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Review

Potential nutritional strategies for the amelioration or prevention of high rigor temperature in cattle – a review

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Abstract. Environmental conditions influence animal production from an animal performance perspective and at the carcass level post-slaughter. High rigor temperature occurs when the animal is hyperthermic pre-slaughter, and this leads to tougher meat. Hyperthermia can result from increased environmental temperature, exercise, stress or a combination of these factors. Consumer satisfaction with beef meat is influenced by the visual and sensory traits of the product when raw and cooked, with beef consumers commonly selecting tenderness of the product as the most important quality trait. High rigor temperature leads to a reduction in carcass and eating quality. This review examines some possible metabolic causes of hyperthermia, with focus on the importance of adipose tissue metabolism and the roles of insulin and leptin. Potential strategies for the amelioration or prevention of high rigor temperature are offered, including the use of dietary supplements such as betaine and chromium, anti-diabetic agents such as thiazolidinediones, vitamin D, and magnesium (Mg) to provide stress relief.

Additional keywords: adipose, fat, heat stress, insulin resistance, leptin, supplementation, TZDs.

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Introduction

Farming and abattoir practices need to adapt to climate change, to prevent or reduce the impact of high environmental temperature on meat quality due to processes such as high rigor temperature (Gregory 2010). In Australia, the 75% of beef carcasses have high rigor temperature (Warner *et al*. 2014*a*), which shows the importance of finding methods to reduce the incidence. One of the main causes of high rigor temperature appears to be a combination of rapid glycolysis and relatively slow cooling, which can lead to high rigor temperatures and potential toughening (Jacob and Hopkins 2014). Moreover, high rigor temperature may be exacerbated by loss of proteolytic potential due to inactivation of proteolytic enzymes (Kim *et al*. 2014; Warner *et al*. 2014*a*). The mechanisms of high rigor temperature in beef carcasses and the effects on meat quality are reviewed in Kim *et al*. (2014) and Jacob and Hopkins (2014) in this issue of *Animal Production Science*. Factors that contribute to elevated body temperature pre-slaughter potentially predispose animals to higher carcass temperature immediately post slaughter (Jacob and Hopkins 2014).

There is mounting evidence that a state of insulin resistance can compromise the ability of an individual to thermoregulate. For example, literature related to rodents and humans suggests that stressors such as infection, inflammation, ageing, heat stress and excessive adipose tissue deposition can all modulate insulin action (Tsiotra and Tsigos 2006). Since all of these stressors are also encountered by beef cattle, we hypothesise that the responses in cattle may be similar. In particular, as cattle fatten and develop intramuscular fat and marbling, which is morphologically and metabolically similar to visceral fat in humans, the responses to stressors may resemble those seen in diabetic humans. In this context, intramuscular fat accumulation has been found to be associated with insulin resistance in humans (Goodpaster *et al*. 1997; Kim *et al*. 2006; Komiya *et al*. 2006). We hypothesise that there are links between insulin resistance, stress and thermoregulation in cattle, and possible links to high rigor temperature.

Effect of age, weight and fatness on plasma metabolites and hormones

Insulin resistance is defined as a reduced ability to respond to the effects of insulin, especially by peripheral tissue such as muscle and adipose tissues. Since cells must have glucose to survive, the body compensates for insulin resistance by producing additional insulin, resulting in hyperinsulinemia and eventually hyperglycemia. In cattle, blood glucose increases with age (Lorenz 2000), while fasting plasma insulin is positively correlated with liveweight (McCann *et al*. 1986). When grazing beef cattle are introduced to concentrate feeding in a feedlot, the response is exacerbated and there is an increase in circulating glucose and insulin with time on feed (Fig. 1) (Henry *et al*. 2000; Hersom *et al*. 2004*c*), which is also associated with increasing fatness and adipocyte cellularity (Hersom *et al*. 2004*a*, 2004*b*, 2004*c*). Feeding of grain is also associated with an increase in core body temperature (Jacob *et al*. 2014). Fat sheep and cattle have higher plasma concentrations of glucose, insulin

and non-esterified fatty acids (NEFA) than their thin counterparts (Table 1) (McCann *et al*. 1989; McCann *et al*. 1997; Henry *et al*. 2000). Moderately fat sheep and cattle exhibit an exaggerated insulin response to intravenous glucose injection (McCann *et al*. 1989; McCann *et al*. 1986). Although these differences are most exaggerated in obese ruminants, they also occur when animals grow older, heavier and fatter. For example, Eisemann *et al*. (1997) found that insulin responsiveness and sensitivity of the hindquarter of beef steers decreased with increasing age and liveweight (275 *v*. 490 kg), indicating insulin resistance of peripheral tissues as steers get heavier and fatter. Metabolism across the hindlimb or hindquarter is generally considered to reflect skeletal muscle metabolism, although these tissue beds also contain adipose tissue (Boisclair *et al*. 1993, 1994; Dunshea *et al*. 1995). Together these data suggest that long periods of grain feeding would increase fat deposition and elevate circulating glucose and insulin, presumably by inducing a state of insulin resistance. This was demonstrated recently, where animals with a higher blood insulin concentration at slaughter had a higher incidence of high rigor temperature (Warner *et al*. 2014*a*).

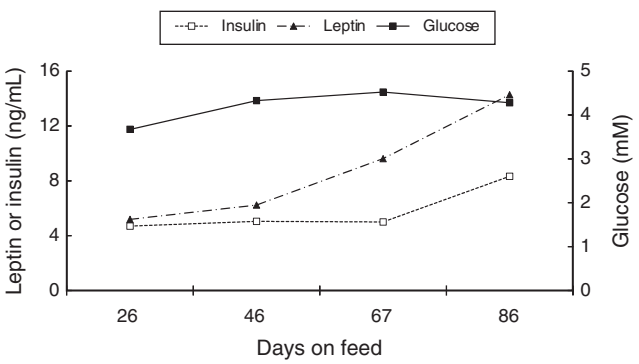

Fig. 1. Effect of days on feed in the feedlot on plasma insulin (□), leptin (▲) and glucose (■) in beef cattle. Data are drawn from the second experiment described in Hersom *et al*. (2004*c*).

