Development and Application of Physiological Markers of Grizzly Bear Health

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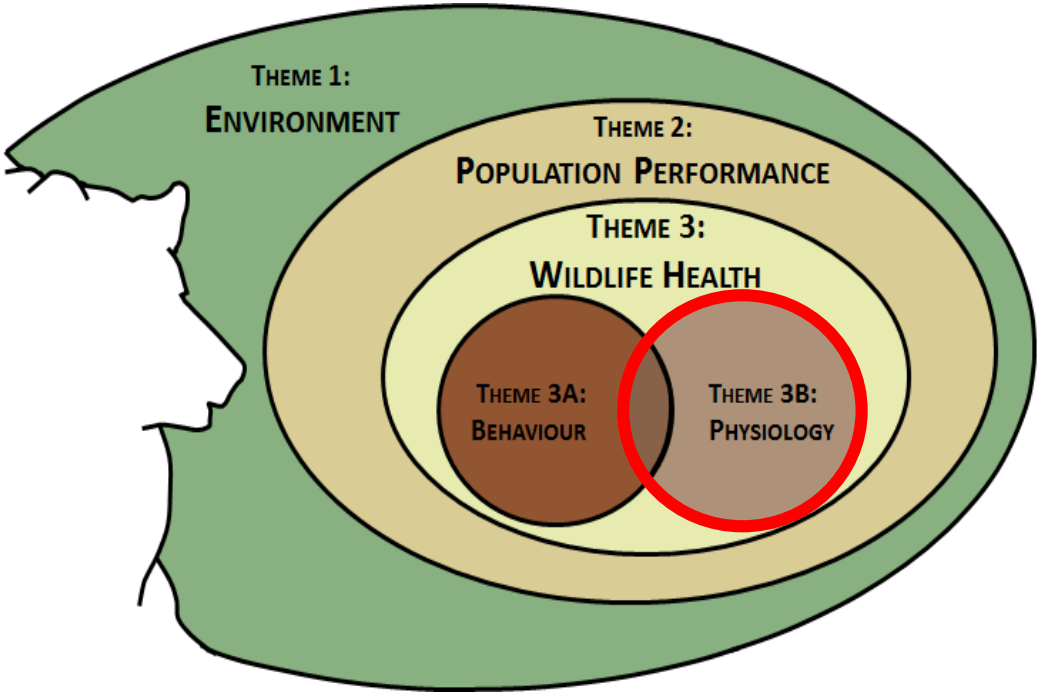
Industrial Research Question	Addressed by Project
4. Have changing landscape conditions associated with natural resource extraction activities resulted in changes in the health of grizzly bears within the study area?	Q3A.1, Q3B.1, Q3B.2

We want to determine if resource extraction activities on the landscape affect the health of individual grizzly bears.



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<u>Objective 1:</u> Determine if long-term stress, represented by **hair cortisol concentration (HCC)**, was associated with landscape variables in a population that doubled from 2004 to 2014.

<u>Objective 2:</u> Develop a liquid chromatography/tandem mass spectrometry/multiple reaction monitoring (LC/MSMS/MRM) method to identify and quantify **proteins in skin** that are associated with reproduction, metabolism, and stress.

Key Question: How do changing landscape conditions influence stress, metabolism, and reproduction in grizzly bears?

Sample:	**Hair** (125mg) barbed wire snag	**Skin** (6mm) biopsy punch
Targets:	**Hormones** (cortisol, estradiol, progesterone, testosterone)	**Proteins** (macromolecules that respond to stimuli)
Method:	Extracted with methanol and measured using an **enzyme-linked immunoassay kit**	Extracted with urea and measured using **LC/MSMS/MRM**

Variables related to **biological attributes (proteins)**, **anthropogenic footprint, food resource availability, and landscape conditions** were tested as predictors of physiological function.

