Published March 28, 2017

Effects of a simulated wolf encounter on brain and blood biomarkers of stress-related psychological disorders in beef cows with or without previous exposure to wolves[1]

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ABSTRACT. This experiment compared mRNA expression of brain-blood biomarkers associated with stress-related psychological disorders, including post-traumatic stress disorder (PTSD), in beef cows from wolf-naïve and wolf-experienced origins that were subjected to a simulated wolf encounter. Multiparous, non-pregnant, non-lactating Angus-crossbred cows from the Eastern Oregon Agricultural Research Center (Burns, OR; CON; n = 10) and from a commercial operation near Council, ID (WLF; n = 10) were used. To date, gray wolves are not present around Burns, OR, and thus CON were naïve to wolves. Conversely, wolves are present around Council, ID, and WLF cows were selected from a herd that had experienced multiple wolf-predation episodes from 2008 to 2015. After a 60-d commingling and adaptation period, CON and WLF cows were allocated to groups A or B (d -1; 5 CON and 5 WLF cows in each group). On d 0, cows from group A were sampled for blood and immediately slaughtered, and samples were analyzed to evaluate inherent differences between CON and WLF cows. On d 1, cows from group B were exposed in pairs (1 CON and 1 WLF cow) to experimental procedures. Cows were sampled for blood, moved to 2 adjacent drylot pens (1 WLF and 1 CON cow/pen) and subjected to a simulated wolf encounter event for 20 min. The encounter consisted of (1) cotton plugs saturated with wolf urine attached to the drylot fence, (2) reproduction of wolf howls, and (3) three leashed dogs that were walked along the fence perimeter. Thereafter, another blood sample was collected and cows were slaughtered. Upon slaughter, the brain was removed and dissected for collection of the hypothalamus, and one longitudinal slice of the medial pre-frontal cortex, amygdala, and Cornu Ammonis (1 region of the hippocampus from both hemispheres). Within cows from group A, expression of *c-Fos proto-oncogene* in hippocampus and amygdala were greater ($P < 0.01$) in WLF vs. CON cows. Within cows from group B, expression of hippocampal *brain-derived neurotrophic factor* mRNA and expression of c-*Fos proto-oncogene* mRNA in hippocampus and amygdala were less ($P \leq 0.04$) in WLF vs. CON cows. These are key biological markers known to be downregulated during stress-related psychological disorders elicited by fear, particularly PTSD. Hence, cows originated from a wolf-experienced herd presented biological evidence suggesting a psychological disorder, such as PTSD, after the simulated wolf encounter when compared with cows originated from a wolf-naïve herd.

Key words: Beef cattle, biomarkers, fear, stress, wolves

 J. Anim. Sci. 2017.95:1154–1163
doi:10.2527/jas2016.1250

INTRODUCTION

The reintroduction of gray wolves into the Yellowstone National Park increased the dispersion of wolf packs into livestock grazing areas within the northwestern US (Larsen and Ripple, 2006), escalat-

[1]Financial support for this research was provided by the Oregon Beef Council.

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Received November 28, 2016.
Accepted January 19, 2017.

ing the incidence of cattle-wolf interactions and cattle predation by wolves in the area (Idaho Department of Fish and Game, 2016). Although the economic implications of predators on livestock systems are mainly associated with animal injury or death (Oakleaf et al., 2003; Breck and Meier, 2004), these parameters are not the only negative impacts that wolf predation causes to beef cattle systems (Laporte et al., 2010).

The mere presence of predators alters stress physiology and behavior of the prey, particularly if the preyed animal was already exposed to similar predation episodes (Boonstra, 2013). Research from our group indicated that the presence of wolves increases excitability and fear-related physiological stress responses in cows previously exposed to wolves, but not in cows unfamiliar with this predator (Cooke et al., 2013). More specifically, Cooke et al. (2013) subjected beef cows from wolf-naïve and wolf-experienced origins to a simulated wolf encounter. This simulation process increased temperament score, body temperature, and plasma cortisol concentration in wolf-experienced cows, which are biological responses known to impair cattle productivity (Cooke, 2014), but not in wolf-naïve cows. These results also suggested that wolf presence alters mental parameters in wolf-experienced cows due to fear memories from previous predation episodes, leading to behavioral and physiological changes equivalent to psychological disorders such as post-traumatic stress disorder (**PTSD;** Sherin and Nemeroff, 2011). To test this latter hypothesis, this experiment compared mRNA expression of brain and blood biomarkers associated with stress-related psychological disorders in wolf-naïve and wolf-experienced cows subjected to a simulated wolf encounter (Cooke et al., 2013).

MATERIALS AND METHODS

This experiment was conducted at the Oregon State University– Eastern Oregon Agricultural Research Center (**EOARC**; Burns, OR). Animals utilized were cared for in accordance with acceptable practices and experimental protocols reviewed and approved by the Oregon State University, Institutional Animal Care and Use Committee. The experimental design, including cattle selection and the simulated wolf encounter, was based on our previous research in this area (Cooke et al., 2013).