Table 1. Effects of bodyweight on plasma hormone and metabolite levels and body composition and organ weights

In addition, body composition analysis was achieved by DXA analysis and organ weights (adapted from Henry *et al*. 2000). Results from body composition analysis were corrected for bodyweight. All data are presented as mean ± (s.e.m.); **P* < 0.05, ***P* < 0.01, ****P* < 0.001, for significance of difference between fat and thin groups

Measurement	Fat	Thin
Plasma hormones and metabolites		
Glucose (mM)	3.48 ± 0.24	2.96 ± 0.06
Insulin (μU/mL)	9.76 ± 1.84	2.51 ± 0.71**
NEFA (μM)	792 ± 101.33	516 ± 35.3***
Leptin (ng/mL)	1.59 ± 0.1	0.31 ± 0.06***
Body composition and organ weights (g/kg)		
Lean body mass	266.46 ± 3.71	290.49 ± 6.04***
Fat mass	155.27 ± 6.5	46.3 ± 5.37***
Abdominal fat	0.08 ± 0.009	0.022 ± 0.008***
Liver	0.008 ± 0.002	0.013 ± 0.0004*

Another hormone associated with increased body fatness is leptin, a hormone produced by adipocytes that can signal the brain as a satiety factor; and mutations in the leptin gene or in the leptin receptor can lead to obesity (see review by Zieba *et al*. 2005). Expression of the leptin gene in adipose tissue and circulating leptin concentrations are highly correlated with percentage of body fat in rodents (Soukas *et al*. 2000) and the degree of obesity in humans (Auwerx and Staels 1998). The concentration of leptin is directly related to the degree of adiposity in cattle (Ehrhardt *et al*. 2000; Hersom *et al*. 2004*c*) and sheep Chilliard *et al*. 2005). Long-fed cattle and obese sheep tend to reduce their feed intake as they become fatter and leptin may act as a satiety factor to inhibit feed intake in fat animals that have accumulated adipose tissue. In this context, central leptin infusion decreases feed intake in *ad libitum* fed feed but not in sheep on a restricted feed intake (Henry *et al*. 1999, 2004).

There is a complex interplay between plasma insulin and leptin. Insulin stimulates plasma leptin in monogastrics such as rodents and humans (Ahima and Flier 2000; Considine 2001), and probably ruminants (Ingvartsen and Boisclair 2001). Plasma leptin appears positively related to plasma glucose and insulin and negatively related to plasma NEFA and somatotropin in periparturient dairy cows (Block *et al*. 2001). Also, Leury *et al*. (2003) found that insulin infusion increased plasma leptin during late pregnancy when cows were in positive energy balance but had little effect during early lactation when cows were in negative energy balance. However, central administration of leptin does not alter plasma insulin (Henry *et al*. 1999), except perhaps in previously well-fed sheep that were fasted for 72 h, in which insulin decreased (Henry *et al*. 2004). Since finishing beef cows have a highly positive energy balance, the increased plasma leptin could be due to a combination of increased body fatness and increased insulin secretion. In high residual feed intake (RFI) (inefficient) steers, plasma insulin concentrations tend to be higher, perhaps indicating that inefficient steers are less sensitive to the actions of insulin (Richardson *et al*. 2004).

Therefore, it is reasonable to suppose that as long-fed cattle accumulate subcutaneous, visceral and intramuscular fat, they also become insulin resistant. As a result, plasma insulin, glucose and leptin increase and animals reduce their ability to cope with stressors such as infection, inflammation and heat stress. Although the focus here has been on adipose tissue production of leptin, it is also possible that several other adipose-tissue-derived factors such as adiponectin, tumour necrosis factor α (TNFα) and resistin may also be involved in the induction of insulin resistance in long fed cattle (Komatsu *et al*. 2005; Roh *et al*. 2006).

Heat stress reduces the *in vitro* expression of leptin and its receptor mRNA in bovine peripheral blood mononuclear cells (Lacetera *et al*. 2009), although the concentrations of leptin and its receptors were increased when lactating dairy cattle were exposed to longer daylengths *in vivo* (Bernabucci *et al*. 2006), similar to those seen in summer months. It is therefore suggested that during heat exposure, the secretion of leptin from adipose tissue is enhanced while the secretion of leptin from other tissues, which contribute less to the overall pool of leptin, may be reduced in a homeostatic mechanism that assists to attenuate less critical processes such as immunity (Lacetera *et al*. 2009).

Insulin resistance and heat tolerance

In addition to the effects that insulin resistance has on tissue carbohydrate metabolism, increasing evidence suggests that diabetic individuals suffer from thermal intolerance, exhibiting an inability to control body temperature (Ohtsuka *et al*. 1995). In part this is because skin blood flow and skin thickness are reduced in diabetic individuals (Forst *et al*. 2006), thereby reducing the ability to thermoregulate. In this context, chronic treatment with the insulin sensitiser rosiglitazone increases skin blood flow and improves the ability to thermoregulate in diabetic individuals (Petrofsky *et al*. 2005).

The fundamental thermoregulatory strategy of any mammal, including beef cattle, is to maintain a body core temperature higher than ambient to allow heat to dissipate via conduction, convection, radiation and evaporation (Collier *et al*. 2006). Conduction, convection and radiation are referred to as sensible routes of heat loss and they require a thermal gradient to operate. On the other hand, evaporation works on a vapour/pressure gradient, and it is defined as insensible heat loss. When ambient temperature conditions approach body temperature, the only viable route of heat loss is evaporation; if ambient conditions exceed body temperature, heat flow will reverse and the animal becomes a heat sink. In order to assist evaporation from the skin during heat exposure, and thereby dissipate heat, there is an increase in blood flow to, and sweating from, the skin of cattle and other ruminants (Choshniak *et al*. 1982). Merino sheep, which are more heat-tolerant than British sheep breeds, appear to exhibit greater insulin sensitivity, at least with respect to fat metabolism, than British breeds (Ponnampalam *et al*. 2005). Interestingly, such differences exist between Merino sheep selected for high and low greasy fleece weights (G. M. Butler, F. R. Dunshea and B. J. Leury, unpubl. data).