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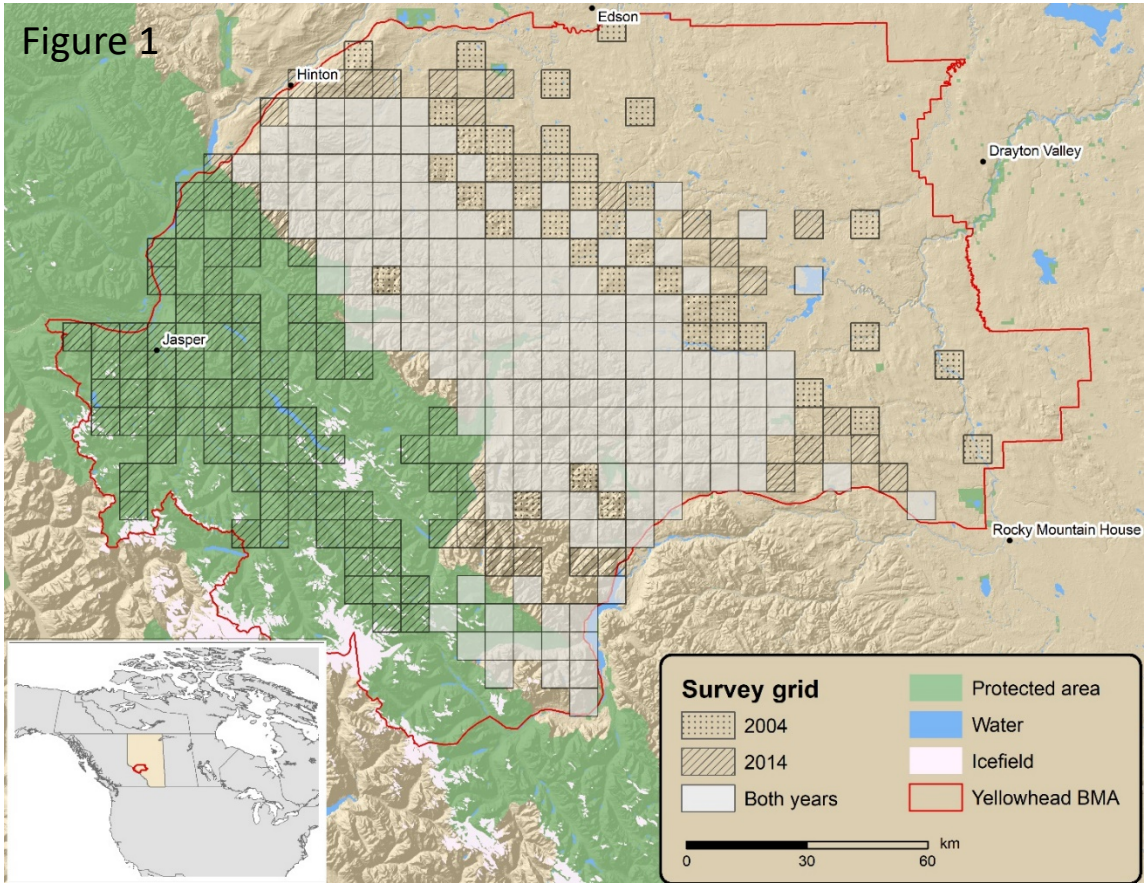

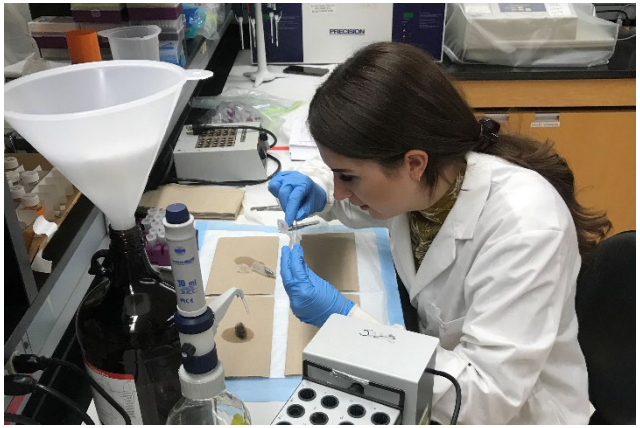

- Hair was collected by **using barbed wire hair snags** placed in 7 km X 7 km grid cells throughout the Yellowhead BMA in 2004 and 2014 (Figure 1).
- Cortisol was extracted from hair using methanol and measured with an **enzyme-linked immunoassay kit**.