Animal Management

Multiparous, non-pregnant, non-lactating Angus-crossbred cows from EOARC (**CON**; $n = 10$) and from a commercial cow-calf operation (**WLF**; $n = 10$) near Council, ID were used. Both locations used domestic herding dogs to move cattle across pastures or to the handling facility, although no work dogs were present at the EOARC during the experimental period. The CON cows (age = 6.2 ± 0.5 yr, BW = 589 ± 12 kg) were selected from the EOARC mature cowherd. The EOARC herd is reared and maintained near Burns and Riley (OR) and to date no known wolf packs exist nor wolf-predation episodes have occurred in this region (Oregon Department of Fish and Wildlife, 2016). Hence, CON cows were considered naïve to wolf presence and predation. The WLF cows (age = 7.1 ± 0.3 yr; BW = 561 ± 15 kg) were selected from the commercial operation located near Council, ID. This region (McCall-Weiser Wolf Management Zone) includes active wolf packs (Idaho Department of Fish and Game, 2016). The herd from which WLF cows were selected have experienced multiple confirmed wolf predation episodes from 2008 to 2015 when grazing summer pasture allotments (USDA-APHIS, Idaho Wildlife Services, Boise, ID); although, none of the experimental WLF cows had been directly predated or injured by wolves. Therefore, WLF cows were considered experienced with wolf presence and predation episodes.

The WLF cows were transported to the EOARC 60 d prior to the beginning of the experiment (d 0). During this period (d -60 to -1), CON and WLF cows were commingled and maintained in a single meadow foxtail (*Alopecurus pratensis* L.) dominated pasture (Wenick et al., 2008) harvested for hay the previous summer, and had ad libitum access to meadow-grass and mineral-vitamin supplement as described by Cooke et al. (2013). All cows were also individually processed through the EOARC handling facility, but not restrained in the squeeze chute, once a week from d -60 to -5 to acclimate WLF cows to the EOARC personnel and facilities (Cooke, 2014). On d -1, cows were ranked within wolf exposure status (CON and WLF) by temperament score (Cooke, 2014; by the same single technician), and allocated to 2 groups of 10 cows each (5 CON and 5 WLF cows in group A and group B) in a manner that groups had similar temperament score (2.65 ± 0.19 and 2.60 ± 0.19 for groups A and B, respectively). All cows from group A were assigned to experimental procedures on d 0, which did not include a simulated wolf encounter (Cooke et al., 2013), to assess inherent differences between CON and WLF cows across the blood-brain biomarkers evaluated herein. All cows from group B were assigned to experimental procedures on d 1, which included the simulated wolf encounter.

Experimental Procedures

Group A. On d 0, cows from group A were moved to a single drylot pen (15 × 30 m) with free choice hay and water, while cows from group B remained on pasture. Cows from group A were individually processed for

blood collection into PAXgene tubes (BD Diagnostics, Sparks, MD), and immediately slaughtered. Cows from group A that were waiting to be processed and all cows from group B were maintained, respectively, 300 and 500 m from the processing-slaughter site to prevent cows from perceiving the sampling and slaughter process (Cooke et al., 2013). All cows were sampled for blood and slaughtered within 4 h. Minimum, maximum, and average environmental temperatures on d 0 were, respectively, 2, 25, and 13°C, and average humidity was 44% with no observed precipitation.

Group B. On d 1, cows from group B were also moved to a single drylot pen (15 × 30 m) with free choice hay and water. Minimum, maximum, and average environmental temperatures on d 1 were, respectively, 1, 26, and 13°C, and average humidity was 43% with no observed precipitation. Two cows, being 1 CON and 1 WLF, were randomly selected and concurrently processed for blood collection into PAXgene tubes (BD Diagnostics).

After blood was collected, these 2 cows were immediately assigned to the simulated wolf encounter described by Cooke et al. (2013). More specifically, cows were moved to 2 adjacent drylot pens separated by a fence line (1 WLF and 1 CON cow in each pen). Pens were 17 × 17 m, located 100 m from the handling facility, and had no feed or water source. After arrival in their respective pens, CON and WLF cows were immediately subjected to a simulated wolf encounter for 20-min as in Cooke et al. (2013). Wolf urine (Harmon Wolf Urine Scent; Cass Creek, Grawn, MI) was applied to 12 cotton plugs (Feminine care tampons; Rite Aid, Camp Hill, PA). The plugs were attached to the drylot fence line every 11 m (6 plugs/pen) before any experimental procedures on d 1, and wolf urine was re-applied to plugs after each pair of cows was exposed to the simulation. After cows were settled within each dry lot pen, wolf howls previously recorded from the wolf packs residing in Wallowa County, OR were continuously reproduced using a stereo system (S2 Sports MP3 CD/Radio Boombox; Sony Corporation of America, San Diego, CA) located 10 m from the dry lot pens; cows had no visual contact with the stereo system. Additionally, 3 dogs were introduced using a leash by 2 technicians outside the drylot perimeter fence during the entire 20-min simulation. The dogs were 2 adult German Shepherd females (BW = 39 ± 2 kg) to represent adult wolves, and 1 adult Border Collie × Alaskan Malamute female (BW = 24 kg) to represent a young wolf. The maximum and minimum distances allowed between dogs and cows were 25 and 5 m, respectively. Dogs did not act aggressively or vocalized during the simulated wolf encounter.