One link between insulin resistance, skin blood flow and thermoregulation may be via nitric oxide synthase (NOS). Chronic diabetes, acute hyperglycaemia and NOS inhibition by N^{ω}-nitro-L-argine (L-NNA) all lower heat tolerance in rats (Swiecki *et al*. 2003). Those authors suggest that, in part, this may be due to the inability to produce heat shock proteins (HSP), as the control rats had greater hepatic and intestinal expression of HSP-72 when subjected to heat stress than the heat-treated diabetic, hyperglycaemic or L-NNA-treated rats. The regulation of blood flow to the skin may be mediated via the central nervous system, at least in part regulated by an isoform of NOS, specifically neuronal NOS (nNOS) (Kellogg *et al*. 2008). Kellogg *et al*. (2008) examined the effects of the specific nNOS inhibitor 7-nitro indazole (7-NI), administered by intradermal microdialysis, on vasodilation induced by whole-body heat stress or local skin warming and found that nNOS inhibition reduced the increases skin blood flow during whole body heat stress but not during local skin warming. The differences between the effects of different isoforms of NOS, as well as the differing responses to systemic and local hyperthermia, highlight how complex the vasodilatory response can be (Cals-Grierson and Ormerod 2004). However, muscle nNOS is reduced in individuals that are insulin-resistant (Bradley *et al*. 2007) but there are few data on skin nNOS activity or contents.

In lactating cattle, heat stress reduced feed intake and increased basal and glucose-stimulated insulin concentrations, while adipose tissue reserves were not mobilised, which is likely a response that allows animals to acclimate to heat, although this is yet to be elucidated (Achmadi *et al*. 1993; Rhoads *et al*. 2009; Shwartz *et al*. 2009; Wheelock *et al*. 2010). We recently reviewed these responses in lactating dairy cattle in depth (Dunshea *et al*. 2013), and thus the responses in lactating cattle will not be covered here. Prolactin modulates sweat gland function (Alamer 2011) and is involved in maintaining electrolyte and water balance in the heat-stressed mammalian gut, kidney and mammary gland (Collier *et al*. 1982; Faichney and Barry 1986). Prolactin increases insulin secretion (Bole-Feysot *et al*. 1998) while also reducing the number of insulin receptors in adipose tissue (McNamara 1991). Therefore, the increase in plasma prolactin concentrations has a multifaceted influence on the heat-stressed mammal and may be partially responsible for heat-associated insulin resistance. Prolactin may also influence metabolism independent of physiological responses (such as respiration rate) to heat, as we recently demonstrated in sheep exposed to a mild level of heat (~36°C for 6–7 h daily) (DiGiacomo 2011).

Leptin and thermogenesis

In rodents, leptin regulates thermogenesis in brown adipose tissue (BAT) directly and indirectly by controlling sympathetic outflow from the brain (Lowell and Spiegelman 2000). In chronically heat-stressed mice, adipose tissue leptin mRNA expression was significantly increased, which was paralleled by an increase in circulating plasma leptin despite the concurrent decrease in feed intake; perhaps indicating that heat treatment itself influences leptin (Morera *et al*. 2012).

In rats, catabolic states such as starvation (Sivitz *et al*. 1999) and lactation (Xiao *et al*. 2004) reduce uncoupling protein 1 (UCP 1) mRNA expression in BAT, and this effect can be reversed by peripheral leptin treatment. Central administration of leptin increases sympathetic nerve activity (Rahmouni and Morgan 2007), while a leptin antagonist blunts high fat feeding induced elevation of UCP 1 mRNA expression (Zhang *et al*. 2007) in BAT of rats, providing compelling evidence that leptin acts on the brain to regulate diet-induced thermogenesis. However, effects of leptin on skeletal muscle thermogenesis are more equivocal.

Leptin treatment was shown to increase oxygen uptake in explants of murine soleus muscle, indicative of thermogenesis (Dulloo *et al*. 2002), providing evidence for a direct effect on muscle. In an elegant study, Henry *et al*. (2008) demonstrated a post-prandial rise in thermogenesis that was evident in gluteal (subcutaneous) fat, visceral (retroperitoneal) fat and skeletal muscle. Importantly, central infusion of leptin markedly increased the post-prandial thermogenesis at all three sites, albeit to varying degrees. Central leptin infusion increased post-prandial thermogenesis to a greater extent in skeletal muscle and visceral fat than in gluteal fat (Henry *et al*. 2008), and this is associated with increased expression of UCP3 and UCP3 mRNA and increased mitochondrial oxygen consumption due to a shift towards uncoupled respiration (Henry *et al*. 2011). Furthermore, skeletal muscle and visceral fat exhibited higher baseline temperatures than gluteal fat. Together these data demonstrated that inherently different thermogenic capacities

are operative in discrete tissues in sheep. Given that skeletal muscle represents a significant mass within the body and its robust thermogenic capabilities, the energy expended by this tissue is of great importance in the determination of daily energy expenditure.

In the context of what might happen in feedlot cattle, the accumulation of adipose tissue lipid and resultant increased plasma leptin may place a post-prandial thermogenic load that could become even more difficult to dissipate because of increased fat cover and reduced skin blood flow resulting from insulin resistance. This could become exacerbated with the resultant stress and the associated catecholamine-induced heat production around transport and slaughter. It is highly likely that even a small increase in body temperature as the animal enters the kill-chain could be enough to ensure that a carcass becomes classed as high rigor temperature.