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Objective 1 Hormones: Study Area and Methods

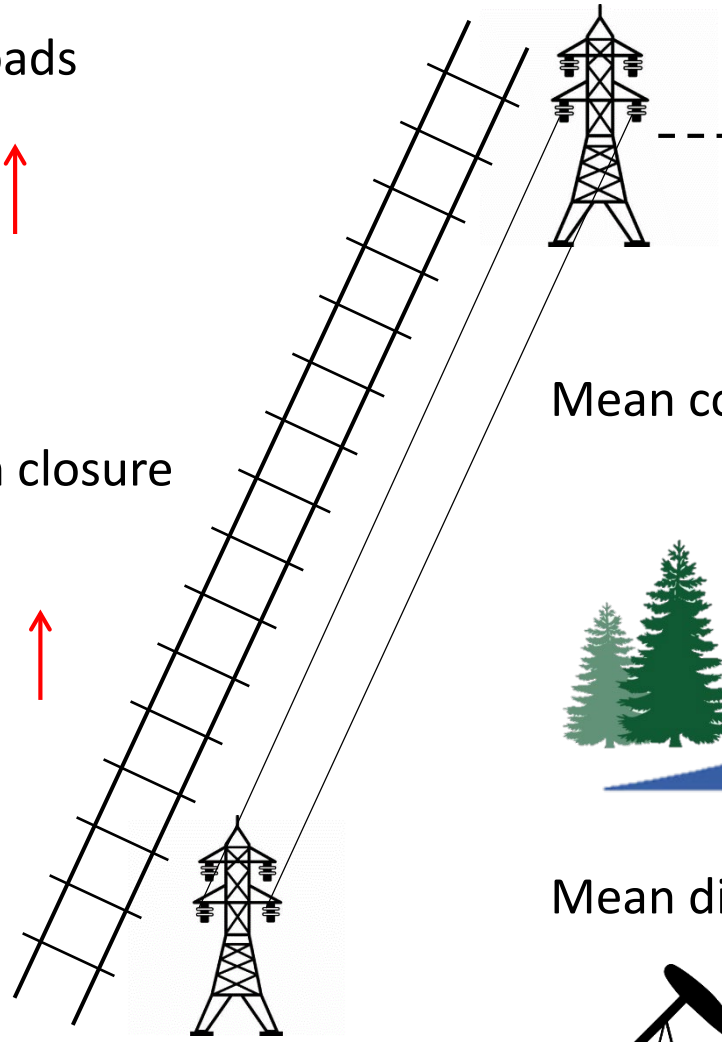
- Variables from remotely sensed data for 2004 and 2014 were extracted at the **7 x 7 km grid cell level (Table 1).**
- **Generalized linear model analysis** was used to determine predictors of HCC and Tukey HSD was used to compare HCC between 2004 and 2014 (α=0.05).

Table 1. Potential drivers of stress in bears

Category	Variable
Anthropogenic Footprint	Roads
	Coal mines
	Railways and powerlines
	Oil and gas well-sites
	Forest harvesting (cutblocks)
	Protected area
Food Resource Availability	Percent conifer
	Crown closure/forest cover
	Land cover
Terrain Conditions	Elevation
	Compound topographic index
	Terrain ruggedness index

Mean distance to roads

HCC ↑



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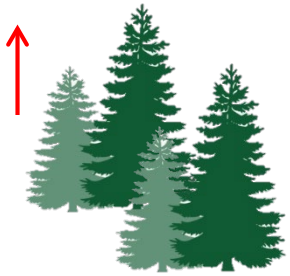
Variability of crown closure



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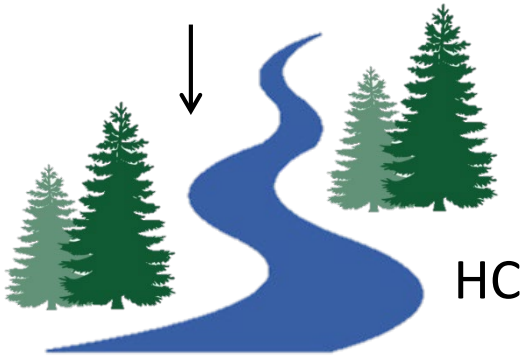
Mean compound topographic index