Immediately after the simulated wolf encounter, another round of blood samples were collected into PAXgene tubes (BD Diagnostics) and cows were slaughtered. Cows from group B that were waiting to be processed were maintained at least 300 m from the site where sampling, simulated wolf encounter, and slaughter were performed to prevent cows from perceiving these procedures (Cooke et al., 2013). In addition, the first blood sampling was performed in a different handling facility as the second blood sampling and slaughter, which were located 200 m apart. Cows from group B were sampled, exposed to the simulated wolf encounter, and slaughtered in pairs (1 CON and 1 WLF cow). All experimental procedures to cows from group B were completed within 8 h.

Slaughter. All cows were slaughtered using the same procedures and in the same site. Slaughter was conducted in accordance with AVMA Guidelines for the Euthanasia of Animals (Leary et al., 2013). Cows were individually restrained and rendered unconscious using a non-penetrative captive bolt stun gun (Cash Special 0.25 Caliber Non-Penetrating Heavy-Duty Stunner with 3.5 grain Power Load; Accles & Shelvoke Inc., West Greenwich, RI) to prevent excessive brain structural damage. Once unconscious, cows were exsanguinated by incision of the ventral aspect of the throat or neck transecting skin, muscle, trachea, esophagus, carotid artery and jugular vein with a sharp knife with a 25-cm rigid blade. Following exsanguination, the brain was removed and immediately dissected for collection of the hypothalamus, as well as 1 longitudinal slice (0.2 cm) of the medial pre-frontal cortex, amygdala, and Cornu Ammonis (**CA**)-1 region of the hippocampus from both cerebral hemispheres. Tissues were immediately stored in 5-mL sterile cryogenic tubes containing 2 mL of RNA stabilization solution (RNAlater, Ambion Inc., Austin, TX), maintained at 4°C for 24 h, and stored at -80°C until further processing. Cows from group A were slaughtered and brain tissues were collected serially, with WLF and CON cows alternately assigned to slaughter to account for a potential slaughter order effect. Pairs from group B were slaughtered within a 5-min interval, and the order of slaughter (CON or WLF cow slaughtered first) also alternated between pairs. Brain was concurrently dissected from group B pairs after both cows were slaughtered. Across both groups, all brain tissues were collected, processed, and immersed into RNA stabilization solution approximately 30 min after slaughter.

Sample Analyses

Brain tissue samples. Total RNA was extracted only from tissue samples using the TRIzol Plus RNA Purification Kit (Invitrogen, Carlsbad, CA). Besides the hypothalamus, samples from the left and right hemispheres were combined for RNA extraction. Quantity and quality of isolated RNA were assessed via UV absorbance

(NanoDrop Lite; Thermo Fisher Scientific, Wilmington, DE) at 260 nm and 260/280 nm ratio, respectively (Fleige and Pfaffl, 2006). Extracted RNA (200 ng) was reverse transcribed using the High Capacity cDNA Reverse Transcription Kit with random hexamers (Applied Biosystems, Foster City, CA). Real-time reverse-transcriptase (**RT**) PCR was completed using the Fast SYBR Green Master Mix (Applied Biosystems) and gene-specific primers (20 pM each; Table 1) with the StepOne Real-time PCR system (Applied Biosystems), according to procedures described by Cooke et al. (2008). At the end of each RT-PCR, amplified products were subjected to a dissociation gradient (95°C for 15 s, 60°C for 30 s, and 95°C for 15 s) to verify the amplification of a single product by denaturation at the anticipated temperature. A portion of the amplified products were purified with the QIAquick PCR purification kit (Qiagen Inc., Valencia, CA) and sequenced at the Oregon State University-Center for Genome Research and Biocomputing to verify the specificity of amplification. All amplified products represented only the genes of interest. Responses were quantified based on the threshold cycle ($\mathbf{C_T}$), the number of PCR cycles required for target amplification to reach a predetermined threshold. The CT responses from genes of interest were normalized to the geometrical mean (Vandesompele et al., 2002) of CT values from *glyceraldehyde-3-phosphate dehydrogenase* and β-*actin* (Tanic et al., 2007; Derks et al., 2008). The CV for the geometrical mean of *glyceraldehyde-3-phosphate dehydrogenase* and β-*actin* CT values was 3.3% for amygdala samples, 2.8% for hippocampus samples, 2.5% for hypothalamus samples, and 2.2% for medial pre-frontal cortex samples. Results are expressed as relative fold change ($2^{-\Delta\Delta CT}$), as described by Ocón-Grove et al. (2008).

Blood samples. Total RNA was extracted from blood samples using the PAXgene Blood RNA Kit (Qiagen). Assessment of quantity and quality of isolated RNA, reverse transcription (120 ng of extracted RNA), and real-time RT-PCR with gene-specific primers (20 pM each; Table 1) were performed as described for tissue samples. Responses from genes of interest were quantified based on C_T and normalized to the geometrical mean of CT values from *glyceraldehyde-3-phosphate dehydrogenase* and β-*actin* (Vandesompele et al., 2002). The CV for the geometrical mean of *glyceraldehyde-3-phosphate dehydrogenase* and β-*actin* CT values samples was 1.8%. Results are expressed as relative fold change ($2^{-\Delta\Delta CT}$) as described by Ocón-Grove et al. (2008).

