



# Can we get information on dairy cows chronic stress biomarkers using milk MIR spectra?

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# Welfare as hot topic

## Le Monde

### Belgium enshrines animal welfare in Constitution

Belgium's Constitution now requires all new decisions to take account of their potential impact on animals. The practical implementation will play out at the regional level.

By Elena Louazon

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Dans un abattoir, près de Bruxelles, le 27 juillet 2017. YVES HERMAN / REUTERS

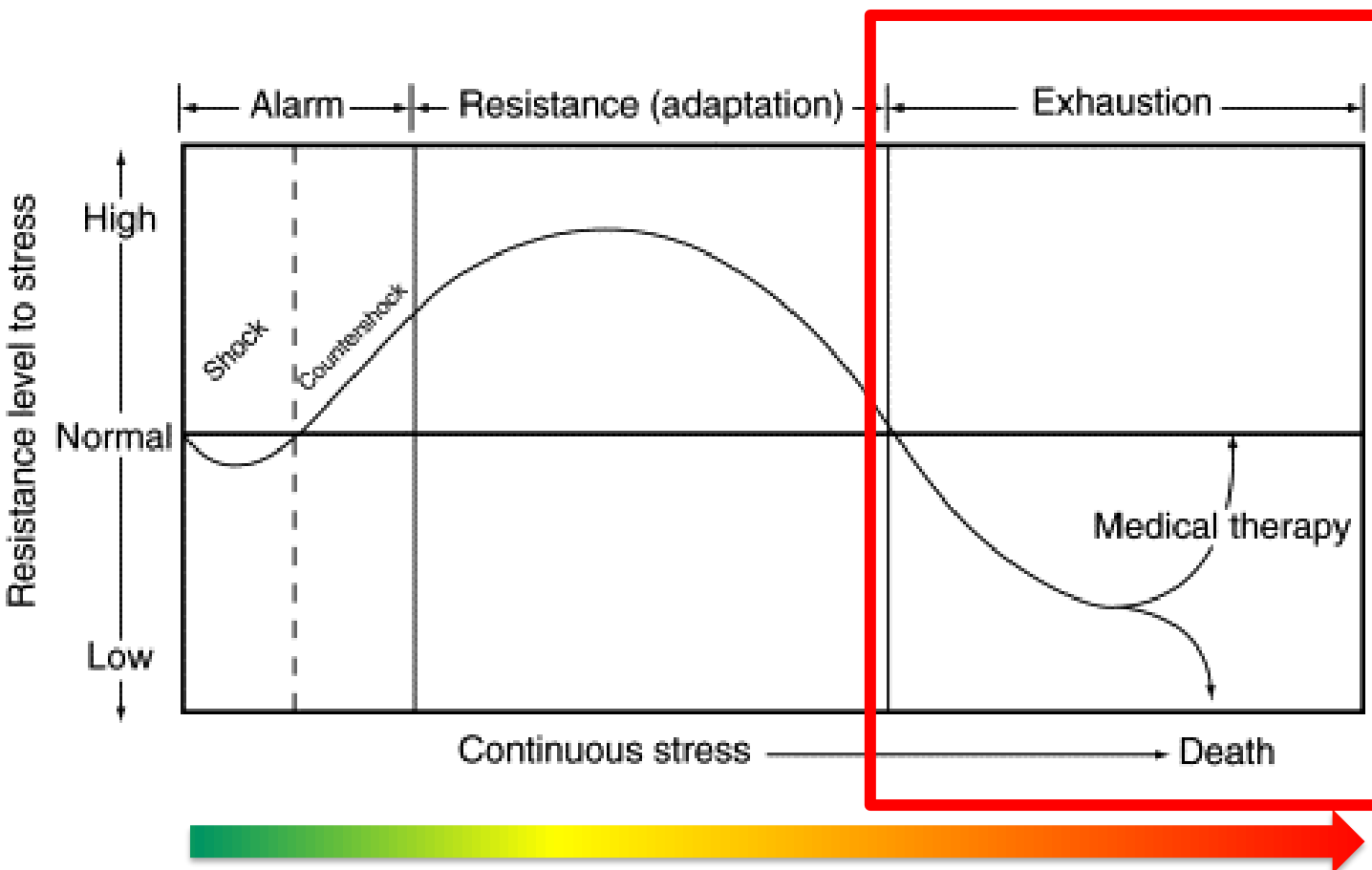
*France - Welfare logo on animal products*



Also in Germany, Slovenia,  
Luxembourg, Italia,  
Autstria, Switzerland,  
Egypt, Brazil and India

# Chronic stress

“stress is the non-specific response of the body to any demand made upon it” (Selye, 1976)



➤ susceptibility to metabolic, inflammatory and infectious diseases (Moberg et al., 1980; Romero, 2004).

➤ fertility troubles (Dobson and Smith, 2000; Walker et al.)

➤ growth disturbances (Eler)

➤ weight (Morm)

➤ milk product

**No consensus in literature regarding chronic stress biomarkers**

➤ production and economics of farms,

➤ welfare of cows

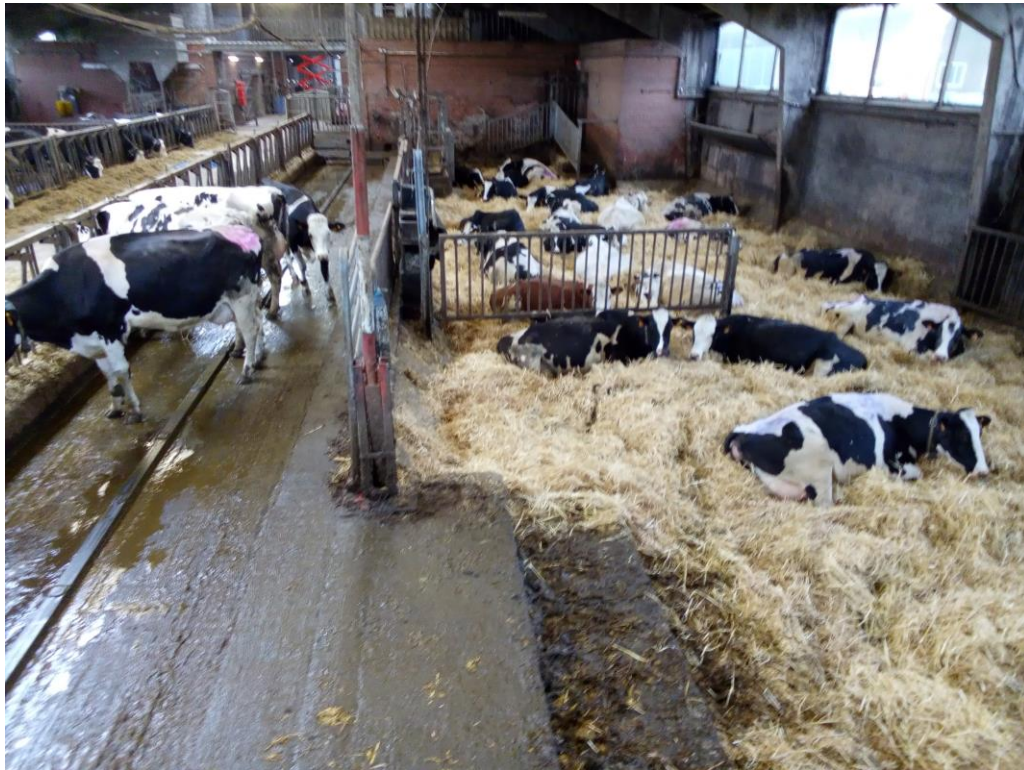
➤ societal perception of dairy production



# 2020 experiment : identify chronic stress biomarkers

## Control group

- 15 cows
- $>10\text{ m}^2$  per cow
- more feed bunks than cows



## Stress group (during 4 weeks)

- 15 cows
- severe overstocking  $< 5\text{ m}^2$  per cow
- restricted access to feed (1 feed bunk for 2 cows)
- punctual unusual events





## Global measures

- MY
- SCC
- weight
- BCS
- MIR predictions  
(milk & blood composition)