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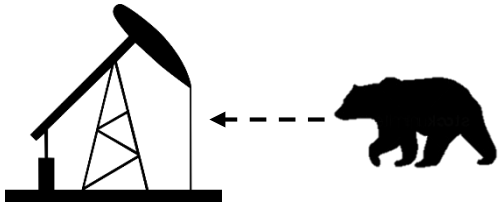
Mean distance to oil/gas well-sites



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Mean distance to railways and powerlines

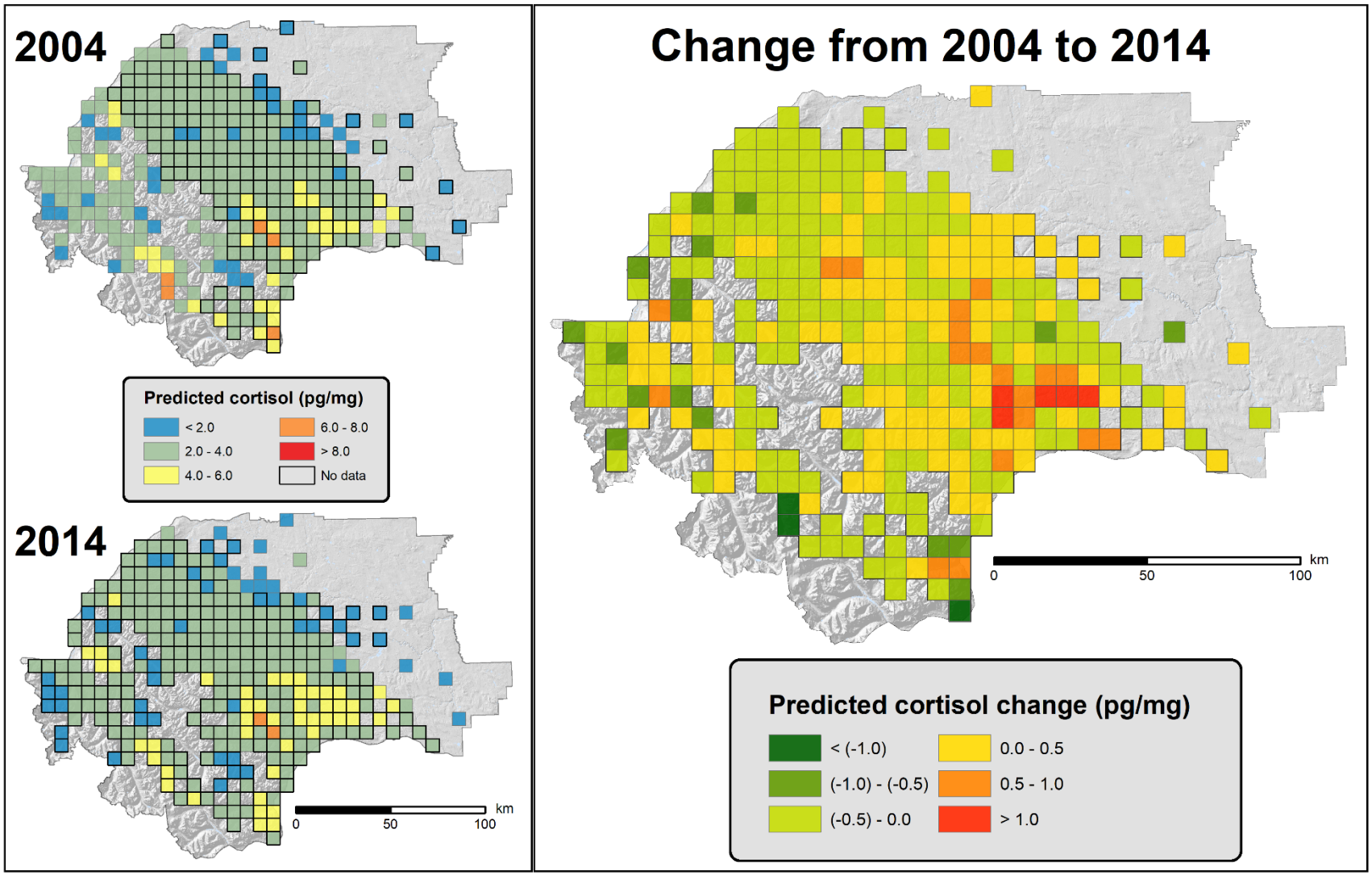


Figure 2. Predicted hair cortisol concentrations in 2004 and 2014 and change over time from landscape variables in the final generalized linear model.