Stress and temperature

A main cause of high rigor temperature appears to be a combination of rapid glycolysis and relatively slow cooling, which can lead to high rigor temperatures and potential toughening (Warner *et al*. 2014*b*). Moreover, high rigor temperature may cause a loss in proteolytic potential due to inactivation of proteolytic enzymes (Kim *et al*. 2014). Factors that contribute to elevated body temperature pre-slaughter would potentially predispose animals to higher carcass temperature immediately post slaughter. In the previous section, evidence was provided for impaired thermoregulatory capacity in cattle fed grain for long periods, due to the progressive development of an insulin-resistant state. Thermoregulatory capacity can also be altered by exposure to certain stressors. For instance, exposure to high ambient temperature, exercise (Warner *et al*. 2005) and psychological stress (Pighin *et al*. 2014) can result in an elevation of body temperature (core and peripheral temperatures) as a consequence of passive and/or active heat accumulation. In nearly all circumstances, an animal responds to external stimuli or stress by making complex adjustments to physiological processes in an attempt to maintain homeostasis. This normally involves activation of the hypothalamic-pituitary-adrenal (HPA) axis and *sympathetic nervous system* (SNS). Thus, an insulin-resistant state and/or stress may reduce the capacity of cattle fed grain for long periods to dissipate heat considerably in the immediate pre-slaughter period.

Effect of heat stress and exercise on body temperature

Exercise and thermal stress are known to increase core body temperature in a range of species, and the combination of the two stressors augments the elevation in body temperature (Febbraio *et al*. 1994). The magnitude of increase in core temperature depends on the severity and duration of each particular stressor. Although little is known about changes in muscle temperature *per se* during exercise and heat stress in ruminants, in humans it is documented that during exercise intramuscular temperature rises in proportion to the increase in workload, and the rise in intramuscular temperature is augmented during combined exercise and heat stress (Febbraio *et al*. 1994; Parkin *et al*. 1999). Warner *et al*. (2005) showed that lambs exercised for 10 min pre-slaughter had higher post-mortem muscle temperatures in the loin, neck and leg. Thus, it seems reasonable to assume that intramuscular temperature is also elevated in ruminants during exercise and thermal stress.

Effect of psychological stress on body temperature

With respect to thermoregulation in animals, recent research in the area of psychological stressors and effects on body temperature may provide some insight into variation in body temperature in livestock. The ‘psychogenic fever’ response has been most notably demonstrated in humans following physical examinations and after watching disturbing films, as well as in laboratory animals (Takakazu *et al*. 2001). This was recently demonstrated in lambs, where a rise in core body temperature occurred in response to the stressors associated with pre-slaughter handling such as mustering and handling (Pighin *et al*. 2014).

Stress can increase core temperature, and this effect can last for weeks, and even years, depending on the severity and duration of the stressor (Godsil *et al*. 2000; Takakazu *et al*. 2001; Pardon *et al*. 2004; Bhatnagar *et al*. 2006). Body temperature is also known to respond to conditioning, where the animal anticipates adverse environments and experiences, resulting in a rise in core temperature (Godsil *et al*. 2000; Pardon *et al*. 2004). This rise in core temperature is caused by a fever-like response, similar to that occurring during illness. An interaction between psychological stress and the fever response is thought to be associated with thermosensitive neurons in the preoptic area of the hypothalamus (that are central to the regulation of body temperature), which are responsive to non-thermal emotions, at least in monkeys (Takakazu *et al*. 2001). The release of stress hormones (i.e. activation of the HPA axis and stimulation of the SNS) mediates some, but not all, of the mechanisms that increase core temperature.

Some of the body temperature responses to social stress are clearly independent of the HPA response. Bhatnagar *et al*. (2006) demonstrated that repeated social stress in rats was associated with an increase in body temperature, presumably independent of activation of the HPA axis, since the elevation in core temperature was sustained well after increases in ACTH (adrenocorticotropic hormone) and corticosterone concentrations had returned to their basal levels following the generalised stress response. Interestingly, this repeated stress reduced bodyweight gain but increased feed intake during different periods, meaning that these changes to bodyweight cannot be explained entirely by the changes to feed intake, and may be the result of increased temperatures. An alternative mechanism may be that enhanced noradrenaline release in the hypothalamus during stress increases production of prostaglandin E_2 (PGE_2), which acts on the central nervous system to increase core temperature (Takakazu *et al*. 2001).

Skin blood flow decreases in response to stress as blood is redirected towards skeletal muscles and/or because of peripheral vasoconstriction (i.e. SNS-induced). Interestingly, this has also been observed with fear-related stress. In rats, tail and paw temperature decreased in response to fear, presumably due to SNS vasoconstrictor activity, although there was no marked reduction in temperature for other body surfaces (Vianna and Carrive 2005). Thus, these responses are quite differential and

probably depend on body shape/conformation, etc.; similar responses have been noted in primates including humans.

Moreover, studies are often conflicting and it is difficult to ascertain whether there are real species differences or the measurement techniques are inadequate or that type of stressor and/or physiological state/animal variation is important. For instance, Ruis *et al*. (2001) found that gilts subjected to social isolation displayed significantly lower temperature, recorded from the ear and rectum by thermometer, which contrasts with most other studies. This indicates that perhaps species-specific responses occur. Re-exposure to fear-inducing situations in rats increases body temperature, possibly due to vasoconstriction limiting heat loss and metabolic effects generating heat via muscle activity and increased metabolism (Vianna and Carrive 2005). Those authors also found that the increase in body temperature following re-exposure to fear was delayed by 7 min and was higher in sham- than fear-conditioned animals. However, this may depend on the type of stressor, as Godsil *et al*. (2000) found that rectal temperatures of sham-conditioned rats did not increase, whereas that of animals who received foot shock increased by ~1°C.

Effects of elevated muscle temperature

Both exercise and heat stress can cause significant changes in cardiovascular and thermoregulatory processes; in combination, this can be a significant strain on animals. There has been considerable research in this area in several species including sheep (e.g. Bell *et al*. 1983) and humans (e.g. Buskirk 1977) but there are virtually no data in large ruminants. By contrast, much less is known about the combined effects of exercise and heat stress on whole animal and muscle metabolism (Febbraio 2001), with most of the available information in the field of human exercise physiology. Both of these stressors *per se* and especially in combination cause relative hyperthermia in humans and decreased exercise performance (Drust *et al*. 2005).