## Heart monitoring



## Blood (Glucose, Fructosamin ,T4, $\beta$ -endorphine, leucocytes)



## Behaviour

- observations
- avoidance distance
- rumination
- activity

## Saliva (cortisol)



## Hair (cortisol)

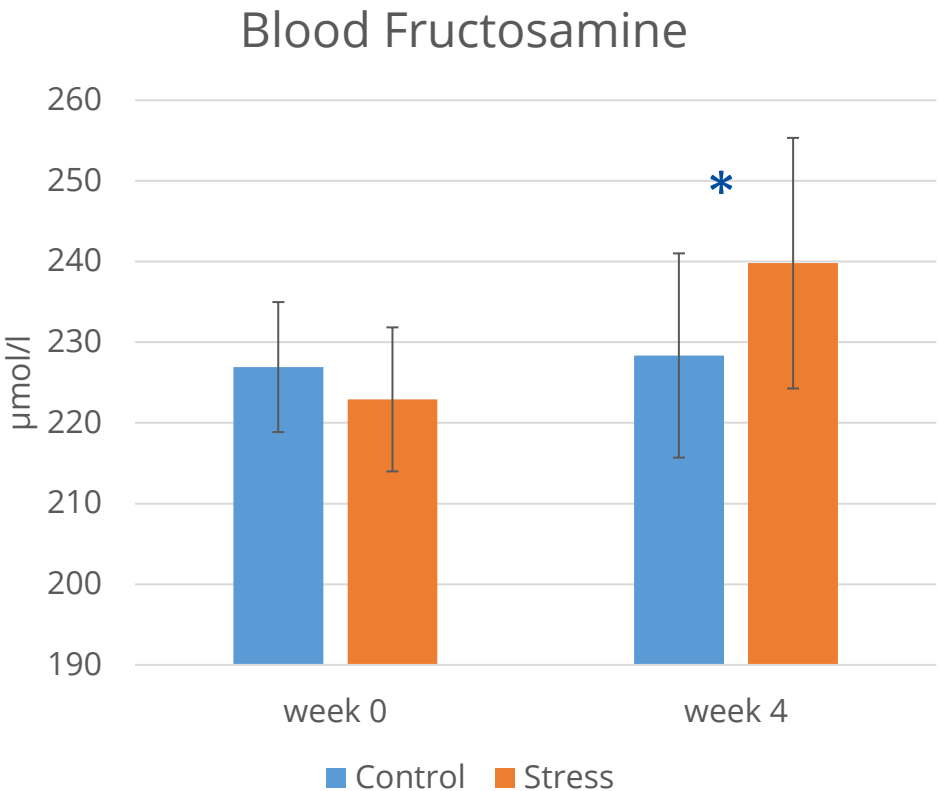
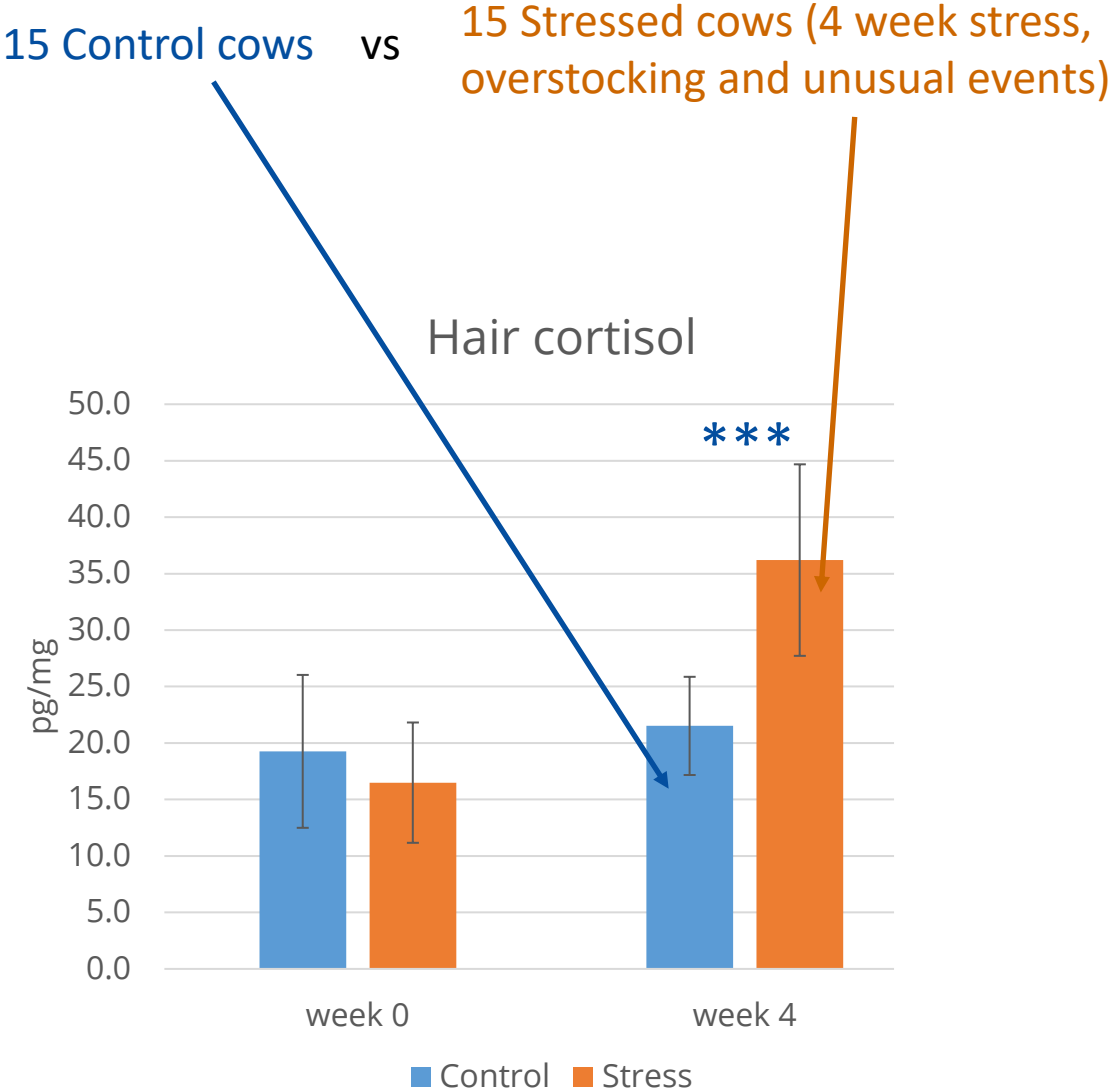


# Chronic stress biomarkers



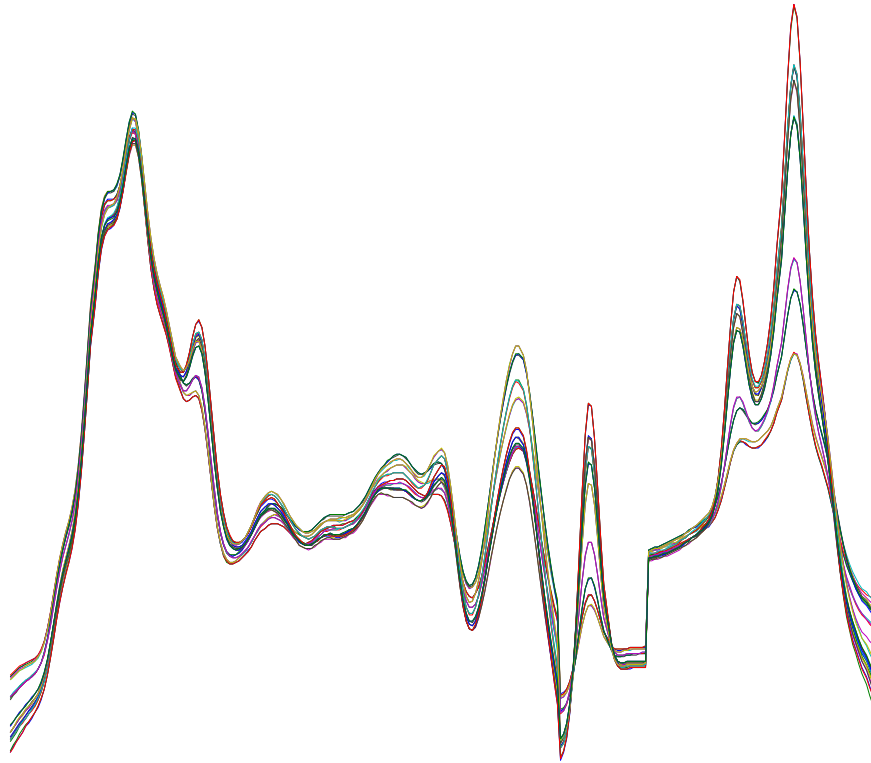
## Identification of chronic stress biomarkers in dairy cows

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(\*)  $P \leq 0.1$   
\*  $P \leq 0.05$   
\*\*  $P \leq 0.01$   
\*\*\*  $P \leq 0.001$

# Can we predict Hair Cortisol and Blood Fructosamine with milk MIR spectra???

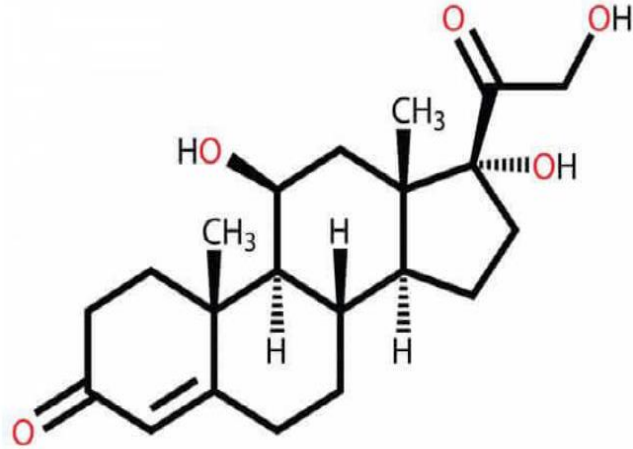


Other indirect phenotypes?

