungi can be present	*Claviceps Pastinaca*	Horse
Range of feed choice small (species-poor, scarce offer)	Hunger lowers perception of APT/ANT	*Senecio*	Horse
Selection confined (feed conserves)	Small particle size lowers APT, not ANT	*Anthox-anthum*	Horse/ cattle
Feed conserve	Conserving lowers APT, not always ANT	*Senecio*	Horse
Animal species	APT/ANT are species-specific in the plant-animal interaction	*Equisetum*	Horse/ cattle
Individual disposition of the animal	Hunger lowers perception of APT/ANT	*Glyceria*	Cattle

Case 1:
Senecio jacobaea. Checking the context

Context of plant poisoning	Fact and effect	Case 1
Failing of preventive or post-ingestive strategies	Animal consumes unlimited amount of ANT or APT	
Exceptional situation	• Curiosity leads to eating (much) • Not adapted animal • No perception of APT/ANT	
Humid climate (Pastures)	Fungi can be present	
Early or late in the year (Pastures)	Fruits and fungi can be present	
Range of feed choice small (species-poor, scarce offer)	Hunger lowers perception of APT/ANT	
Selection confined (feed conserves)	Small particle size lowers APT, not ANT	
Feed conserve	Conserving lowers APT, not always ANT	
Animal species	APT/ANT are species-specific in the plant-animal interaction	
Individual disposition of the animal	Hunger lowers perception of APT/ANT	

Case 2:
Claviceps purpurea. Checking the context

Context of plant poisoning	Fact and effect	Case 2
Failing of preventive or post-ingestive strategies	Animal consumes unlimited amount of ANT or APT	
Exceptional situation	• Curiosity leads to eating (much) • Not adapted animal • No perception of APT/ANT	
Humid climate (Pastures)	Fungi can be present	
Early or late in the year (Pastures)	Fruits and fungi can be present	
Range of feed choice small (species-poor, scarce offer)	Hunger lowers perception of APT/ANT	
Selection confined (feed conserves)	Small particle size lowers APT, not ANT	
Feed conserve	Conserving lowers APT, not always ANT	
Animal species	APT/ANT are species-specific in the plant-animal interaction	
Individual disposition of the animal	Hunger lowers perception of APT/ANT	

Case 3:
Pastinaca sativa. Checking the context

Context of plant poisoning	Fact and effect	Case 3
Failing of preventive or post-ingestive strategies	Animal consumes unlimited amount of ANT or APT	
Exceptional situation	• Curiosity leads to eating (much) • Not adapted animal • No perception of APT/ANT	
Humid climate (Pastures)	Fungi can be present	
Early or late in the year (Pastures)	Fruits and fungi can be present	
Range of feed choice small (species-poor, scarce offer)	Hunger lowers perception of APT/ANT	
Selection confined (feed conserves)	Small particle size lowers APT, not ANT	
Feed conserve	Conserving lowers APT, not always ANT	
Animal species	APT/ANT are species-specific in the plant-animal interaction	
Individual disposition of the animal	Hunger lowers perception of APT/ANT	

