A Re-evaluation of the Optimal Liver Copper Concentrations for Health, Performance and Fertility of Replacement Holstein–Friesian Heifers

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Abstract:

Excessive copper (Cu) supplementation is common on dairy farms worldwide, despite a growing body of research highlighting the risks of over-supplementation, including liver damage, impaired growth, and reduced fertility. However, diagnosing Cu toxicity remains challenging due to the liver's allostatic regulation of blood Cu levels and debate surrounding toxicity thresholds. This study utilised secondary data from a longitudinal study conducted between September 2016 and September 2018 involving eighty replacement Holstein-Friesian heifers. Data was utilised to generate receiver operating characteristic curves which established liver Cu thresholds associated with suboptimal liver function and fertility. Results indicated that hepatic Cu concentrations exceeding 167 mg/kg of dry matter (DM) were associated with reduced conception rates to first service, while concentrations above 260 mg/kg of DM reduced conception probability to first and second services. Hepatic Cu concentrations exceeding 322 mg/kg of DM were linked to impaired liver function, as evidenced by elevated serum glutamate dehydrogenase activity. In contrast, a toxicity threshold value could not be generated for plasma Cu, underscoring its inadequacy as a biomarker. The fertility thresholds identified in this study may be more closely related to optimizing Cu levels for animal performance rather than indicative of liver Cu toxicity, suggesting the need for further research.

Keywords:

Dairy cattle, Fertility, Liver, Glutamate dehydrogenase, Plasma copper.

Introduction:

Copper (Cu) is an essential trace element required by all living organisms [1]. In mammals, Cu plays a critical role within metalloproteins, cofactors, and the function of metalloenzymes [2]. Decades of research has highlighted the adverse consequences of Cu deficiency within ruminants, as exemplified by impaired immune function [3], decreased haematological parameters [4] and ovarian inactivation leading to potential infertility [5]. In contrast, there is increasing evidence of an over supplementation of dietary Cu on dairy farms, particularly when cattle are continuously housed. For example, Sinclair and Atkins [6] surveyed early lactation diets from 50 farms across central and northern England, reporting a mean overall dietary Cu concentration of 27.9 mg/kg of dry matter (DM), which was 16.9 mg/kg of DM above the nutritional recommendation to meet cow requirements proposed by the National Research Council (NRC [7]) in 2001, and 17.9 mg/kg of DM above the revised guidelines proposed in 2021 [8]. The UK is not alone in reporting excessive levels of Cu supplementation on farm with Castillo et al. [9] surveying 39 Californian dairy herds and reporting a mean Cu intake of 18.0 mg/kg of DM, almost 1.9 times above NRC [7] recommendations. Similarly, Duplessis et al. [10] reported that 52% of surveyed Canadian dairy herds were feeding above NRC [7] guidelines, whilst 65% were feeding above the European equivalents [11,12]. This over supplementation is further

reinforced by Kendall et al. [13] who reported that 40% of UK dairy cull cows had hepatic Cu concentrations exceeding 508 mg/kg of DM, a threshold above which cattle are generally considered to suffer from chronic Cu poisoning [14]. However, it should be taken into consideration that much debate surrounds the threshold for Cu toxicity with a considerable range in the proposed hepatic thresholds from 350 to 1500 mg/kg of DM [14–16].

The reasons for this increased Cu supplementation on-farm are unclear but could be multi-factorial [17]. It may simply be that in the absence of clinical Cu toxicity, there is a perception that "more is better!" from those within the industry leading to excess levels of Cu supplementation on-farm [18]. However, recent evidence would also suggest that other factors such as basal forage type (e.g. grass versus maize silage; [19]), rumen pH (e.g. high versus low; [20]), and copper source (e.g. oxide versus sulfate; [21,22]) can greatly alter dietary Cu absorption. There are various clinical signs which can present due to excessive Cu loading, including liver damage [5] impaired growth [23], and death due to haemolytic crisis [24], with the Animal and Plant Health Agency reporting 80 cases of clinical Cu toxicity in UK cattle between 2016 and 2023 [25].