- ✓ Molecules in blood (Luke et al., 2019)
- ✓ Dry Matter intake (McParland et al., 2011)
- ✓ Methane emissions (Dehareng et al., 2012)

# Can we predict Hair Cortisol and Blood Fructosamine with milk MIR spectra???

## Hair cortisol



✚ Gold standard chronic stress biomarker

(Comin et al., 2013; Burnett et al., 2015; Heimbürge et al., 2019; Vesel et al., 2020; Tallo-Parra et al., 2017b).

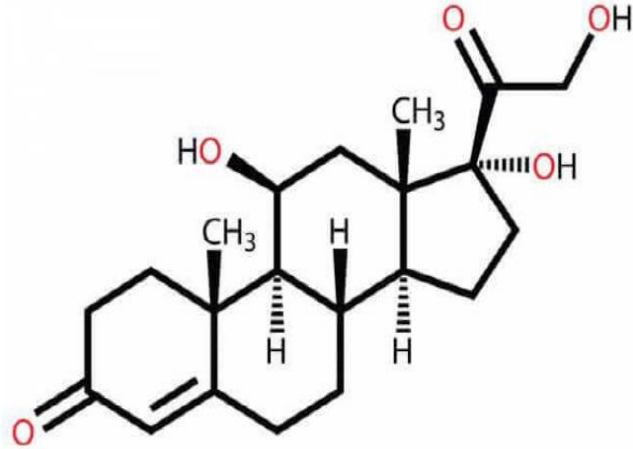
✖ ~ 20 ng/g

Low probability to have an associated signal in MIR



# Can we predict Hair Cortisol and Blood Fructosamine with milk MIR spectra???

## Hair cortisol

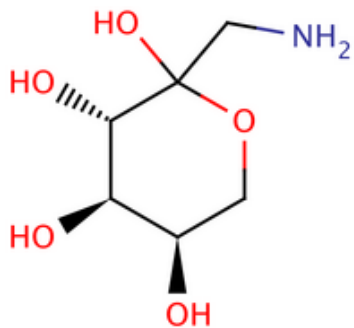


**+** Gold standard chronic stress biomarker

(Comin et al., 2013; Burnett et al., 2015; Heimbürge et al., 2019; Vesel et al., 2020; Tallo-Parra et al., 2017b).

**-** ~ 20 ng/g  
Low probability to have an associated signal in MIR

## Blood fructosamine



**-** Indicator of 3 weeks glycemia, rather « unknown indicator » with few informations

- High: chronic stress impact on energy metabolism (higher content of circulating blood glucose)
- Low: undernutrition, energy deficit, fatty liver in dairy cows (Caré, 2018; Mostafavi, 2014)

**+** Linked with energy metabolism: better probability to have information with MIR

# Large scale sampling protocol

Countries	Partners	n cows
Austria	LKVAustria	159
	AWE	35
Belgium	CRAW	170
	ILVO	39
France	BCO	104
	Doubs	111
	LTN	117
	PDD	56
	Rhone	55
	Seenorest	131
	Seenovia	145
Germany	LKVBW	180
Luxembourg	Convis	36
		1338



- ✓ 78 herds
- ✓ All parities
- ✓ All lactation stages
- ✓ Grazing and winter seasons
- ✓ Mountain and plain areas
- ✓ 7 Breeds (*Holstein, Crossed, Montbeliarde, Simmental, Vorderwalder, Brown Swiss, Jersiaise*)

# Large scale sampling protocol

## Recommendations

- 25% cows with good welfare
- 75% cows potentially suffering from chronic stress for at least one month

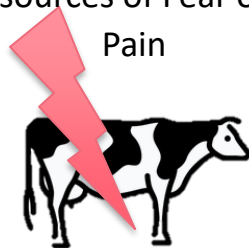
Overstocking



Chronic diseases



Permanent  
sources of Fear or  
Pain



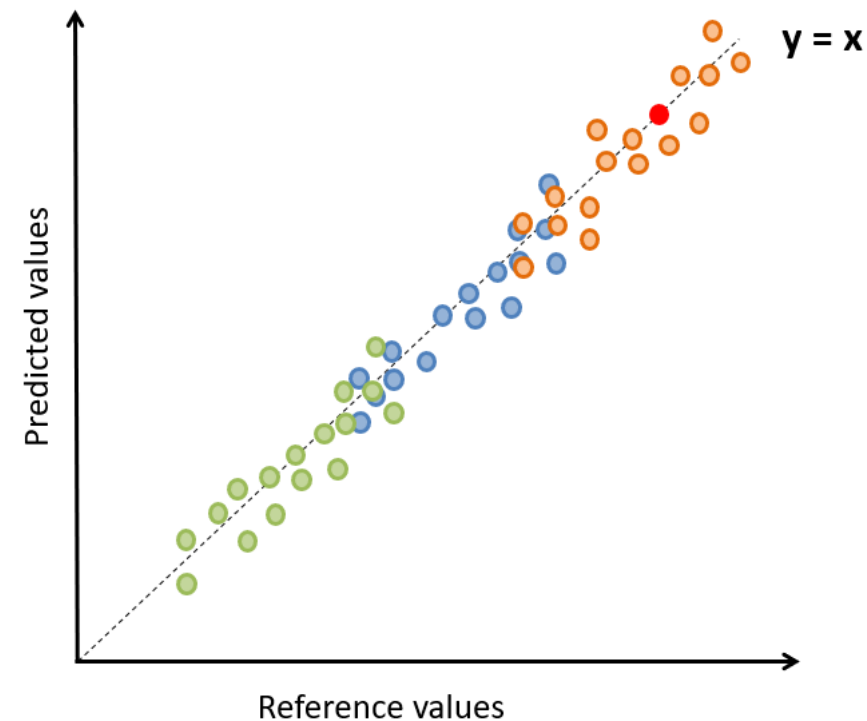
Environmental discomfort



Limited access to  
food or water



Inadequate barn  
design or location





# Large scale sampling protocol

**At the same the day** (or day before)

- ✓ 1 milk sample
- ✓ 1 blood sample
- ✓ 1 hair sample



## Milk sample for MIR analysis

Analyzed locally on 22 MIR instruments (15 Foss, 2 Delta, 5 Bentley)

Standardized with CRA-W/EMR method



## Blood sample for fructosamine analysis

Tail vein (*vena caudalis*) with Yellow dry tubes (*Serum separating gel*)

Centrifugation (2500 g for 10 min at 2-8°C)

Serum pipetting

Analysis at Synlab (Liège, Belgium) with spectrophotometric methods (Westgard et al., 2017)