Wilson et al., 2019 Ecosphere, *in review*

Objective 1 Hormones: Results


- 139 skin samples (112 individuals) from 2013 to 2019 have been **collected by ear plug and/or skin biopsy.**
- We have identified over 600 proteins in grizzly bear skin, **19 of which were chosen as targets** (37 peptides) because of their roles in physiological function.
- LC/MSMS/MRM assay has been **developed and validated** for targets.

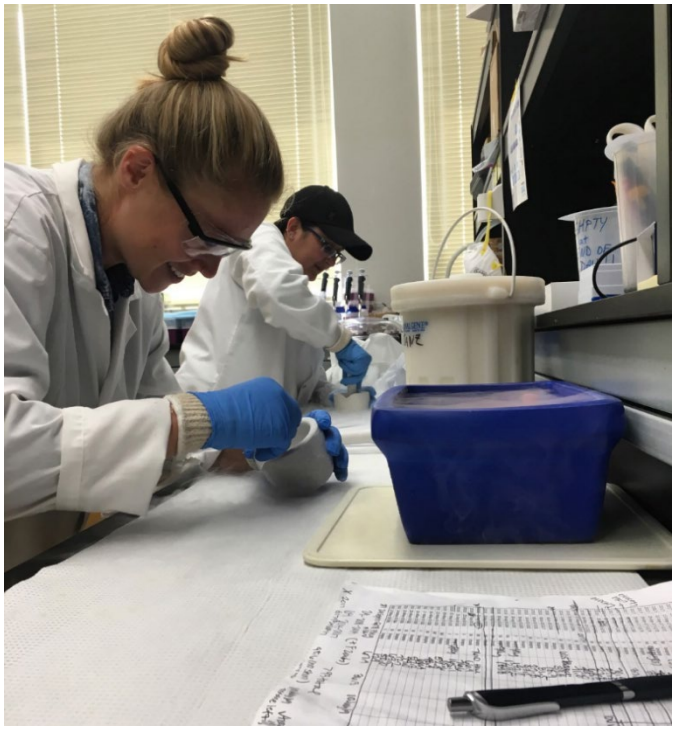
Objective 2 Proteomics: Workflow

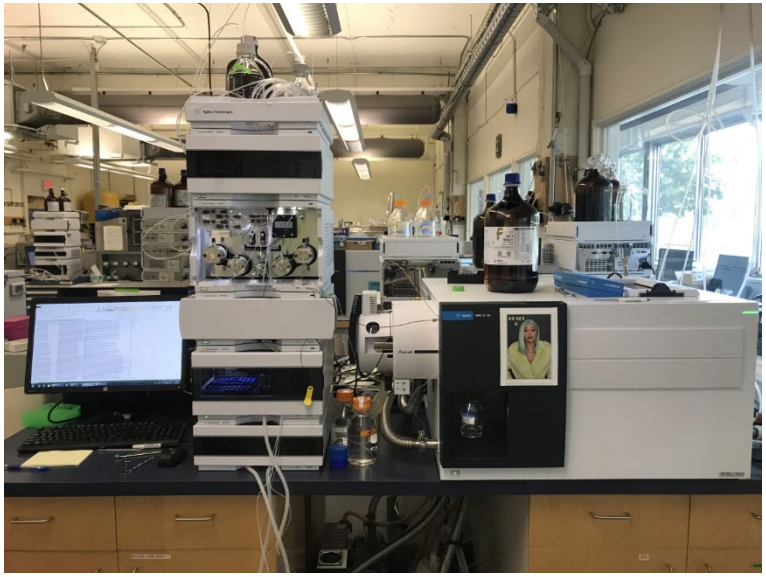
Table 2. Target proteins identified and quantified in grizzly bear skin.