Statistical Analysis

Cow was considered the experimental unit. All data were analyzed using the MIXED procedure of SAS (SAS Inst. Inc., Cary, NC; version 9.3) and Satterthwaite approximation to determine the denominator degrees of freedom for the tests of fixed effects. The model statement for all brain samples contained the fixed effects of wolf exposure status (CON and WLF), group (A or B), and the interaction, with cow (wolf exposure status × group) as random variable. The model statement for blood samples from group A contained the fixed effects of wolf exposure status, with cow (wolf exposure status) as random variable. The model statement for blood samples from group B contained the fixed effects of wolf exposure status, time (pre- and post-simulation assessments), the resultant interaction, with cow (wolf exposure status) as random variable. The specified term used in the repeated statement for blood samples from group B was time, the subject was cow (wolf exposure status), and the covariance structure utilized was autoregressive, which provided the best fit for these analyses according to the Akaike information criterion. All models also included sampling or slaughter order as an independent covariate. Significance was set at $P \leq 0.05$, and tendencies were determined if $P > 0.05$ and ≤ 0.10. Sampling and slaughter order were not significant covariates ($P \geq 0.35$) in their respective analyses, and were removed from models. Therefore, results were reported least squares means and separated using protected LSD. Results were reported according to main effects if no interactions were significant, or according to the highest-order interaction detected.

RESULTS AND DISCUSSION

Results from our previous research (Cooke et al., 2013) suggested that exposing wolf-experienced cows to a simulated wolf encounter elicited behavioral and physiological changes comparable with stress-related psychological disorders, including PTSD symptoms (Sherin and Nemeroff, 2011). However, the same outcome was not detected in cows unfamiliar with this predator, perhaps due to the lack of fear and traumatic memories from past predation episodes (Creel and Christianson, 2008; Boonstra, 2013). Comparable fear-related stress models were adopted to investigate PTSD biomarkers in rodents (Ressler et al., 2011; Zhang et al., 2015; Dong et al., 2016), including predator-scent stress using feline urine (Kozlovsky et al., 2007). Collectively, research evaluating PTSD in human and rodent models identified biomarkers expressed in blood cells, as well as hypothalamus, amygdala, pre-frontal cortex, and CA1 region of the hippocampus (Kozlovsky et al., 2007; Le-Niculescu et al., 2011; Sherin and Nemeroff, 2011). Together, these regions constitute the brain "fear network", and have been directly implicated in PTSD-like stress responses (Gorman et al., 2000; Bremner, 2006; Sherin and Nemeroff, 2011). To our knowledge, no other research has evaluated similar parameters in

Table 1. Primer sequences and accession number for all gene transcripts analyzed by real-time reverse-transcriptase PCR[1]

Target gene	Primer sequence	Accession no.
Genes of interest		
Adenylate cyclase activating polypeptide 1		
Forward	TTAATAAGGCCTACCGCAAAGTG	NM_001046555.1
Reverse	TGAGCGTCTGCAGGTGATCT	
Adenylate cyclase activating polypeptide 1 receptor type 1		
Forward	CCCTGGGATGTGGGACAA	XM_010804298.1
Reverse	GGCAACTGACCAGGACCATCT	
ATPase H+ transporting accessory protein 1		
Forward	GCCCTCTCTTTGGCAGATGA	NM_175806.2
Reverse	GGAGAACTTCTTGTCTGTCACATTG	
Brain-derived neurotrophic factor		
Forward	GCCCAAGGTGGGTTCAAGA	XM_005216336.3
Reverse	CGATCACGTGTTCAAAAGTGTCA	
Caspase 3		
Forward	GCCATGGTGAAGAAGGAATCA	XM_010820245.2
Reverse	TCCCCTCTGAAGAAACTTGCTAA	
Caspase 9		
Forward	GTGTCCGTCGAGAGAATTGTGA	XM_005217016.3
Reverse	GCTTGGGCTTCCCTCTCAAG	
Carnitine palmitoyltransferase 1B		
Forward	ACACATCTACCTGTCCGTGATCA	XM_015471456.1
Reverse	CCCCTGAGGATGCCATTCT	
Down syndrome cell adhesion molecule		
Forward	TGAATGGCATCATCCGAAAG	XM_015462162.1
Reverse	GGCTTCAAACTCGCTGATCAC	
Fos proto-oncogene		
Forward	CTGCTCGCGATCATGATGTT	NM_182786.2
Reverse	TGCAGCGGGAGGAGGAT	
Heat shock 70kDa protein 1A		
Forward	CCCTGGATTGCTCATGTTTGT	NM_203322.2
Reverse	TCAACATCTCAAACAGCTTGCA	
Neurotrophic tyrosine kinase receptor type 2		
Forward	TGGTGCGGCGATCCTT	XM_005210377.3
Reverse	TGTTCTCAGGATCTGCACTGGTA	
Telomeric repeat binding factor 1		
Forward	ATTTCCTCTGCGTTTCGCTTT	XM_005215517.3
Reverse	GTCGCGAGTGCGATGGA	
Telomeric repeat binding factor 2		
Forward	CCTGGAGAGTCACCTGGATGA	XM_005218478.3
Reverse	GGCGGAGGACTCAGATTTCA	
X-box binding protein 1		
Forward	GAGAGCGAAGCCAATGTGGTA	NM_001271737.1
Reverse	ACTGTGAATTCAGGGTGATCTTTCT	
Reference genes		
β-actin		
Forward	CTGGACTTCGAGCAGGAGAT	NM_173979.3
Reverse	GGATGTCGACGTCACACTTC	
Glyceraldehyde-3-phosphate dehydrogenase		
Forward	ACCCAGAAGACTGTGGATGG	NM_001034034.2
Reverse	CAACAGACACGTTGGGAGTG	