A striking metabolic alteration with exercise, at least for submaximal exercise in humans, is the preferential shift towards muscle glycogen utilisation at the expense of triglyceride use. and this appears to involve both oxidative and non-oxidative energy pathways (Febbraio 2001). Accumulation of muscle lactate and plasma lactate is also increased during exercise and heat stress in humans (e.g. Parkin *et al*. (1999)) and dogs (Kozlowski *et al*. 1985). This appears related to increased intramuscular glycogenolysis and not to increased uptake and oxidation of blood glucose. More recent studies have shown that while oxidation by muscle of exogenous glucose is reduced under these conditions (Jentjens *et al*. 2002), a combination of ingested glucose and fructose may improve oxidation of carbohydrate in muscle (Jentjens *et al*. 2006). Only limited information is available on muscle metabolism during heat stress and/or exercise in large ruminants. Glucose availability in ruminants relies predominantly on gluconeogenesis, so glucose sparing might be expected. However, Pethick *et al*. (1987) demonstrated that glucose was preferentially absorbed and oxidised over NEFA in the hindlimb of exercising sheep under thermo-neutral conditions. How the imposition of heat stress *per se* or in combination with exercise influences the pattern of substrate oxidation in ruminants is not known.

The mechanisms responsible for alterations in metabolism during combined exercise and heat stress or heat stress *per se* have not need elucidated. Several possible mechanisms have been proposed, including a direct temperature effect on enzyme reaction rates (the Q10 effect; Young 1990), reduced oxygen and substrate delivery to muscle as a result of competing demands for blood-flow delivery to the periphery to aid in heat dissipation (Rowell 1974), and an increase in circulating epinephrine concentrations (Yaspelkis *et al*. 1993).

Probably one of the most important implications of elevated intramuscular temperature *per se* is the potential effect on key enzymes that ultimately influence muscle metabolism, the so-called Q10 effect (Young 1990). A Q10 value represents the *x*-fold increase in enzyme reaction rate for every 10°C increase in temperature; this is usually ~2–3 for enzyme-mediated reactions. Thus, for increases in intramuscular temperature of ~1°C, enzyme reaction rates could increase by ~15–20%. The implication for elevated carcass temperature immediately post-slaughter is obvious if glycogen is preferentially utilised pre-slaughter and its metabolism accelerated post-slaughter because of the elevated temperatures.

Stress and heat shock proteins

Considerable research has also recently focused on the role of intracellular HSPs in protecting cell integrity during exposure to stress, in particular hyperthermia. Heat shock proteins are a family of highly conserved proteins of varying molecular weight and are found in nearly all cells and tissues (Lindquist 1986). Some are expressed constitutively in non-stressful situations and others are induced by a range of different stressors, including heat stress and exercise.

One of the most important of the HSPs is HSP70 and its inducible form, HSP72. Activation of the HPA and SNS axes are likely to play an important role in stimulating the synthesis of HSPs (Fleshner and Johnson 2005). These proteins are rapidly induced in response to a range of stressors, including heat stress (Ruell *et al*. 2004), exercise in humans (Febbraio *et al*. 2002; Whitham *et al*. 2006) and psychological stress in rats (Fleshner *et al*. 2004). Small HSPs are involved in muscle cell maintenance and repair, and the upregulation of some of these proteins, such as HSP27, is related to improved tenderness, juiciness and flavour in beef (Bernard *et al*. 2007); in transported pigs, a decline in HSP27 expression is associated with a decline in meat quality (Yu *et al*. 2009). Not only are HSPs rapidly induced intracellularly, but they can also appear in blood, although the exact mechanism for translocation into blood has not been identified, and the importance of elevated circulating HSPs has not been established. Nonetheless, they may be a valuable biomarker of stress in animals.

Heat shock proteins and thermo-tolerance

It is conceivable that variation in the rapidity and extent of induction of HSPs between animals in their response to stressors such heat and exercise, and psychological stress, may translate into variation in thermo-tolerance. In humans for instance, heat intolerance appears related to transcriptional malfunction associated with HSP induction, along with other stress-related proteins, which suggests some inherent basis for

thermoregulatory dysfunction (Moran *et al*. 2006). This may have important implications for animal health and performance, but equally, it may have significant implications for intracellular protein integrity (e.g. protection of proteolytic enzymes from heat-induced denaturation) in the carcass post-slaughter. For instance, some animals may be more sensitive to elevated body temperature and have enhanced upregulation of HSP synthesis in the pre-slaughter period, which subsequently protects muscle cell integrity in the carcass. This may help to reduce high rigor temperature and other meat quality defects associated with elevated carcass temperature post-slaughter. For example, in Duroc boars, differences in the genotype at the HSP72 gene appear to influence backfat deposition under different environmental temperatures, although this appears to be most prominent in cooler months (Huang *et al*. 2004).

In addition to any inherent variation between animals in the induction of HSPs and thermo-tolerance, recent evidence suggests that it may be possible to pre-condition animals to thermal stress, enabling them to cope with subsequent heat-stress events. Although the exact mechanisms for this have not been elucidated, short-term (30-min) hyperthermia preconditioning in mice was associated with enhanced survival rates and elevated hepatic levels of HSP70 (King *et al*. 2002).