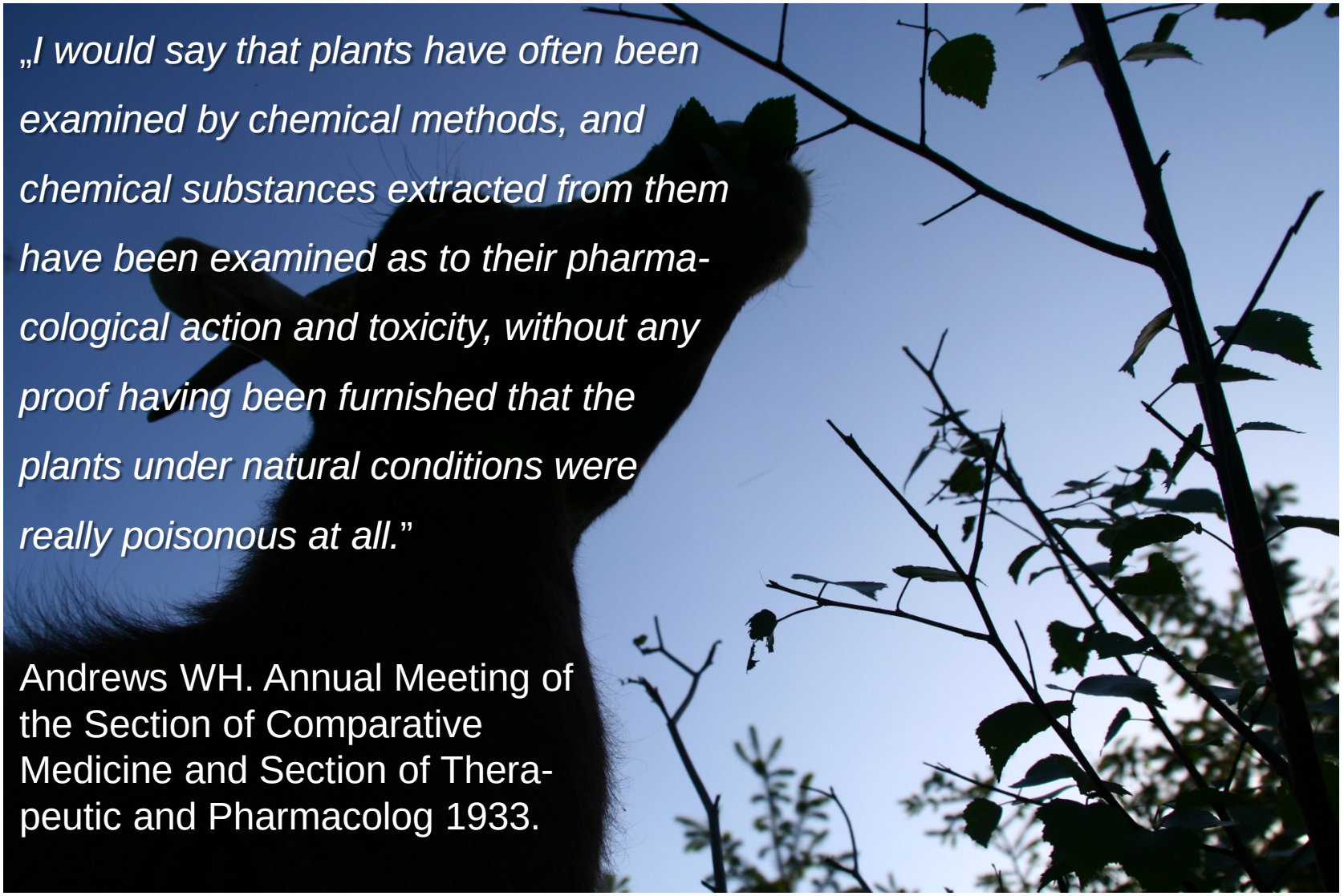
Case 4:
Glyceria maxima. Checking the context

Context of plant poisoning	Fact and effect	Case 4
Failing of preventive or post-ingestive strategies	Animal consumes unlimited amount of ANT or APT	
Exceptional situation	• Curiosity leads to eating (much) • Not adapted animal • No perception of APT/ANT	
Humid climate (Pastures)	Fungi can be present	
Early or late in the year (Pastures)	Fruits and fungi can be present	
Range of feed choice small (species-poor, scarce offer)	Hunger lowers perception of APT/ANT	
Selection confined (feed conserves)	Small particle size lowers APT, not ANT	
Feed conserve	Conserving lowers APT, not always ANT	
Animal species	APT/ANT are species-specific in the plant-animal interaction	
Individual disposition of the animal	Hunger lowers perception of APT/ANT	

Is there anything to conclude in general?

In which context does a plant have a toxic effect on animals?