# Large scale sampling protocol

## Hair sample for cortisol analysis

Hairs collected at the tail switch

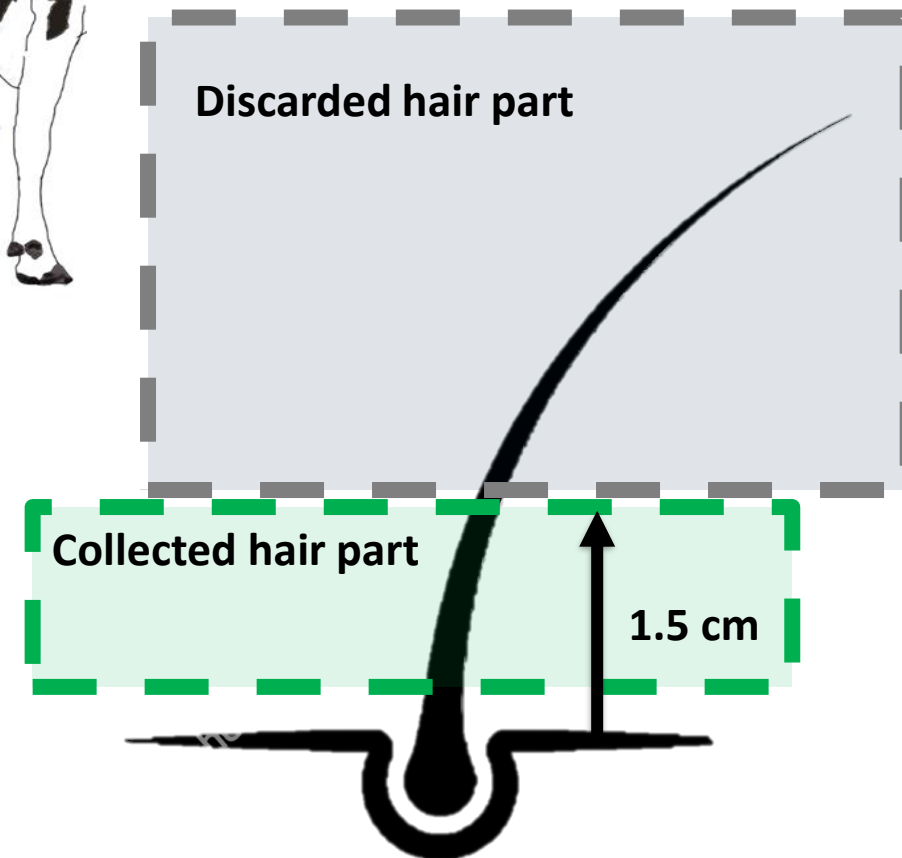
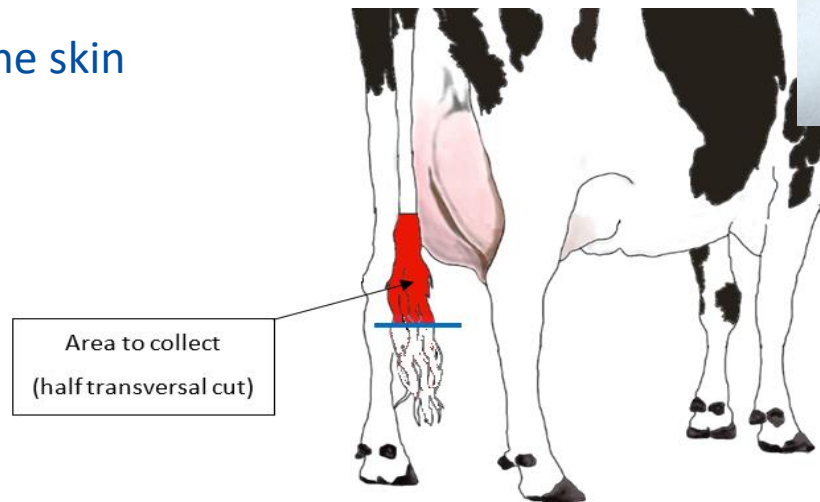


Collected part: 1.5 cm starting from the skin  
(corresponding to 1 month growth)

250 mg

Analysis at CRA-W

- Sieving
- Cleaning
- Grinding
- Extraction
- Elisa test (Salimetrics extended range)



# Data editing

## Reference data cleaning

- Hair cortisol CV < 12%
- Blood hemolysis  $\leq 2$
- DIM < 365
- Fructosamine  $100 < X < 300$
- Missing values

## Spectral data cleaning

- No GH threshold
- Fat differences between predictions and lab analysis < 0.3 g/100ml

## Data handling

- Breed: dummy variable with Holstein (1) or others (0)
- Hair color: dummy variable with dark (1) or light hairs (0)

After editing

n=1004 for fructosamine

n=1104 for cortisol



# Quantitative models

- MIR (212 wavenumbers and first derivative )
  - +DIM
  - +DIM+DIM<sup>2</sup>
  - +DIM+DIM<sup>2</sup>+parity
  - +DIM+DIM<sup>2</sup>+parity+parity<sup>2</sup>
  - +DIM+DIM<sup>2</sup>+parity+parity<sup>2</sup>+MY
  - +DIM+DIM<sup>2</sup>+parity+parity<sup>2</sup>+MY+breed
  - +DIM+DIM<sup>2</sup>+parity+parity<sup>2</sup>+MY+breed+color
- [MIR] reduced in 12 or 14 LV after PLS
  - +DIM
  - +DIM+DIM<sup>2</sup>
  - +DIM+DIM<sup>2</sup>+parity
  - +DIM+DIM<sup>2</sup>+parity+parity<sup>2</sup>
  - +DIM+DIM<sup>2</sup>+parity+parity<sup>2</sup>+MY
  - +DIM+DIM<sup>2</sup>+parity+parity<sup>2</sup>+MY+breed
  - +DIM+DIM<sup>2</sup>+parity+parity<sup>2</sup>+MY+breed+color

Combined with :

- ✓ PLS
- ✓ SVM (support vector machine), better ability to handle non linear data

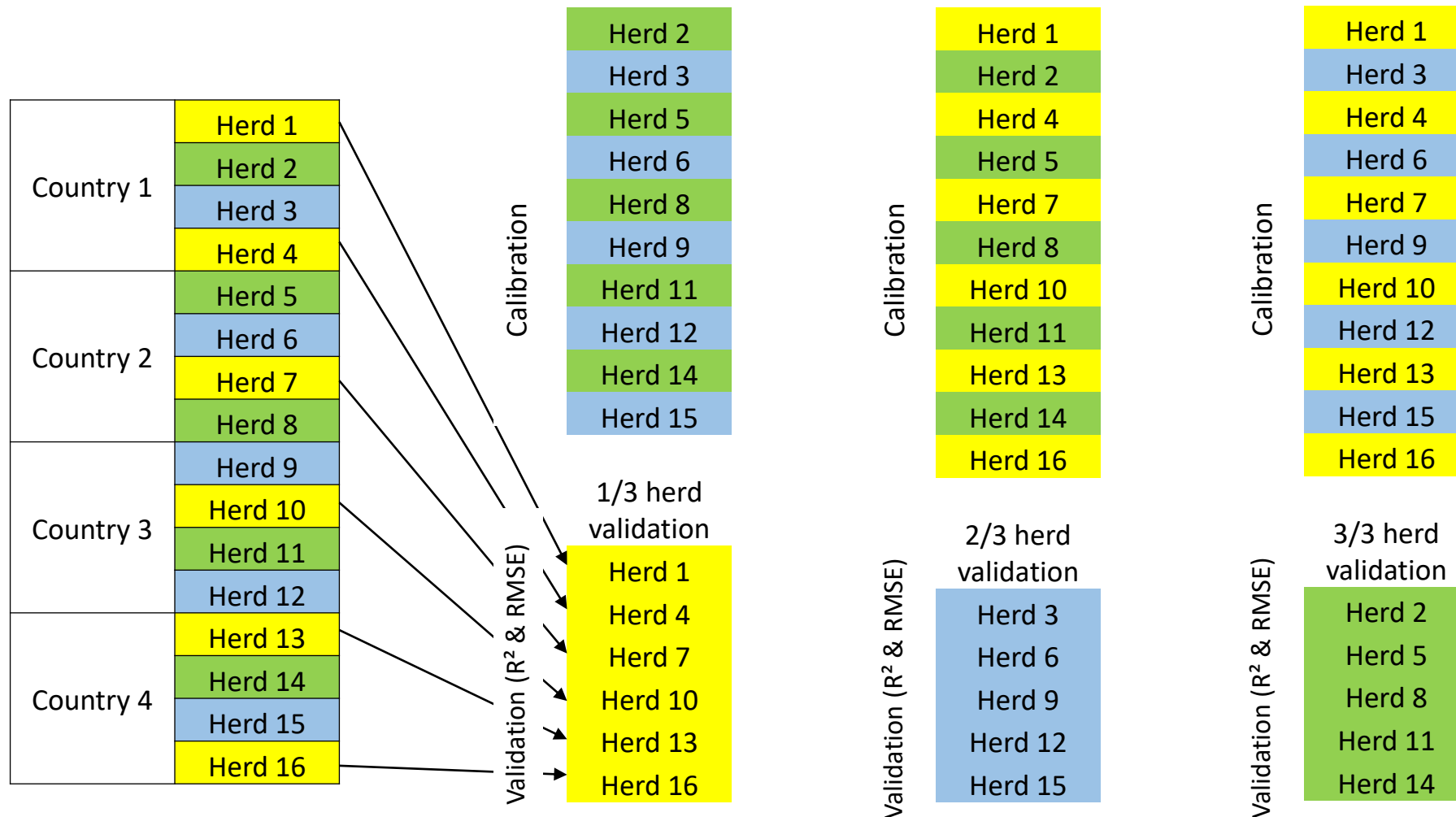
**BUT 212 MIR variables may dilute the other information (Fearn, 2010)**

Combined with :