In addition to hyperthermia pre-conditioning, there is now ample evidence in rodents that other forms of pre-conditioning, either alone or in combination with hyperthermia pre-conditioning, may offer significant protection for tissues and cells from subsequent exposure to a range of stressors. One important example is the demonstrated role of L-glutamine (L-Gln) in potentiating the induction of HSP following subsequent heat stress, injury or sepsis (Wischmeyer *et al*. 2001; Ropeleski *et al*. 2005; Morrison *et al*. 2006; Wang *et al*. 2007). As one of the most abundant amino acids in plasma, L-Gln is synthesised and stored in several tissues, including both lung and skeletal muscle. L-Gln becomes conditionally essential in states of severe stress in which intracellular L-Gln levels can fall by ~50% and plasma concentrations by 30% (Kovacevic and McGivan 1983). A study by Singleton and Wischmeyer (2006) showed that in rats previously administered with L-Gln orally and then exposed to hyperthermic conditions, HSP expression in gut tissues was enhanced, gut permeability was reduced, and plasma endotoxin concentrations were lower than in rats exposed to hyperthermia without L-Gln supplementation. The integrity of the gastrointestinal epithelium is highly susceptible to heat stress, and thus it is not surprising that HSP72 is highly expressed in gut tissue even under thermo-neutral conditions (Ruell *et al*. 2004), and protection of gut tissue under these conditions plays a vital role in allowing animals to cope with elevated body temperature. By contrast, some tissues decrease HSP expression in response to stress. Rats exposed to restraint stress had decreased HSP90 in liver and spleen tissues, while brain and some peripheral tissues (thymus, adrenal glands and testes) showed no change to their HSP70/90 steady-state levels (Vamvakopoulos *et al*. 1993).

Heat-stressed cells have compromised mitochondrial function, and thus aerobic metabolism is adversely affected, causing cells to rely more heavily upon glycolytic energy metabolism (Welch 1992). Intriguingly, chaperone proteins (HSPs) are well documented to protect against obesity-induced insulin resistance (e.g. Kurucz *et al*. 2002; McCarty 2006; Chung *et al*. 2008; Gupte *et al*. 2011). While the exact mechanism behind this phenomenon is unidentified, it is thought to involve reduced serum insulin and IGF-1 levels (McCarty 2006) and it is also suggested to be due to the protective effects of HSPs limiting the actions of plasma free radicals (Kurucz *et al*. 2002). Furthermore, HSPs may offer mitochondrial protection by preventing damage to oxidation processes (Gupte *et al*. 2008). Intriguingly, people with an inherited reduced capacity to synthesise HSPs, as well as ageing populations, have an increased risk of developing insulin resistance (McCarty 2006; Kavanagh *et al*. 2009).

Chung *et al*. (2008) showed the expression of HSP70, but not HSP90, protein in human skeletal muscle to be markedly reduced in insulin-resistant populations, and in the skeletal muscle of type 2 diabetic humans, HSP70 expression was decreased. In the same study, heat therapy protected mice from basal hyperglycemia and hyperinsulinemia otherwise induced by consuming a high-fat diet (Chung *et al*. 2008). Moreover, diabetic rodents and humans with higher concentrations of HSP70 have higher survival rates (Kavanagh *et al*. 2009). This leads to the interesting notion that HSP induction may combat insulin resistance. Furthering this idea, liver samples from diabetic animals had significantly less heat shock factor 1 (HSF1, a transcription factor that regulates the expression of HSP) and somewhat less HSP70 than normal animals (Kavanagh *et al*. 2009). Human skeletal muscle HSP70 is strongly correlated with numerous insulin-stimulated metabolic changes such as decreased lipid oxidation and increased uptake, oxygenation and storage of glucose (Kurucz *et al*. 2002). In adipose cells, prolonged oxidative stress negatively influenced insulin-stimulated GLUT4 actions, therefore limiting insulin-stimulated increases in glucose transport (Kurucz *et al*. 2002). Nevertheless, involvement of HSPs in insulin signalling and nutrient partitioning requires further investigation, which may provide insights into how heat affects substrate metabolism at a cellular level.

Dietary interventions to reduce high rigor temperature

Betaine

The most compelling dietary intervention to date is the use of betaine (trimethylglycine), as reviewed by Dunshea *et al*. (2005). Mammals utilise betaine for two major functions. The first is as a methyl donor (via S-adenosyl-methionine (SAM)), thereby sparing methionine and increasing the available substrates for protein synthesis (Matthews *et al*. 2001). Second, when not catabolised, betaine is used as an organic osmoprotectant (Fernández *et al*. 1998; Huang *et al*. 2007). Bacterial studies show that betaine is able to assist the organism to survive high osmolarities by restoring changes to enzymes and stabilising and assisting in thermoprotection (Caldas *et al*. 1999). As an osmolyte, betaine accumulates within osmotically stressed cells (Eklund *et al*. 2005) and betaine uptake can be via active (Na^+ Cl^-) or passive transporter methods (Craig 2004). This is an important role, as loss of water within a cell can lead to cell damage or catabolism, with osmotic stress interrupting the cell cycle and halting cell proliferation while causing cells to initiate apoptosis.

Dietary betaine has also been shown to improve the growth performance of pigs by reducing the maintenance energy requirement due to its osmolyte properties reducing the need for sodium/potassium pumps (Schrama *et al*. 2003; Suster *et al*. 2004). Betaine supplementation (0.125%) significantly lowered serum urea nitrogen in pigs, while total protein levels significantly increased (Huang *et al*. 2007), and IGF-1, free thyronine (FT_3), free thyroxine (FT_4) and insulin levels all significantly increased by 40–50% in response to betaine supplementation (Huang *et al*. 2006).

Betaine can also improve water-holding capacity and reduce drip loss in meat (Dunshea *et al*. 2005), although in meat lambs, betaine supplementation did not alter the hydration status of the animals at slaughter (Pearce *et al*. 2008). Also, dietary betaine can improve the integrity of gut mucosal cells and reduce the severity of some enteric infections in poultry (Matthews and Southern 2000; Klasing *et al*. 2002). Cronje (2005) has suggested that that heat stress and its manifestations are a disease of the gut and that dietary betaine may assist by improving gut integrity.