Category	Protein	Biological Function
Metabolism	Adiponectin	Regulates glucose, transports nutrients, and clears cellular debris
	Clusterin	
	Apolipoprotein B-100	
	Alpha-1-acid glycoprotein	
	Transthyretin	
	Vitamin D-binding protein	
Reproduction	Ceruloplasmin	Transports nutrients for pregnancy, clears pathogens, and initiates parturition
	Fetuin-B	
	Complement C3	
	Afamin	
	Prostaglandin (PG) F synthase 1	
	Serpin B5 (Maspin)	
Stress	78 kDa glucose-regulated protein	Transports stress hormones, maintains protein function, and reduces inflammation
	Endoplasmin (HSP90)	
	Superoxide dismutase	
	Corticosteroid-binding globulin	
	Alpha-2-macroglobulin	
	Kininogen-1	
	Annexin	

- Samples were ground to a fine powder under **liquid nitrogen** using pre-chilled mortars and pestles.

- Proteins were extracted from skin using urea and digested with trypsin **to generate peptides.**





Heavy isotope-labeled peptides were added to each sample and the resulting peptide mixtures were analyzed using an Agilent 1290 Infinity II LC system coupled with an Agilent 6495 Triple Quadrupole LC/MS.

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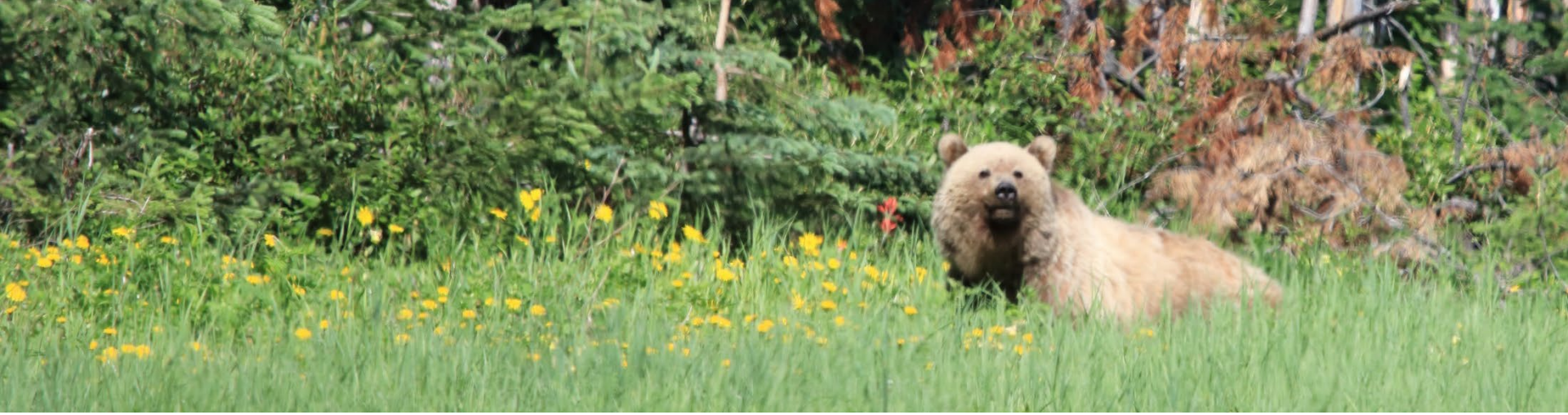
Objective 2 Proteomics: Workflow



Generalized linear models (GLM) were used to evaluate the **effects of biological attributes** on the mean expression by category and of each of the 19 proteins.