[1]Primers for β-*actin* and *glyceraldehyde-3-phosphate dehydrogenase* obtained, respectively, from Gifford et al. (2007) and Cerri et al. (2012) . All other primer sequences were designed based on the bovine gene sequences deposited in the National Center for Biotechnology Information and using the Primer Express v. 3.0.1 software (Applied Biosystems, Foster City, CA).

beef cattle. Hence, this experiment was based on the research design described by Cooke et al. (2013), and focused on psychological and PTSD-related biomarkers established in the rodent and human literature.

Amygdala Samples

No differences were detected ($P \geq 0.14$) between WLF and CON, either in group A or B, for mRNA expression of *caspase 3*, *caspase 9*, *carnitine palmitoyltransferase 1B*, and *down syndrome cell adhesion molecule* (Table 2), which have all been associated with stress and PTSD-pathogenesis in rodent and human models. More specifically, *caspase 3* and *caspase 9* play critical roles in cell apoptosis, and their expression increased in the amygdala of rats exposed to a PTSD-eliciting model (Xiao et al., 2011). *Carnitine palmitoyltransferase 1B* expression, an enzyme in the fatty acid metabolism, was overexpressed in the amygdala of rats considered with PTSD compared with non-stressed controls (Zhang et al., 2015). *Down syndrome cell adhesion molecule*, which is widely expressed in the amygdala and is associated with neural development, has also been downregulated in humans diagnosed with PTSD (Logue et al., 2015).

The *c-Fos proto-oncogene* is a transcription factor involved in cellular reactivity to external stress, and has been used to assess neuronal activation (Le-Niculescu et al., 2011). In rats exposed to unconditioned or conditioned fear, *c-Fos* mRNA expression in the amygdala was less compared to cohorts not exposed to fear stimulus (Dayas et al., 2001; Day et al., 2008). In the present experiment, a wolf exposure status × group interaction ($P < 0.01$) was detected for *c-Fos* mRNA expression. The WLF cows had greater ($P < 0.01$) mRNA expression of *c-Fos proto-oncogene* compared with CON cows in group A, which were not exposed to the simulated wolf encounter. The reason for this outcome is unknown, as cows were managed similarly prior to slaughter, but could be associated with cow origin and previous history of management and stressful events (Cooke et al., 2013). Conversely, mRNA expression of *c-Fos proto-oncogene* was less ($P = 0.02$) in WLF cows compared with CON cows from group B after the simulated wolf encounter. One can speculate that simulated wolf encounter (Cooke et al., 2013) elicited greater fear and downregulated *c-Fos proto-oncogene* mRNA expression in WLF cows compared with CON, corroborating with equivalent research with rodents (Dayas et al., 2001; Day et al., 2008; Kaouane et al., 2012). Yet, from all amygdala genes evaluated herein that have been associated with PTSD in rodents and humans (Table 2), only *c-FOS proto-oncogene* mRNA expression supported our hypothesis. Nevertheless, *c-FOS* has been classified as a top candidate gene for anxiety conditions including PTSD; hence, a key brain biomarker for psychological disorders (Le-Niculescu et al., 2011).

Table 2. Expression of amygdala genes from cows experienced with the presence of wolves (WLF; $n = 10$) or naïve to wolves (CON; $n = 10$), and subjected to a simulated wolf encounter[1,2]

Item	WLF	CON	SEM	*P*-value
Caspase 3				
Group A	2.56	2.01	0.41	0.39
Group B	2.34	3.26	0.46	0.16
Caspase 9				
Group A	1.72	1.27	0.19	0.14
Group B	1.13	1.48	0.21	0.24
Carnitine palmitoyltransferase 1B				
Group A	3.11	3.25	0.56	0.87
Group B	1.56	2.26	0.62	0.42
Down syndrome cell adhesion molecule				
Group A	3.58	3.16	0.84	0.74
Group B	5.00	5.52	0.94	0.69
c-Fos proto-oncogene				
Group A	2.76	1.31	0.29	< 0.01
Group B	1.31	2.41	0.33	0.02

[1]Values are expressed as relative fold change compared to threshold cycle of reference genes analyzed within the same sample (Ocón-Grove et al., 2008). Simulated wolf encounter consisted in olfactory (wolf urine; Harmon Wolf Urine Scent; Cass Creek, Grawn, MI), auditory (wolf howls reproduced on a stereo system (S2 Sports MP3 CD/Radio Boombox; Sony Corporation of America, San Diego, CA), and visual (3 adult female dogs conducted by leash, being 2 German Shepherd and 1 Border Collie × Alaskan Malamute) for 20 min.