- ✓ MLR (multiple linear regression)
- ✓ SVM (support vector machine)

# Validation & stats

- External-herd-validation, removing 33% of herds and iterating 3 times the process to validate with all herds

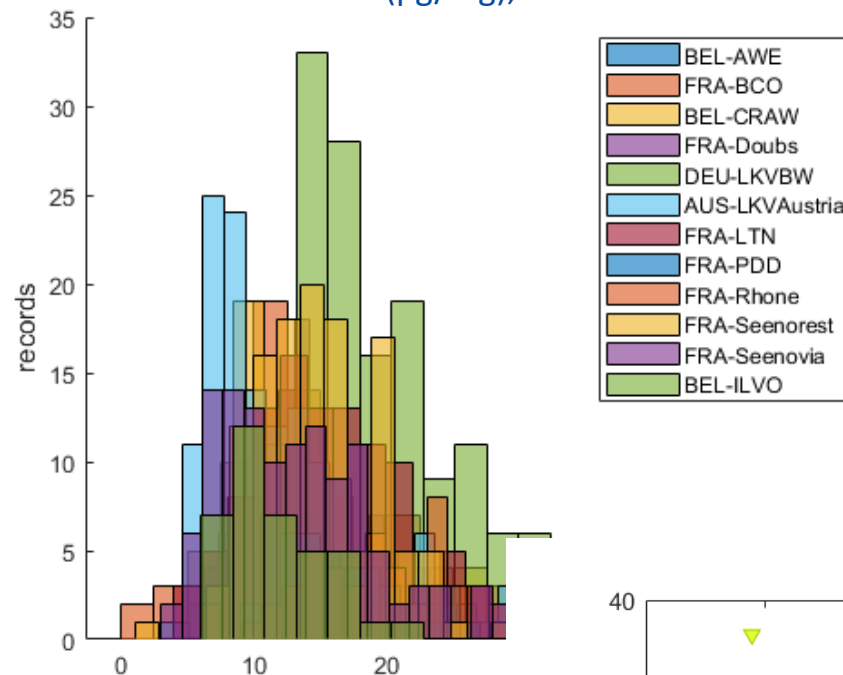


Final stats:

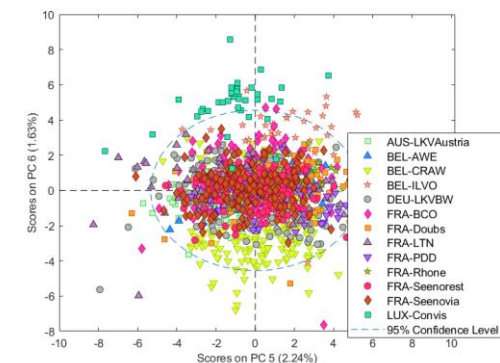
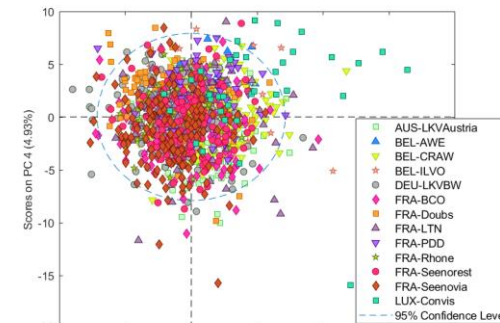
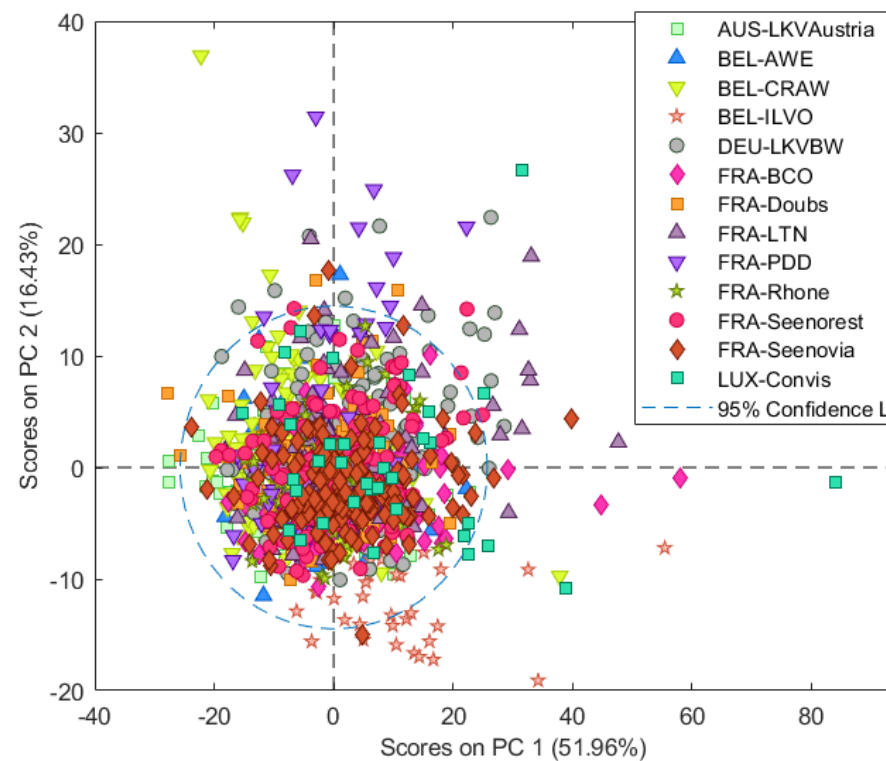
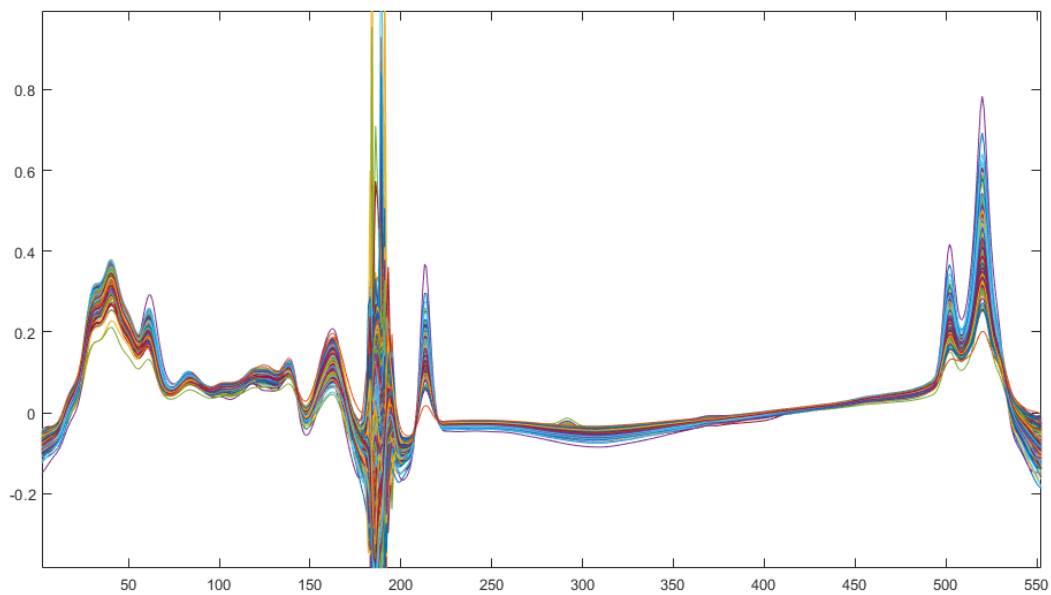
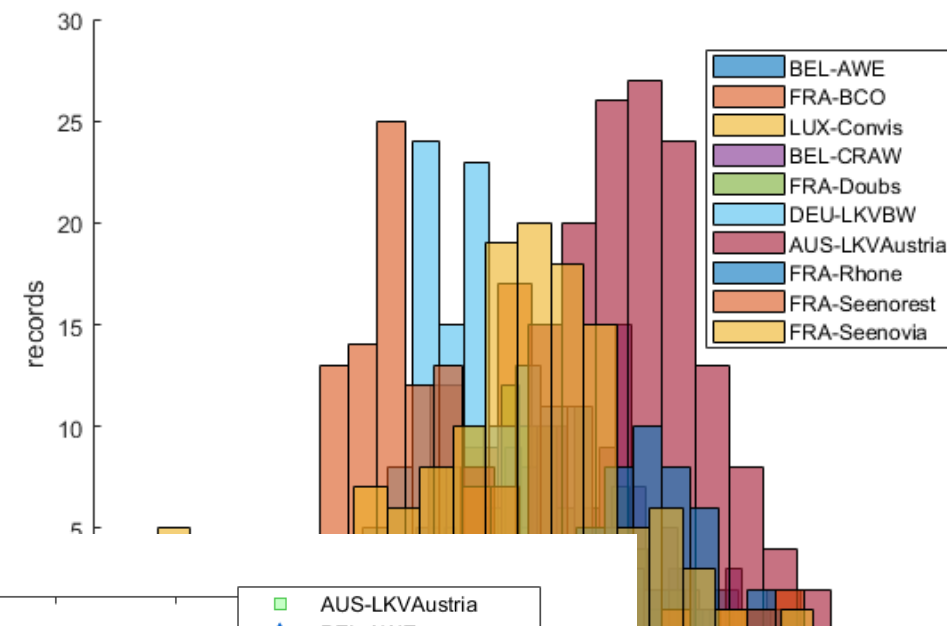
- Mean R<sup>2</sup><sub>v</sub>
- Mean RMSE<sub>v</sub>