1. **Sex:** male, solitary female and adult female with cubs
2. **Age:** ≥5 years old=Adult; <5 years old=Sub-Adult
3. **Location/population:** samples collected across 6 BMAs
4. **Season** (Nielsen et al., 2004; Carlson et al., 2016)
   - Hypophagia (period from den emergence (April) to 14 June)
   - Early hyperphagia (15 June to 7 August)
   - Late hyperphagia (8 August to den entry (November))
5. **Year** (2013-2019)
6. **Sampling type:** ear plug or biopsy

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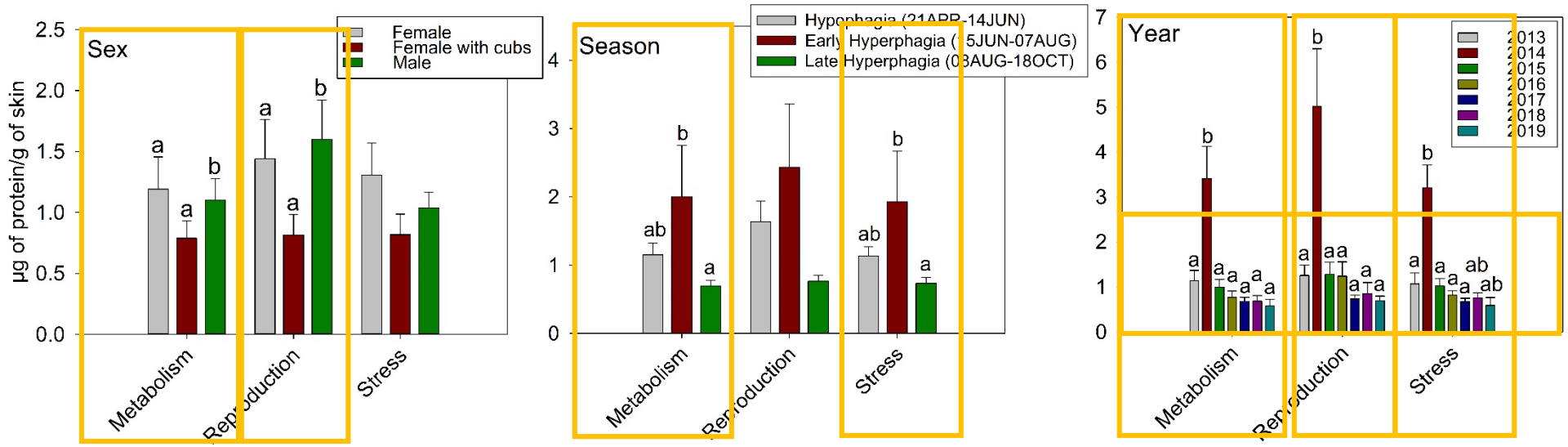
Objective 2 Proteomics: Workflow

Figure 3. Mean (±SEM) ug of protein per g of skin for proteins related to metabolism, reproduction, and stress across biological attributes. Bars with different letters within a category were different ($P < 0.05$).



- Functional categories demonstrated changes in protein expression with respect to differences in **sex, season, and year** (Figure 2).
- Protein expression did not differ among age class, population, or sample type.
- Further analysis among both age class and sex groups is required.

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Objective 2 Proteomics: Preliminary Results

Table 3A. Effects of biological attributes on individual proteins within a functional category (greatest in listed attribute within metric; $P < 0.05$).

Category	Protein	Biological effect		
		Sex	Age Class	Season
Metabolism*	Adiponectin		Subadult	
	Clusterin	Male		Early Hyperphagia
	Apolipoprotein B-100	Male		
	Alpha-1-acid glycoprotein			
	Transthyretin			Early Hyperphagia
	Vitamin D-binding protein	Male		Early Hyperphagia

*Regulates glucose, transports nutrients, and clears cellular debris

Table 3B. Effects of biological attributes on individual proteins within a functional category (greatest in listed attribute within metric; $P < 0.05$).

Category	Protein	Biological effect		
		Sex	Age Class	Season
Reproduction*	Ceruloplasmin	Male		
	Fetuin-B	Male	Subadult	Early Hyperphagia
	Complement C3	Male		
	Afamin	Male		Early Hyperphagia
	Prostaglandin (PG) F synthase 1	Female	Subadult	
	Serpin B5 (Maspin)		Subadult	