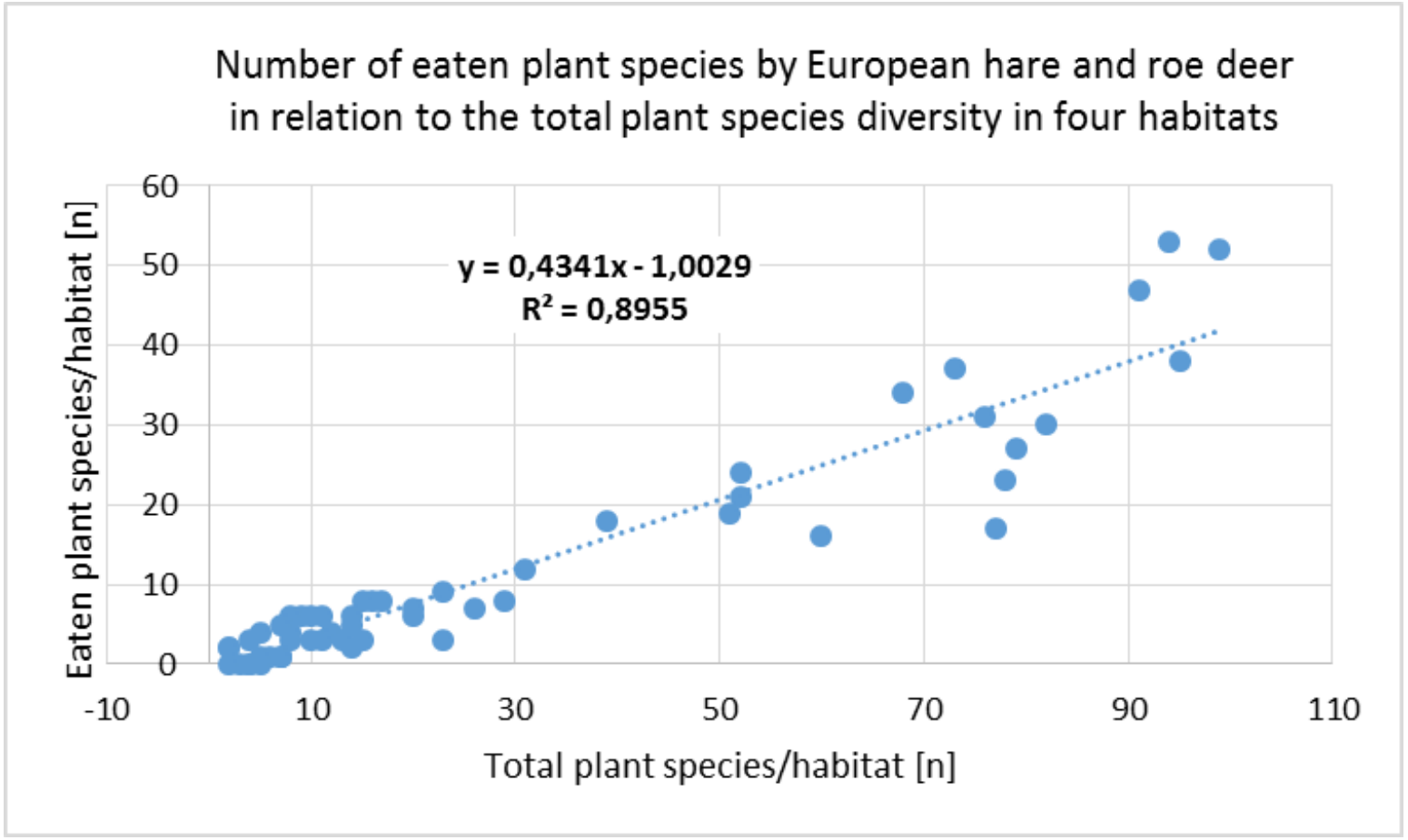
Highlight 1: Survival strategies of large herbivores
→ How to avoid plant poisoning?



„I would say that plants have often been examined by chemical methods, and chemical substances extracted from them have been examined as to their pharmacological action and toxicity, without any proof having been furnished that the plants under natural conditions were really poisonous at all.”

Andrews WH. Annual Meeting of the Section of Comparative Medicine and Section of Therapeutic and Pharmacolog 1933.

Highlight 1: Survival strategies of large herbivores
→ How to avoid plant poisoning?



Four habitats are considered: Field-borderline (n=14), maize field (n=14), oat field (n=16), fallow (n=10)

Finishing with answering the questions

1. What is a toxic plant?

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2. What can be done best in order to shield animals from poisoning?

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3. What happens if animals come into contact with toxic plants?

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4. In which context does a plant have a toxic effect on animals?

Answer of question 4 - or „How to identify, estimate and avoid a plant poisoning“

1. The more confined the feeding conditions (species-poor, scarce offer, selection confined, conserve) the more APT and ANT become less perceptible.
2. The less APT and ANT are perceptible, the higher the risk of poisoning is.
3. Thus, a toxic non-risk plant such as *Senecio* can be turned into a high-risk plant due to confined feeding conditions. Make the conditions as natural as possible.
4. Keep attention to any exceptional situation, humid climate (pasture), sensitivity of the animal species and individual disposition.
5. A young animal should be allowed to taste of everything little in order to develop a diverse microflora in its gut and a well-equipped immune system.

Did I forget something?

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Shall I put my goat into the stable?

Context of plant poisoning
Failing of preventive or post-ingestive strategies
Exceptional situation
Humid climate (Pastures)
Early or late in the year (Pastures)
Range of feed choice small (species-poor, scarce offer)
Selection confined (feed conserves)
Feed conserve
Animal species
Individual disposition of the animal