# Results

Cortisol (pg/mg), final n= 1071



Fructosamine ( $\mu\text{mol/L}$ ), final n=940



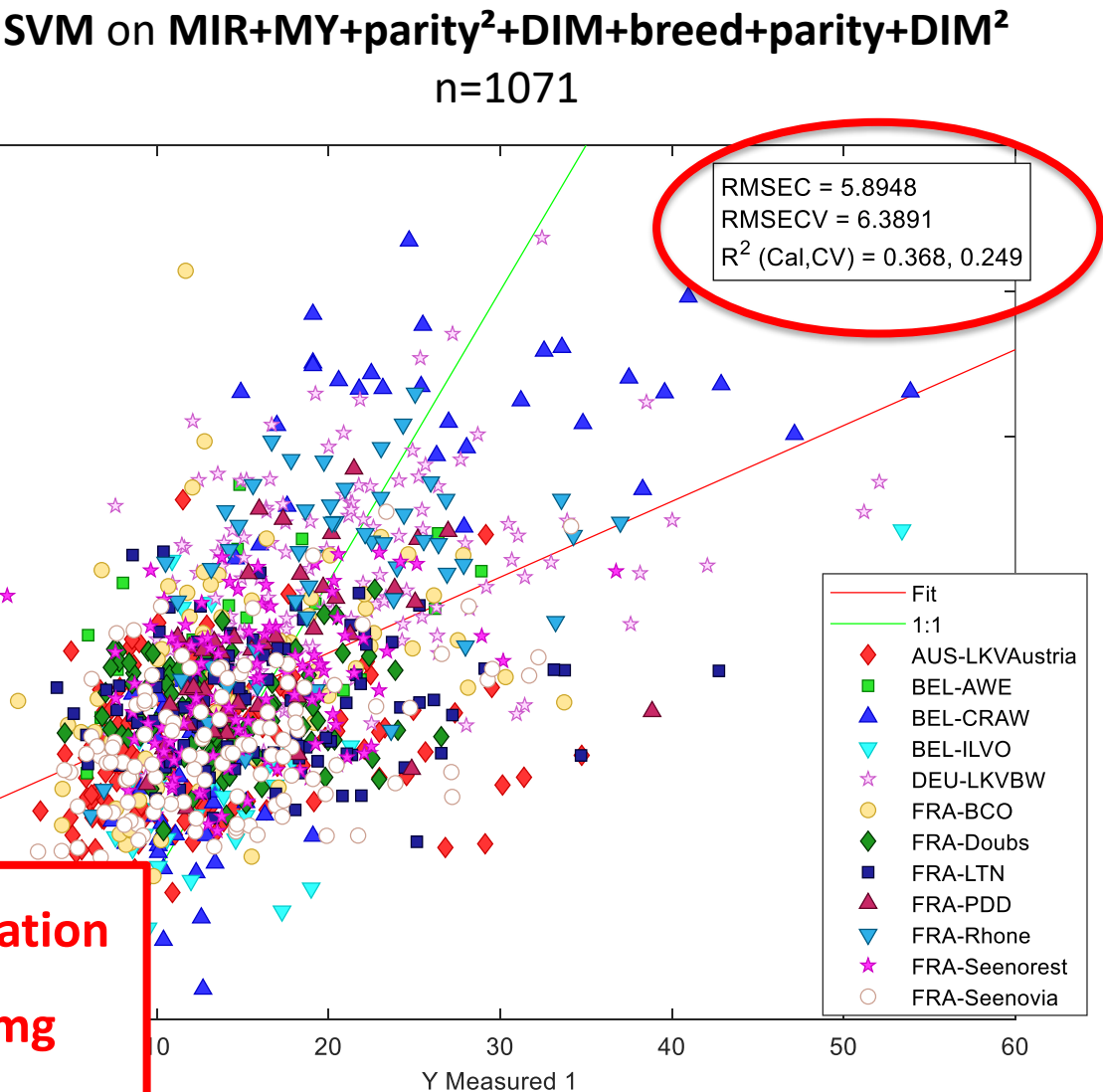


# Cortisol quantitative models

	Calibration RMSE			
	PLS	SVM	MLR-PLS	SVM-PLS
MIR	6.0	5.9	6.0	6.0
MIR+MY	6.0	5.9	6.0	6.0
MIR+MY+parity <sup>2</sup>	6.0	5.9	5.9	5.9
MIR+MY+parity <sup>2</sup> +DIM	6.0	5.8	5.9	5.9
MIR+MY+parity <sup>2</sup> +DIM+breed	6.0	5.9	5.9	5.9
MIR+MY+parity <sup>2</sup> +DIM+breed+parity	6.0	5.9	5.9	5.9
<b>MIR+MY+parity<sup>2</sup>+DIM+breed+parity+DIM<sup>2</sup></b>	6.0	5.8	5.9	5.9
MIR+MY+parity <sup>2</sup> +DIM+breed+parity+DIM <sup>2</sup> +color	6.0	5.7		