Most investigations utilising dietary betaine as a nutritional supplement involve pigs and poultry, although there is evidence that dietary betaine can reduce heat stress and improve feed intake and growth performance in beef cattle (Cronje 2005; Loxton *et al*. 2007). Growing lambs supplemented with 2 g/kg of betaine had significantly reduced fat thickness, while liveweight and muscle area did not differ (Fernández *et al*. 1998). Importantly, albeit with small numbers, Loxton *et al*. (2007) found that dietary betaine supplementation reduced the incidence of heat toughening (defined as passing through the MSA temperature–pH window) from 5/6 carcasses to 1/7 carcasses. In sheep, no change to bodyweight and lean muscle content was seen in response to betaine supplementation (Fernández *et al*. 2000). We recently showed dietary betaine supplementation (0, 2 and 4 g betaine/day) to have dose-dependent physiological responses in sheep exposed to heat (36−43°C) and thermo-neutral conditions (22°C) (DiGiacomo 2011), such that regardless of environmental temperature, rectal temperatures were lower in sheep supplemented 2 g betaine/day and higher in those supplemented 4 g betaine/day compared with control sheep (39.6, 39.8 and 39.7°C, respectively, $P = 0.002$). Similar responses were seen for respiration rate (113, 102 and 116 breaths/min for 0, 2 and 4 g betaine/day, respectively) and heart rate (95, 88 and 99 beats/min) (DiGiacomo *et al*. 2012).

In lactating dairy cows, plasma NEFA concentrations decreased linearly with increasing dietary betaine supplementation levels, which may be the direct result of increased insulin concentrations inhibiting NEFA production (Wang *et al*. 2010*a*). Increased circulating NEFA concentrations are involved in insulin resistance, and therefore a decrease in circulating NEFA levels due to dietary betaine supplementation may be beneficial (Boston *et al*. 2008). In mice fed a high-fat diet, dietary betaine supplementation inhibits lipogenesis and reduces insulin resistance by improving hepatic function via improved adipose tissue function and reduced hepatic damage (Wang *et al*. 2010*b*). Betaine supplementation reverses insulin resistance in an *in vitro* model, while in both *in vitro* and *in vivo* models, betaine normalises downstream signalling pathways involved in gluconeogenesis, perhaps by improving phosphorylation of early steps in the insulin signalling cascade (Kathirvel *et al*. 2010). Dietary betaine has been shown to improve insulin resistance in humans, again via the amelioration of hepatic injury (Borgschulte *et al*. 2008). Finally, human patients suffering non-alcoholic fatty liver disease have improved hepatic function when given a dietary supplementation of betaine (Patrick 2002; Borgschulte *et al*. 2008;Abdelmalek *et al*. 2009). Although the mode of action via which betaine elicits these effects is contentious, betaine is thought to ameliorate endoplasmic reticulum stress (Wang *et al*. 2010*b*) by converting homocysteine into methionine (a fatty acid) (Abdelmalek *et al*. 2009), although this is not always the case (Borgschulte *et al*. 2008).

Other dietary strategies should be directed towards alleviating insulin resistance or the stress around slaughter, for example, the use of antidiabetic agents such as thiazolidinediones (TZD), chromium, zinc and vanadium.

Type II diabetes strategies (chromium, Zn, TZD, vanadium)

Given that our hypothesis is largely based around the premise that the insulin resistance associated with fattening in cattle grain fed long-term can predispose the animal towards thermal instability, then possible solutions may be some of the antidiabetic compounds such as chromium, zinc, TZD and vanadium.

Dietary chromium has been suggested to increase insulin sensitivity (Steele *et al*. 1977), and over the last 20 years or so, it has been occasionally revisited as a potential means of manipulating fat deposition in humans and farm animals. While chromium chloride has been successful in improving growth performance of poultry (Steele and Rosebrough 1979; Uyanik *et al*. 2002) or protein-deficient rats (Mertz and Roginski 1969), effects in pigs (Page *et al*. 1993; Mooney and Cromwell 1995; Shelton *et al*. 2003) and ruminants (Kegley *et al*. 1997*a*; Kegley *et al*. 2000) have been more equivocal. In part, this may reflect variability in absorption of chromium, since the absorption and utilisation of chromium may depend on incorporation into organic molecules (Evans and Johnson 1980; Kegley *et al*. 1997*b*). However, when organic forms of chromium have been used, there have been consistent improvements in insulin sensitivity in ruminants fed dietary chromium. For example, heifers supplemented with chromium propionate had lower serum insulin concentrations following a glucose infusion (in non-fasted animals), although this did not appear to alter glucose uptake, perhaps indicating increased tissue insulin sensitivity (Spears *et al*. 2012). Holstein calves supplemented with chromium in summer had a reduced insulin response (~30%) to a glucose infusion compared with control animals, suggesting improved efficiency of insulin (Yari *et al*. 2010). Furthermore, dry matter intake, milk fat and protein yield increased while serum insulin and NEFA concentrations decreased in chromium-supplemented, early-lactation Holsteins during summer, again suggesting an increase in insulin sensitivity (Nikkhah *et al*. 2011).

Supplemental chromium picolinate increased glucose clearance rate and decreased glucose half-life in calves fed diets based on corn–cottonseed hulls (Bunting *et al*. 1994). In calves fed milk replacer, supplemental chromium nicotinate

slowed the return to basal glucose concentration after an insulin infusion (Kegley *et al*. 1997*b*). Several other studies with calves and young cattle have shown improved glucose clearance after either glucose or insulin challenges (Bunting *et al*. 2000; Depew *et al*. 1998; Kegley *et al*. 2000). While little or no work appears to have been done in older cattle, Gardner *et al*. (1998) found that dietary chromium chelavite reduced GR fat depth and increased the activity of ATP citrate lyase, a marker of the glucose/insulin axis, by 30% in relatively mature sheep (2-year-old) sheep. Therefore, these data suggest that dietary chromium supplementation may be a means of improving insulin sensitivity in cattle fed grain long-term and improve thermoregulation. However, it should be noted that supplemental chromium did not affect rectal temperature after challenge with infectious bovine rhinotracheitis virus in steers. In a succinct review by Hua *et al*. (2012), the potential mechanisms driving chromium's effect on improving insulin resistance are explored, and it is noted that recent studies have found chromium to improve the actions of insulin and the insulin-signalling pathway while inhibiting downregulators of insulin signalling.