Determining an elevated Cu status on-farm can be difficult, predominantly due to the allostatic control of blood Cu concentration by the liver, which results in plasma or serum concentrations that are a poor indicator of hepatic Cu status [26]. For example, Dias et al. [27] conducted a meta-analysis to determine if plasma Cu could be utilised as an indicator of animal status, concluding that it may only be useful when animals experience either exceptionally high or low hepatic Cu concentrations. Alternative indicators of an increased Cu status rely on blood enzymes, although not a direct indicator of hepatic Cu status, glutamate dehydrogenase (GLDH) has been shown to be a sensitive indicator of hepatotoxicity which could result from increased Cu levels [28]. However, misdiagnosis may be problematic as other conditions can produce similar enzyme profiles [29]. It is widely accepted that liver biopsies are the most accurate method for assessing Cu status, but sampling is an invasive procedure requiring veterinary training, with cost also being a limiting factor on farms [30–32]. However, utilizing cull cattle for liver sampling may help mitigate these challenges by providing a less invasive and more cost-effective alternative [30]. Overall, the difficulty in assessing Cu status may serve to perpetuate the existence of chronic Cu poisoning as a silent epidemic within the dairy industry. Further to this, emerging studies are now raising concerns of harmful subclinical consequences associated with an elevated Cu status. For example, McCaughern et al. [28] reported that Cu supplementation above requirements but below typical farm levels resulted in a 17.5 % reduction in the conception rate (73.7 % versus 91.2 %) of replacement Holstein-Friesian heifers. Additionally, practicing vets have suggested anecdotal evidence of a link between increased dairy cow Cu status with a concurrent increase in disease incidence [28].

In conclusion, there is an oversupply of Cu within the dairy industry, which exceeds the nutritional requirements of cattle. When this environment is combined with recent evidence that liver copper concentrations below historic toxicity thresholds can negatively impact dairy cattle health and performance, there arises a need to reassess hepatic thresholds pertaining to optimal hepatic Cu concentrations within dairy cattle. The objectives of the current study were therefore to re-evaluate the associations between hepatic Cu concentration and the health and performance of replacement Holstein-Friesian heifers, with a view to determining critical thresholds at which these parameters are negatively affected.

Materials and methods

This article is the second paper from a study conducted between September 2016 and September 2018 at the Harper Adams University Dairy Unit, Newport, Shropshire, UK. The initial study evaluated the effect of Cu supply during the rearing phase on the health, performance and fertility of replacement Holstein-Friesian heifers [28]. The aim of this paper was to utilise the secondary data generated to determine the hepatic Cu thresholds above which the fertility, performance and health of Holstein-Friesian dairy heifers is affected.

Briefly, the animal management undertaken by McCaughern et al. [28] can be summarised as follows. A longitudinal study was completed, where Eighty Holstein-Friesian heifers with a liveweight of 137 ± 2.4 kg (mean ± standard error) at 4.1 ± 0.1 months of age where fed either recommended (16 mg/kg of DM; n = 40) or high (32 mg/kg of DM; n =40) total dietary Cu concentrations until six weeks before calving. The recommended level was provided to avoid deficiency and be marginally in excess of animal requirements [7], whilst the higher dietary concentration was selected to reflect the mean Cu concentration reported on UK farms by Sinclair and Atkins [6]. All heifer rearing was performed to reflect typical commercial management conditions in the UK.

Data collection

Liver biopsies were performed at 12.4 months of age according to Davies and Jebbett [33], by insertion of a needle through the 11th intercostal space. Biopsy samples were immediately snap-frozen in liquid nitrogen and stored at -80°C. Liver Cu concentration was determined by inductively coupled plasma mass-spectrometry (ICP-MS) as described by McCaughern et al. [20]. Blood samples were collected via jugular venipuncture using Becton Dickson vacutainers containing silica gel to determine serum GLDH and dipotassium EDTA to determine plasma minerals. Initial blood samples were collected in the week prior to commencing the study and continued at eight-week intervals throughout. After collection, all samples were centrifuged and stored at -20°C until analysis. Serum GLDH was measured using Randox Laboratories kits (GLDH: Catalogue No. GL441) and analyzed with a Cobas Miras Plus

autoanalyzer (ABX Diagnostics). Plasma Cu concentrations were determined using ICP-MS, according to McCaughern et al. [20].

Heifers were weighed and body condition scored (BCS; 1-5 scale with 0.25 increments) fortnightly throughout the duration of the study. Oestrus detection commenced at five months of age as stated by Van Erdenburg et al. [34] whereby behavioural characteristics were assigned points with the sum of these triggering artificial insemination. The breeding period began after 13 months of age, with insemination performed 12 hours after observed oestrus. Conception rate was calculated as the proportion of inseminated heifers confirmed pregnant by trans-rectal ultrasound. Heifers diagnosed as non-pregnant were returned to mating or excluded from the study if 260 days beyond the start of mating.

Statistical analysis

All statistical analyses were carried out using R (version 4.4.1) [35]. Packages included: lme4[36], ROCit [37], mvnormtest [38], mgcv [39], OptimalCutpoints [40], Outliers [41], magrittr [42], EnvStats [43], and nlme [44].