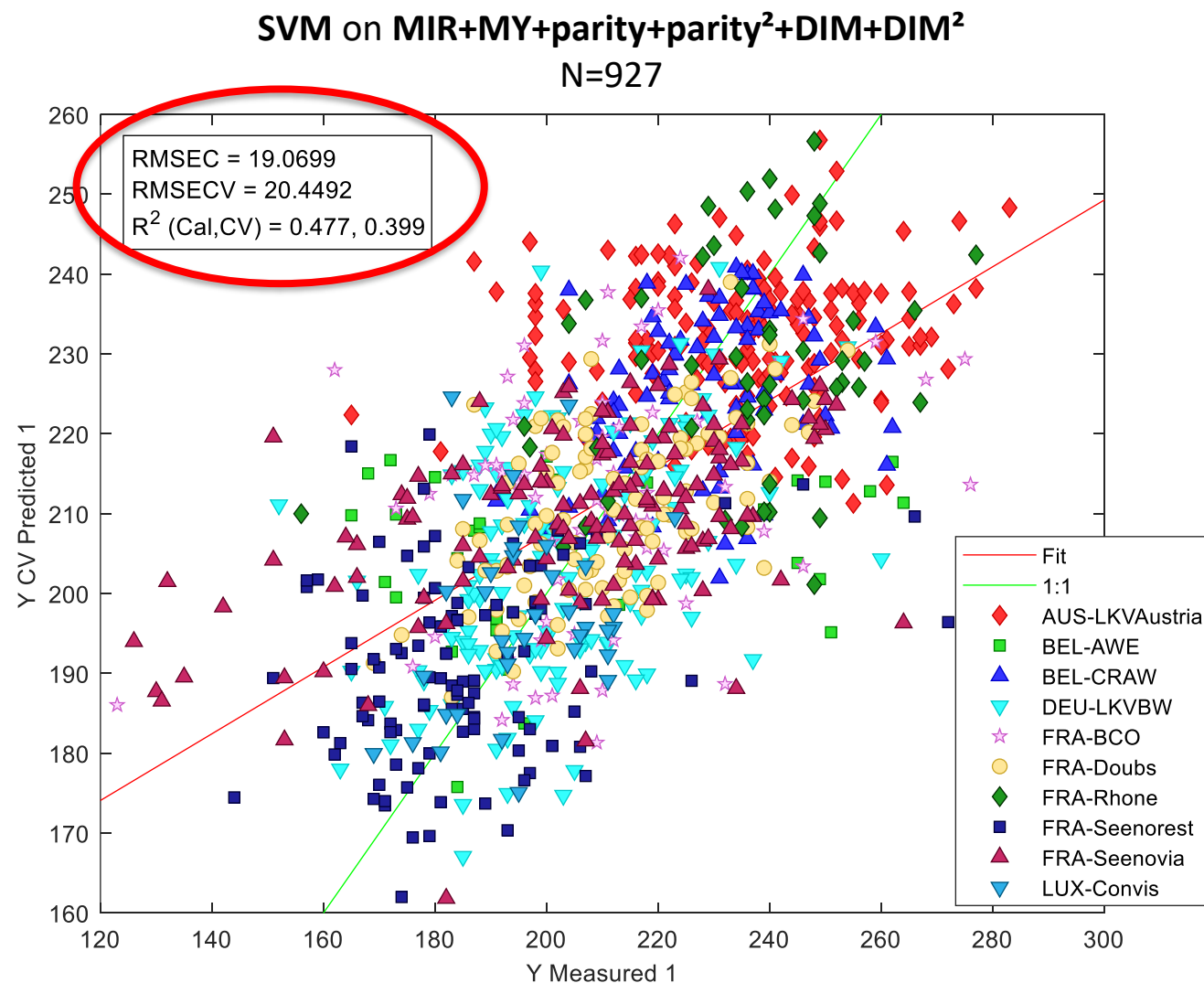
  

	Calibration R <sup>2</sup>			
	PLS	SVM	MLR-PLS	SVM-PLS
MIR	0.32	0.35	0.33	0.35
MIR+MY	0.32	0.35	0.33	0.34
MIR+MY+parity <sup>2</sup>	0.32	0.36	0.34	0.35
MIR+MY+parity <sup>2</sup> +DIM	0.32	0.37	0.34	0.36
MIR+MY+parity <sup>2</sup> +DIM+breed	0.33	0.35	0.34	0.35
MIR+MY+parity <sup>2</sup> +DIM+breed+parity	0.33	0.37	0.34	0.36
<b>MIR+MY+parity<sup>2</sup>+DIM+breed+parity+DIM<sup>2</sup></b>	0.33	0.37	0.34	0.35
MIR+MY+parity <sup>2</sup> +DIM+breed+parity+DIM <sup>2</sup> +color	0.33	0.40		



**External Herd Validation**  
**RMSEv = 6.9 pg/mg**  
**R<sup>2</sup>v = 0.13**

# Fructosamine quantitative models



**External Herd Validation**

**RMSEv = 25.4  $\mu$ mol/L**

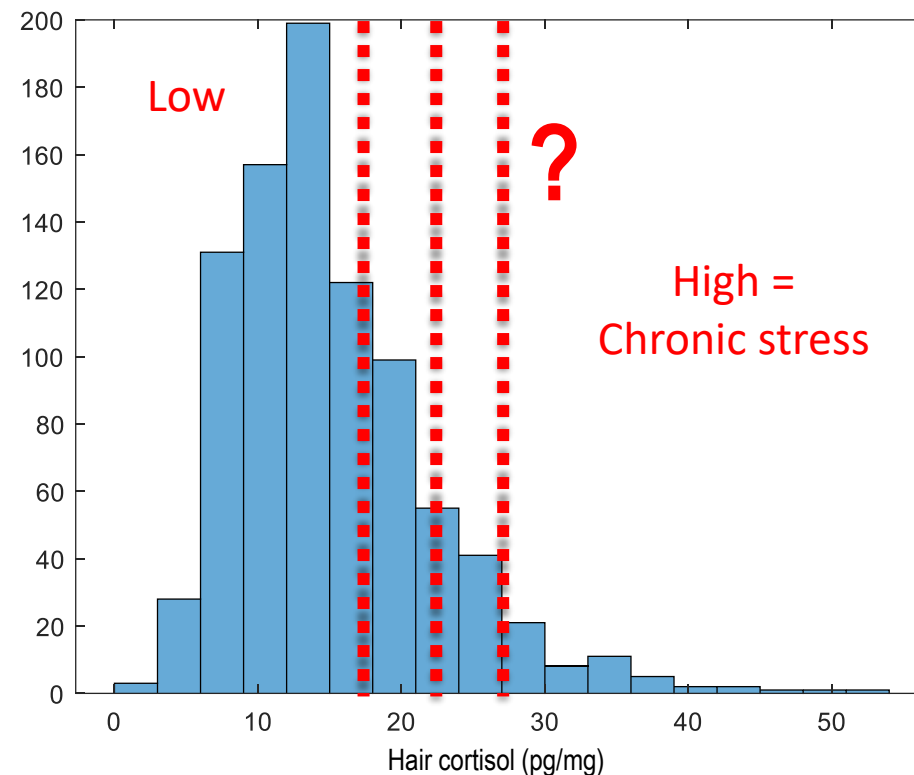
**$R^2_v = 0.20$**

# Discriminant models

- Alternative option, less precise → Low vs High
- But which threshold? No documentation
- Test and optimization of thresholds on model SENSIBILITY
- Mathematical threshold but no physiological meaning!!

- MIR (212 wavenumbers and first derivative )

+DIM  
+DIM+DIM<sup>2</sup>  
+DIM+DIM<sup>2</sup>+parity  
+DIM+DIM<sup>2</sup>+parity+parity<sup>2</sup>  
+DIM+DIM<sup>2</sup>+parity+parity<sup>2</sup>+MY  
+DIM+DIM<sup>2</sup>+parity+parity<sup>2</sup>+MY+breed  
+DIM+DIM<sup>2</sup>+parity+parity<sup>2</sup>+MY+breed+color



Combined with :

✓ PLS-DA

# Cortisol discriminant models

- Identify the best threshold : 22 pg/mg
- Identify the best model : MIR+MY+parity<sup>2</sup>+DIM+breed+parity+DIM<sup>2</sup>

## Optimization of the SENSIBILITY (detection of high cortisol)

	Threshold low vs high (in pg/mg)					
	14	16	18	20	<b>22</b>	24
MIR	47%	48%	48%	48%	53%	45%
MIR+MY	48%	49%	47%	48%	55%	43%
MIR+MY+parity <sup>2</sup>	48%	49%	47%	48%	54%	45%
MIR+MY+parity <sup>2</sup> +DIM	49%	50%	48%	49%	55%	45%
MIR+MY+parity <sup>2</sup> +DIM+breed	49%	50%	46%	50%	55%	45%
MIR+MY+parity <sup>2</sup> +DIM+breed+parity	47%	49%	46%	48%	54%	44%
<b>MIR+MY+parity<sup>2</sup>+DIM+breed+parity+DIM<sup>2</sup></b>	48%	50%	48%	49%	<b>57%</b>	46%
MIR+MY+parity <sup>2</sup> +DIM+breed+parity+DIM <sup>2</sup> +color	48%	50%	46%	49%	54%	46%

# Cortisol discriminant models

- Identify the best threshold : 22 pg/mg
- Identify the best model : MIR+MY+parity<sup>2</sup>+DIM+breed+parity+DIM<sup>2</sup>
- Step 2 : apply the model on the entire dataset

## Cross validation

	Low cortisol	High cortisol	
Predicted Low	719	73	
Predicted High	172	107	
	<b>891</b>	<b>180</b>	<b>1071</b>

Sensibility	Specificity	Accuracy
59.4%	81%	77%

## External-Herd-Validation

	Low cortisol	High cortisol	
Predicted Low	656	71	
Predicted High	235	109	
	<b>891</b>	<b>180</b>	<b>1071</b>

Sensibility	Specificity	Accuracy
60.6%	74%	71%



# Fructosamine discriminant models

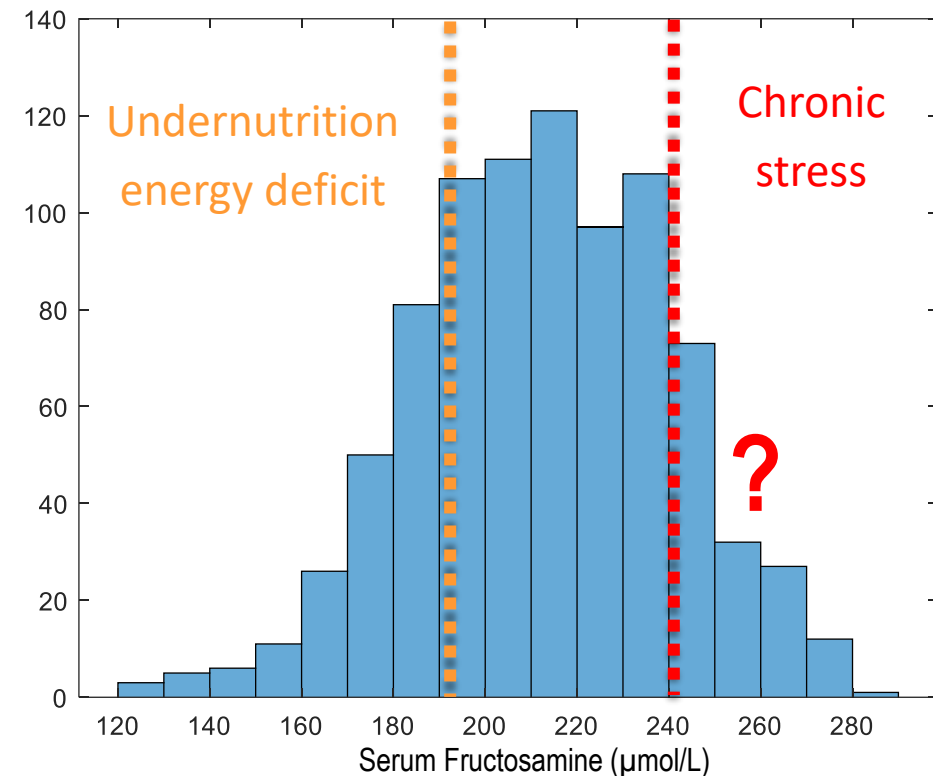
Rather « new indicator », few informations

- High → chronic stress
- Low → undernutrition/energy deficit

Need for 2 thresholds?