[2]Cows from group A ($n = 10$, being 5 CON and 5 WLF) were slaughtered for tissue collection without being exposed to a simulated wolf encounter to represent baseline differences between CON and WLF cows. Cows from group B ($n = 10$, being 5 CON and 5 WLF) were slaughtered for tissue collection immediately after the simulated wolf encounter.

Hippocampus Samples

No differences ($P \geq 0.40$) were detected between WLF and CON, either in group A or B, for mRNA expression of *down syndrome cell adhesion molecule* in the CA1 region of the hippocampus (Table 3), which corroborates with results from amygdala samples. No differences ($P \geq 0.17$) were also detected between WLF and CON across groups for mRNA expression of *adenylate cyclase activating polypeptide 1*, *neurotrophic tyrosine kinase receptor type 2*, and *telomeric repeat binding factors 1* and *2* (Table 3). *Adenylate cyclase activating polypeptide 1* encodes the pituitary adenylate cyclase-activating polypeptide, which is involved in the abnormal cellular stress responses underlying PTSD (Ressler et al., 2011). *Telomeric repeat binding factors 1* and *2* are the 2 major proteins among telomere-binding proteins that negatively regulate telomere length, whereas telomere was shorter in the humans diagnosed with PTSD (Zhang et al., 2014). Thus, mRNA expression of *telomeric re-*

Table 3. Expression of genes from the Cornu Ammonis-1 region of the hippocampus from cows experienced with the presence of wolves (WLF; n = 10) or naïve to wolves (CON; n = 10), and subjected to a simulated wolf encounter[1,2]

Item	WLF	CON	SEM	P-value
Adenylate cyclase activating polypeptide 1				
Group A	18.0	39.9	15.6	0.33
Group B	146.5	157.6	15.6	0.62
Brain-derived neurotrophic factor				
Group A	12.6	36.9	10.7	0.13
Group B	107.7	146.7	10.7	0.02
Down syndrome cell adhesion molecule				
Group A	2.00	2.50	0.42	0.40
Group B	5.96	6.46	0.42	0.41
c-Fos proto-oncogene				
Group A	4.60	2.76	0.37	< 0.01
Group B	1.84	3.00	0.37	0.04
Neurotrophic tyrosine kinase receptor type 2				
Group A	2.09	1.95	0.21	0.65
Group B	3.14	3.40	0.21	0.40
Telomeric repeat binding factor 1				
Group A	2.46	1.95	0.25	0.17
Group B	1.30	1.55	0.25	0.49
Telomeric repeat binding factor 2				
Group A	1.74	1.57	0.14	0.39
Group B	1.52	1.61	0.14	0.65

[1]Values are expressed as relative fold change compared to threshold cycle of reference genes analyzed within the same sample (Ocón-Grove et al., 2008). Simulated wolf encounter consisted in olfactory (wolf urine; Harmon Wolf Urine Scent; Cass Creek, Grawn, MI), auditory (wolf howls reproduced on a stereo system (S2 Sports MP3 CD/Radio Boombox; Sony Corporation of America, San Diego, CA), and visual (3 adult female dogs conducted by leash, being 2 German Shepherd and 1 Border Collie × Alaskan Malamute) for 20 min.

[2]Cows from group A (n = 10, being 5 CON and 5 WLF) were slaughtered for tissue collection without being exposed to a simulated wolf encounter to represent baseline differences between CON and WLF cows. Cows from group B (n = 10, being 5 CON and 5 WLF) were slaughtered for tissue collection immediately after the simulated wolf encounter.

peat binding factors 1 and *2* were greater in rats exposed to a PTSD-eliciting model (Dong et al., 2016).

A wolf exposure status × group interaction (P < 0.01) was detected for *c-Fos* mRNA expression. The WLF cows had greater (P < 0.01) mRNA expression of *c-Fos proto-oncogene* in group A, but less (P = 0.02) expression in group B compared with CON cows (Table 3). These results support outcomes detected in amygdala samples (Table 2) and further suggests that simulated wolf encounter elicited greater PTSD-like fear in WLF compared with CON, particularly because hippocampal *c-Fos* yielded the greatest convergent functional genomics score among a multitude of brain–blood genes evaluated by Le-Niculescu et al. (2011). A wolf exposure status × group interaction (P < 0.01) was also detected for mRNA expression of *brain-derived neurotrophic factor*. Expression of this factor was similar (P = 0.13) between WLF and CON cows in group A, but greater (P = 0.02) in CON cows compared with WLF cows in group B (Table 3). *Brain-derived neurotrophic factor* regulates growth and function of several neuronal systems including learning and memory processes (Hyman et al., 1991), and has been implicated in the neurobiological mechanisms underlying the clinical manifestations of PTSD (Kozlovsky et al., 2007). Accordingly, these latter authors exposed rats to litter with or without feline urine as a PTSD-eliciting model for predator-scent stress, and reported that rats exposed to the urine-containing litter had less mRNA expression of *brain-derived neurotrophic factor* in the CA1 hippocampal region, as well as greater excitability and greater plasma corticosterone concentrations. Therefore, results from Kozlovsky et al. (2007) corroborates that the simulated wolf encounter elicited PTSD-like responses in wolf-experienced cows herein and in our previous research effort (Cooke et al., 2013).