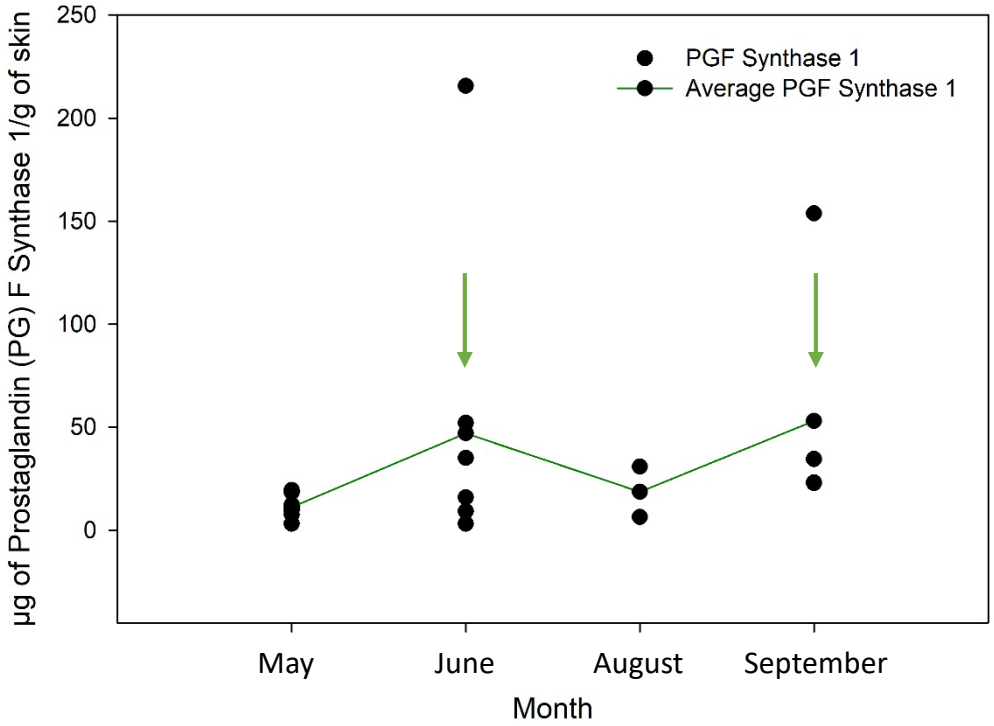
*Transports nutrients for pregnancy, clears pathogens, and initiates parturition

Table 3C. Effects of biological attributes on individual proteins within a functional category (greatest in listed attribute within metric; $P < 0.05$).

Category	Protein	Biological effect		
		Sex	Age Class	Season
Stress*	78 kDa glucose-regulated protein		Subadult	
	Endoplasmin (HSP90)		Subadult	Early Hyperphagia
	Superoxide dismutase			
	Corticosteroid-binding globulin		Subadult	
	Alpha-2-macroglobulin	Male		
	Kininogen-1	Male		Early Hyperphagia
	Annexin		Subadult	

*Transports stress hormones, maintains protein function, and reduces inflammation

Category	Protein	Biological Function
Reproduction	Ceruloplasmin	Transports nutrients for pregnancy, clears pathogens, and initiates parturition
	Fetuin-B	
	Complement C3	
	Afamin	
	Prostaglandin (PG) F synthase 1	
	Serpin B5 (Maspin)	



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4. Have changing landscape conditions associated with natural resource extraction activities resulted in changes in the health of grizzly bears within the study area?	Q3A.1, Q3B.1, Q3B.2

Goal: Create novel tools to monitor species-at-risk on changing landscapes that can be communicated efficiently with industry partners.

1. Yes, there is evidence to suggest that some measures of **health are impacted by landscape variables.**
2. Linking landscape conditions with hair cortisol concentrations provides reliable information regarding potential **drivers of long-term stress** over time and at the population level, and may be used as an initial monitoring approach to **identify areas of increased stress.**
3. Measuring protein expression in skin provides information regarding **physiological function** related to metabolism, reproduction, and stress.
4. These techniques can be used to **monitor species-at-risk** on changing landscapes.

1. Determine if landscape conditions influence **stress, metabolism, and reproduction, measured by protein expression,** in grizzly bears.
2. Compare the **reproductive and stress physiology** of free-ranging grizzly bears captured in the Yellowhead from 2015-2017.
3. Understand **downstream effects of long-term stress** on reproduction and physiological function in grizzly bears.

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Next steps

Questions?

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Thank you!