Test and see from 170 to 250  $\mu\text{mol/L}$

Mathematical threshold but no physiological meaning!!



# Fructosamine discriminant models

Low threshold to discriminate undernutrition/energy deficit

- Best threshold is 200  $\mu\text{mol/L}$
- Best model is MIR+MY+parity+parity<sup>2</sup>+DIM+DIM<sup>2</sup>+breed

## Cross validation

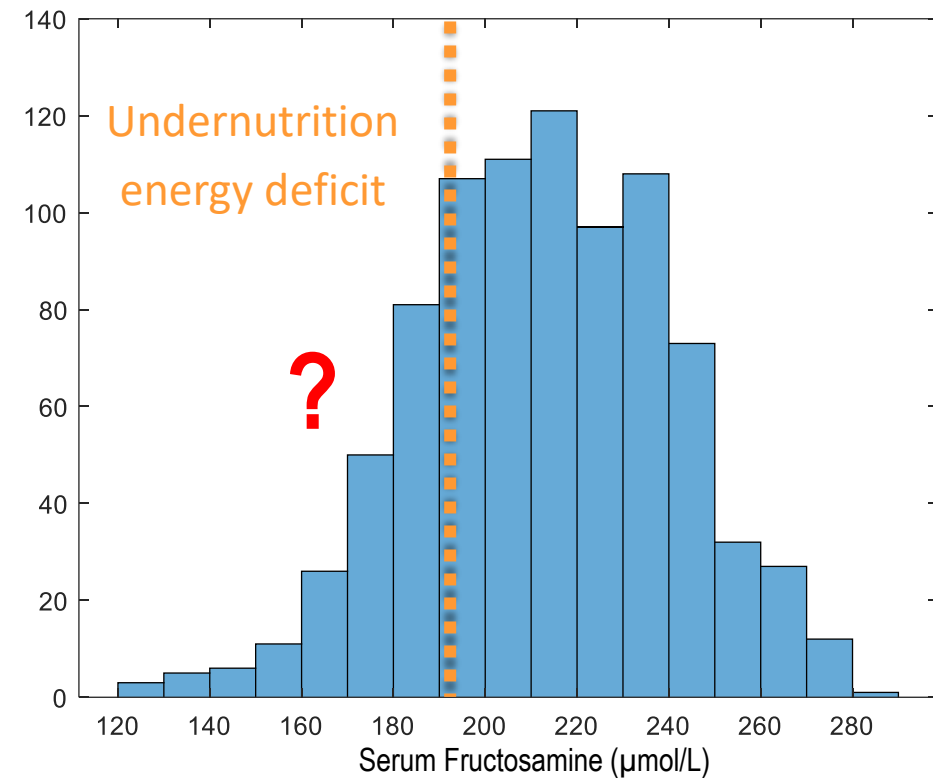
	Low fructosamine	High fructosamine	
Predicted Low	246	126	
Predicted High	82	486	
	<b>328</b>	<b>612</b>	<b>940</b>

Sensibility    Specificity    Accuracy  
79.4%            75%            78%

## External-Herd-Validation on Entire dataset

	Low fructosamine	High fructosamine	
Predicted Low	195	177	
Predicted High	133	435	
	<b>328</b>	<b>612</b>	<b>940</b>

Sensibility    Specificity    Accuracy  
59.5%            71.1%            67.0%



# Fructosamine discriminant models

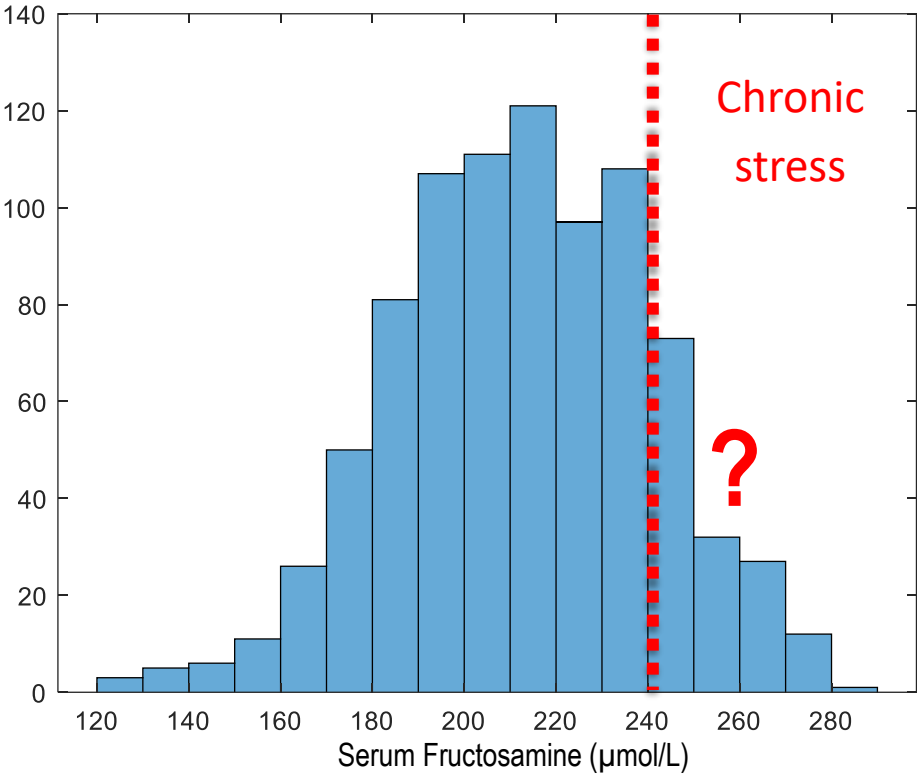
High threshold to discriminate chronic stress

- Best threshold is 230  $\mu\text{mol/L}$
- Best model is with MIR only

## Cross validation

	Low fructosamine	High fructosamine	
Predicted Low	513	58	
Predicted High	175	194	
	<b>688</b>	<b>252</b>	<b>940</b>

Sensibility	Specificity	Accuracy
	75%	75%
77.0%		



## External-Herd-Validation

	Low fructosamine	High fructosamine	
Predicted Low	467	77	
Predicted High	221	175	
	<b>688</b>	<b>252</b>	<b>940</b>

Sensibility	Specificity	Accuracy
	67.9%	68.3%
69.4%		

# Fructosamine discriminant models

- And with the 2 thresholds?
- 1 model with 2 thresholds (3 classes) did not work well (not shown)
- Combination of model « High » and model « Low »

model « High »

model « Low »

	Low value	Medium or High Value
Low or Medium value	Low	Medium
High value	Medium	High

- External-Herd-Validation

	Low (undernutrition)	Medium	High (stress)
Pred Low (undernutrition)	169	110	38
Pred Medium	100	136	46
Pred High (stress)	59	114	168
	328	360	252

sensibility  
specificity  
accuracy

52%  
76%  
67%

38%  
75%  
61%

67%  
75%  
73%

# Can we get information on dairy cows chronic stress biomarkers using milk MIR spectra?

- ✓ Poor quantitative models for both hair cortisol and blood fructosamine
- ✓ Discrimination
  - ✓ Low vs high cortisol: 71% accuracy
  - ✓ Low vs high fructosamine: 73% accuracy
  - ✓ To use at the herd level (proportions)
  - ✓ Thresholds to be optimized (on a physiological basis?)
  - ✓ To combine both biomarkers?
  - ✓ To include a grey zone (with no prediction) to improve classification
  - ✓ To be used in research projects (Holicow...)
  - ✓ To test the model in commercial farms, explore how they behave, do they really enable to highlight stress?





**Thank you for  
your attention!**

**Thanks to our technical staff!**