Kozlovsky et al. (2007) also reported that greater mRNA expression of *neurotrophic tyrosine kinase receptor type 2* in the CA1 hippocampal region, which is the brain-derived neurotrophic factor receptor, in rats exposed to the urine-containing litter. Kozlovsky et al. (2007) acknowledged this outcome as a compensatory response to the stress-induced downregulation of its ligand. In the present experiment, however, no differences ($P \geq 0.40$) were detected between WLF and CON across groups for mRNA expression of *neurotrophic tyrosine kinase receptor type 2* (Table 3). Nevertheless, Nibuya et al. (1999) also reported less *brain-derived neurotrophic factor* mRNA expression but similar expression of *tyrosine kinase receptor type 2* mRNA in the hippocampus of rats exposed to a repeated-stress challenge.

Medial Pre-frontal Cortex and Hypothalamus Cortex Samples

No differences ($P \geq 0.25$) were detected between WLF and CON, either in group A or B, for mRNA expression of *adenylate cyclase activating polypeptide 1* in hypothalamic samples (Table 4), as well as its *receptor type 1*, *heat shock protein 1A*, and *x-box binding protein 1* in medial pre-frontal cortex samples (Table 4). These proteins have also been associated with PTSD pathogenesis in rodent and human models. Lack of differences in *adenylate cyclase activating polypeptide 1* expression in hypothalamic tissue corroborates with results from hippocampal samples (Table 3). However, mRNA expression of *adenylate cyclase activating polypeptide 1 receptor type 1* in medial pre-frontal cortex were greater in rodents exposed to fear-conditioning PTSD models (Ressler et al., 2011). *Heat shock protein 1A*, as key component of the cellular heat shock response to stressors, has also

Table 4. Expression of genes from the medial pre-frontal cortex and hypothalamus from cows experienced with the presence of wolves (WLF; n = 10) or naïve to wolves (CON; n = 10), and subjected to a simulated wolf encounter[1,2]

Item	WLF	CON	SEM	*P*-value
Medial pre-frontal cortex				
Adenylate cyclase activating polypeptide 1 receptor type 1				
Group A	1.64	1.51	0.22	0.68
Group B	1.73	1.85	0.22	0.73
Heat shock 70kDa protein 1A				
Group A	2.48	1.81	0.84	0.58
Group B	2.64	3.99	0.79	0.25
X-box binding protein 1				
Group A	3.98	3.73	0.88	0.84
Group B	2.88	3.30	0.88	0.74
Hypothalamus				
Adenylate cyclase activating polypeptide 1				
Group A	2.03	2.38	0.35	0.49
Group B	1.47	1.39	0.35	0.88

[1]Values are expressed as relative fold change compared to threshold cycle of reference genes analyzed within the same sample (Ocón-Grove et al., 2008). Simulated wolf encounter consisted in olfactory (wolf urine; Harmon Wolf Urine Scent; Cass Creek, Grawn, MI), auditory (wolf howls reproduced on a stereo system (S2 Sports MP3 CD/Radio Boombox; Sony Corporation of America, San Diego, CA), and visual (3 adult female dogs conducted by leash, being 2 German Shepherd and 1 Border Collie × Alaskan Malamute) for 20 min.

[2]Cows from group A (n = 10, being 5 CON and 5 WLF) were slaughtered for tissue collection without being exposed to a simulated wolf encounter to represent baseline differences between CON and WLF cows. Cows from group B (n = 10, being 5 CON and 5 WLF) were slaughtered for tissue collection immediately after the simulated wolf encounter.

Table 5. Expression of genes in blood cells from cows experienced with the presence of wolves (WLF; n = 10) or naïve to wolves (CON; n = 10), and subjected to a simulated wolf encounter[1,2]

Item	WLF	CON	SEM	*P*-value
ATPase H+ transporting accessory protein 1				
Group A	1.34	1.32	0.06	0.83
Group B				
Before simulated wolf encounter	2.01	1.57	0.19	0.13
After simulated wolf encounter	2.47	2.32	0.19	0.59
c-Fos proto-oncogene				
Group A	2.43	2.06	0.45	0.58
Group B				
Before simulated wolf encounter	2.64	1.67	0.50	0.19
After simulated wolf encounter	3.38	2.92	0.50	0.52

[1]Values are expressed as relative fold change compared to threshold cycle of reference genes analyzed within the same sample (Ocón-Grove et al., 2008). Simulated wolf encounter consisted in olfactory (wolf urine; Harmon Wolf Urine Scent; Cass Creek, Grawn, MI), auditory (wolf howls reproduced on a stereo system (S2 Sports MP3 CD/Radio Boombox; Sony Corporation of America, San Diego, CA), and visual (3 adult female dogs conducted by leash, being 2 German Shepherd and 1 Border Collie × Alaskan Malamute) for 20 min.