At relatively high levels, zinc exerts potent insulin-mimetic effects, and so dietary zinc has been proposed as a means of manipulating adipogenesis and marbling in cattle (Kawachi 2006). Sasaki *et al*. (2002) showed that insulin promoted adipocyte proliferation in ovine preadipocyte cells, while Tanaka *et al*. (2001) found that zinc enhanced adipogenesis in 3T3-L1 adipose cells. Yano *et al*. (2004) investigated the effect of zinc, either alone or in combination with insulin, on markers of adipogenesis (glycerol-3-phosphate dehydrogenase activity or lipid content) in bovine preadipocytes and found that zinc increased adipogenesis in a dose-dependent manner, although the effect was not as great as insulin. Whether these effects translate into *in vivo* action or occur in other insulin-dependent tissues is unknown. Also, supplemental zinc has had no effect on plasma glucose Wang (2006) or glucose clearance (Droke *et al*. 1993) in lambs.

Vanadium has also been reported to possess insulin-mimetic activity on various types of cells and so has been suggested as a means of increasing adipogenesis (Kawachi 2006; Shukla and Bhonde 2008). Although vanadium is used as a treatment for diabetes and it may improve insulin sensitivity in cattle, concerns about toxicity and contamination of the food chain would probably ensure that it is not used (Gummow *et al*. 2005). However, given that it is used as a low-dose, orally active antidiabetogenic agent in humans (Rustenbeck 2007), if proof-of-concept studies demonstrate that insulin resistance is involved in high rigor temperature, then vanadium may be worth considering as a dietary supplement.

Thiazolidinediones are potent, synthetic ligands for peroxisome proliferator-activated receptor gamma (PPARγ) that potentiate the action of insulin in peripheral tissues, and are used as orally active treatments for diabetes in humans (Krentz and Bailey 2005). Kushibiki *et al*. (2001) administered TZD to steers injected with TNF-α to induce insulin resistance and observed a decrease in plasma concentrations of NEFA, insulin and glucagon, suggesting that administration of TZD ameliorated insulin action. Administration of TZD in the prepartum period for dairy cattle increased PPARγ mRNA expression and decreased leptin mRNA expression (Schoenberg *et al*. 2011). Also, Smith *et al*. (2007) injected TZDs (intrajugular) to lactating cows, which are normally very insulin-resistant, and observed an improvement in insulin sensitivity as evidenced by a decrease in plasma NEFA. Therefore, TZD may be an effective means of improving insulin resistance in cattle fed grain long-term, although it is not known whether TZDs survive the rumen. Also, the use of TZDs for production animals is likely to be cost-prohibitive.

Vitamin D deficiency is associated with increased insulin resistance (Reis *et al*. 2005), and there is some evidence that vitamin D supplementation in humans may improve insulin sensitivity. Normally sufficient vitamin D is produced after exposure to sunlight, and intuitively, lot-fed cattle in Australia are unlikely to receive no exposure to sunlight. However, dark-skinned individuals need much more sunlight to produce sufficient vitamin D, and dark-skinned or dark-haired cattle are more likely to absorb heat than light-skinned or light-haired cattle. It would also be of interest to observe whether carcasses form dark cattle are more likely to be of high rigor temperature that light cattle. Several studies have shown that vitamin D supplementation can improve tenderness in beef through elevation of serum calcium, calcium being a potent activator of proteases involved in tenderisation (Swanek *et al*. 1999; Montgomery *et al*. 2004).

Magnesium to reduce catecholamine secretion and activation (stress relief)

Magnesium (Mg) has a relaxant effect on skeletal muscle and it has been shown to depress skeletal muscle activity by antagonising calcium, which is required for neurotransmitter release and muscle contraction (see review by Dunshea *et al*. 2005). Studies have shown that dietary magnesium supplementation alleviates the effects of stress by reducing plasma cortisol, norepinephrine, epinephrine and dopamine concentrations (Niemack *et al*. 1979; Kietzmann and Jablonski 1985). Consequently, studies have been conducted to investigate the influence of dietary Mg supplementation on reducing the effects of stress and improving pork quality. Given that the stress around slaughter may result in a loss of thermoregulation, dietary Mg may assist in reducing heat toughening.

Magnesium supplementation (MgO) has also been shown to reduce the loss of glycogen from muscle in Merino lambs during the post-farm period leading up to slaughter (Gardner *et al*. 2001). This has led to some supply chains in Australia insisting on high-energy rations containing supplemental MgO being fed to Merino lambs in the 2–4 weeks pre-slaughter. This logic is based on the accepted fact that the Merino breed, on average, has a higher incidence of dark cutting (or high pHu meat) due to greater stress sensitivity and so increased rate of glycogen loss caused by the post-farm stressors that occur immediately pre-slaughter (Gardner *et al*. 1998). Therefore, dietary Mg may be worth investigating as a means of reducing the heat generation in response to pre-slaughter stress of cattle.

Other

Other targets may include manipulation of NOS through use of NO donors such as arginine or the use of dietary L-Gln to

potentiate HSPs. Other targets that are more difficult to manipulate are leptin and UCPs.

Conclusion

A main cause of high rigor temperature appears to be a combination of rapid glycolysis and relatively slow cooling, which can lead to high rigor temperatures and potential toughening. Moreover, high rigor temperature may be exacerbated by a loss in proteolytic potential due to inactivation of proteolytic enzymes. Factors that contribute to elevated body temperature pre-slaughter potentially predispose animals to higher carcass temperature immediately post-slaughter. There is mounting evidence that a state of insulin resistance can compromise the ability of an individual to thermoregulate and this can be exacerbated by stressors. Several dietary treatments might be investigated as means of reducing heat toughening. Most promising are dietary betaine, chromium and magnesium either alone or in combination.

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