[2]Cows from group A (n = 10, being 5 CON and 5 WLF) were not exposed to the simulated wolf encounter; hence, samples were collected to represent inherent differences between CON and WLF cows. Samples from group B were collected immediately before and after the simulated wolf encounter.

been considered a top candidate gene and brain–blood biomarker for psychological disorders (Le-Niculescu et al., 2011). *X-box binding protein 1* appears to modulate stress-induced apoptosis and the relationship between atrophy of the medial pre-frontal cortex and PTSD, and its mRNA expression were greater in rats exposed to single-prolonged stress to elicit PTSD symptoms (Li et al., 2015). Collectively, all the hypothalamic and medial pre-frontal cortex biomarkers evaluated herein were statistically similar among WLF and CON cows, differing from results obtained in amygdala and hippocampal samples.

Blood Samples

No differences ($P \geq 0.13$) were detected between WLF and CON in group A, as well as in group B prior to and after the simulated wolf encounter, for mRNA expression of *ATPase H+ transporting accessory protein 1* (Table 5). This protein constitutes an enzyme involved in regulation of neuroendocrine secretory granules, and its expression is greater in blood of humans diagnosed with PTSD (Logue et al., 2015). No differences ($P \geq 0.19$) were also detected between WLF and CON across groups for mRNA expression of *c-Fos proto-oncogene* (Table 5). Blood *c-FOS* expression was reported to be greater in PTSD patients (Segman et al., 2005), and is also considered a key blood biomarker for anxiety conditions including PTSD (Le-Niculescu et al., 2011). Hence, the lack of statistical differences among WLF and CON cows in mRNA expression of blood PTSD biomarkers also failed to ratify results obtained from amygdala and hippocampal samples.

Overall Discussion

Across all blood-brain biomarkers associated with fear-related psychological disorders and PTSD evaluated herein, only expression of hippocampal *brain-derived neurotrophic factor* and expression of *c-Fos proto-oncogene* in CA1 hippocampus and amygdala differed between WLF and CON cows after the simulated wolf encounter. Nevertheless, these biomarkers were also downregulated in rodents exposed to fear stimulus to elicit PTSD symptoms (Kozlovsky et al., 2007; Day et al., 2008), whereas hippocampal *c-Fos proto-oncogene* yielded the greatest convergent functional genomics score among all the brain–blood genes evaluated by Le-Niculescu et al. (2011). As previously mentioned, the reason why mRNA expression of *c-Fos proto-oncogene* in amygdala and hippocampal samples

were greater ($P < 0.01$) in WLF vs. CON from group A cows is unknown, and may be related to inherent differences among cattle including cow origin and previous history of management and stressful events (Cooke et al., 2013). Yet, the opposite outcome detected in amygdala and hippocampal samples in group B suggest that the simulated wolf encounter differentially regulated mRNA expression of these fear-related PTSD biomarkers between WLF and CON cows. Moreover, WLF and CON cows were assigned to this experiment approximately 120 d after returning from summer pastures, where WLF grazed areas with active wolf packs. This suggests that previous interactions with wolves appears to have long-term impacts on *brain-derived neurotrophic factor* and *c-Fos proto-oncogene* mRNA regulation on subsequent wolf encounters.

Neural responses to fear appear to be initiated in the amygdala, with direct stimuli from the hippocampus when fear-related memories are present, followed by projections into other brain region and across the blood-brain barrier (Gorman et al., 2000). This latter rationale may help explaining why differences between WLF and CON cows in group B were only noted in amygdala and hippocampal samples; perhaps sampling design adopted herein was not appropriate to detect similar outcomes in hypothalamic, medial pre-frontal cortex, and blood samples. This include the time elapsed between the simulated wolf encounter and slaughter, as well as interval between previous grazing season and the beginning of this experiment; although research is warranted to investigate these assumptions. Further, cattle temperament, body temperature, and plasma cortisol concentrations were not evaluated herein due to limited statistical power, which needed at least 50 WLF and 50 CON cows based on the G*power 3 software (Faul et al., 2007) and Cooke et al. (2013). Yet, the main goal of the present experiment was to focus on mRNA expression of PTSD-related biomarkers established by the human and rodent literature, and provide novel information regarding the impacts of wolf predation on beef cattle welfare beyond injury and death. Therefore, additional research with a greater number of cows, based on the experimental population described by similar human and rodent research, is warranted to corroborate the novel findings reported herein and perhaps yield further statistical differences among WLF and CON cows.

Conclusion

Collectively, results from this experiment indicate that the simulated wolf encounter downregulated mRNA expression of hippocampal *brain-derived neurotrophic factor* and *c-Fos proto-oncogene* in hippocampus and amygdala, which are key biological markers of stress-related psychological disorders elicited primarily by fear. These outcomes are corroborated by comparable research with rodents (Dayas et al., 2001; Kozlovsky et al., 2007; Day et al., 2008), and suggest that altered behavior and physiological changes in wolf-experienced cows exposed to the simulated wolf encounter by Cooke et al. (2013) can be associated with PTSD symptoms. Hence, the presence of wolf packs near cattle herds may negatively impact beef production systems via predatory activities and subsequent death and injury of animals, as well as by inducing fear-related psychological disorders that impair cattle welfare and productivity when packs are in close proximity to previously predated herds. Given these findings, future research should explore options for scientifically based methods aimed at reducing the likelihood of exposure between predators and beef cattle, or perhaps cost-effective ways of inoculating cattle to native predators early in their development to prevent or decrease stress responses later in life.

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