Rosner's test was utilised to identify and remove outliers, while the Shapiro–Wilk test was conducted to assess the data distribution for each variable. Variables that deviated from a Gaussian distribution were transformed using an inverse cubic root. Subsequently, a Mann–Whitney U test was applied to compare hepatic Cu concentrations at 12.4 months of age with the values reported for the UK cattle population by Kendall et al. [13]. Descriptive statistics were also performed to demonstrate the data spread for each continuous variable, consisting of a mean, standard deviation, range, and interquartile range. In contrast, percentages were presented for the dichotomous variables in addition to the underlying numerical counts.

Generalised Additive Models (GAMs) were employed to examine the relationships between hepatic Cu concentrations at 12.4 months of age and dependant variables related to health or performance. The models calculated either the adjusted coefficient of determination (R^2_{adj}) for continuous variables, or McFadden's coefficient of determination for dichotomous variables ($R^2_{McFadden}$). Following Gupta et al. [45], an $R^2_{adj} > 0.5$ was interpreted as indicative of a strong significant relationship, whilst, in line with McFadden [46] an $R^2_{McFadden} > 0.3$ was considered significantly important.

Dependent variables that demonstrated significant relationships with hepatic Cu concentrations were carried forward into receiver operator characteristic (ROC) curves to identify subclinical disease thresholds. These ROC curves evaluated the sensitivity and specificity of hepatic Cu concentrations in predicting disease outcomes as follows:

153 $$\text{Sensitivity}=\frac{Number\ of\ true\ positives}{Number\ of\ true\ positives+number\ of\ false\ negatives}\times 100$$

154

155 $$\text{Specificity}=\frac{Number\ of\ true\ negatives}{Number\ of\ true\ negatives+number\ of\ false\ positives}\times 100$$

156

157 For the fertility ROC curves, heifers confirmed to be in calf were considered the 'healthy' outcome. For
158 serum GLDH activity < 16.0 U/L defined the healthy population as reported by Hunter et al. [47]. In
159 contrast, for plasma Cu concentration two thresholds of > 25 or > 50 μmol/l were examined in
160 accordance with current published thresholds [15,48]. Finally, Youden's *J* statistic was calculated by
161 finding the maximal vertical distance between the ROC curve and a 45° diagonal line passing through
162 the origin using:

163 $$J=\text{Sensitivity}+\text{Specificity}-1$$

164 The hepatic Cu concentration yielding the maximum *J* was deemed the threshold at which disease
165 breakdown occurred, thereby affecting the dependent variable.

166 **Results**

167 Seventy-five animals completed the study, with no signs of clinical hepatic Cu toxicity or deficiency
168 observed throughout the duration of the study. The Mann–Whitney U test determined that the mean
169 hepatic Cu concentration of 298 ± 124 mg/kg of DM at 12.4 months (Table 1) was in line with UK
170 values published by Kendall et al. [13].

171

Table 1. Descriptive characteristics of the replacement Holstein-Friesian dairy heifer population used to model potential relationships between liver copper status and animal health, performance, and fertility.

Items	Lower bound (minimum)	Lower quartile (Q1)	Mean ± SD	Upper quartile (Q3)	Upper bound (maximum)
Hepatic copper (mg/kg of DM) [a]	83.9	236	298 ± 124	356	669
Liver function					
Glutamate dehydrogenase (U/L) [a]	3.40	17.9	30.5 ± 22.8	30.5	120
Plasma copper (µmol/L) [a]	13.0	14.4	15.6 ± 1.63	16.3	22.2
Fertility					
Days to first observed oestrus	210	280	327 ± 64.7	353	549
BCS at PSM [b]	2.50	3.00	3.09 ± 0.29	3.25	3.75
LW at PSM (kg) [b]	408	500	532 ± 44.9	555	650
Days between PSM [b] and conception	6.00	62.0	110 ± 60.6	152	262
Conception rate to first service [c]			51.3% (38/74)		
Conception rate to first and second service [c]			82.4% (61/74)		

Abbreviations: BCS, body condition score; PSM, planned start of mating; LW, live weight.

[a] Data collected from the heifers at 12.4 months of age.

[b] Planned start of mating occurred at 13.0 months of age.

[c] Presented as percentage of animals pregnant within the total population. Parentheses denote the number of animals who conceived out of the total number of animals served.

172

173 *Liver copper concentration and plasma glutamate dehydrogenase*

174 Generalised additive modelling determined that hepatic Cu concentrations at 12.4 months of age
175 explained 67.6% of the variation in serum GLDH activity during this period (R^2_{adj}= 0.649, $P < 0.001$).
176 An ROC curve analysis combined with Youden's *J* statistic, identified a hepatic Cu threshold of 322
177 mg/kg of DM (Figure 1a), above which serum GLDH activity exceeded 16 U/L [47].

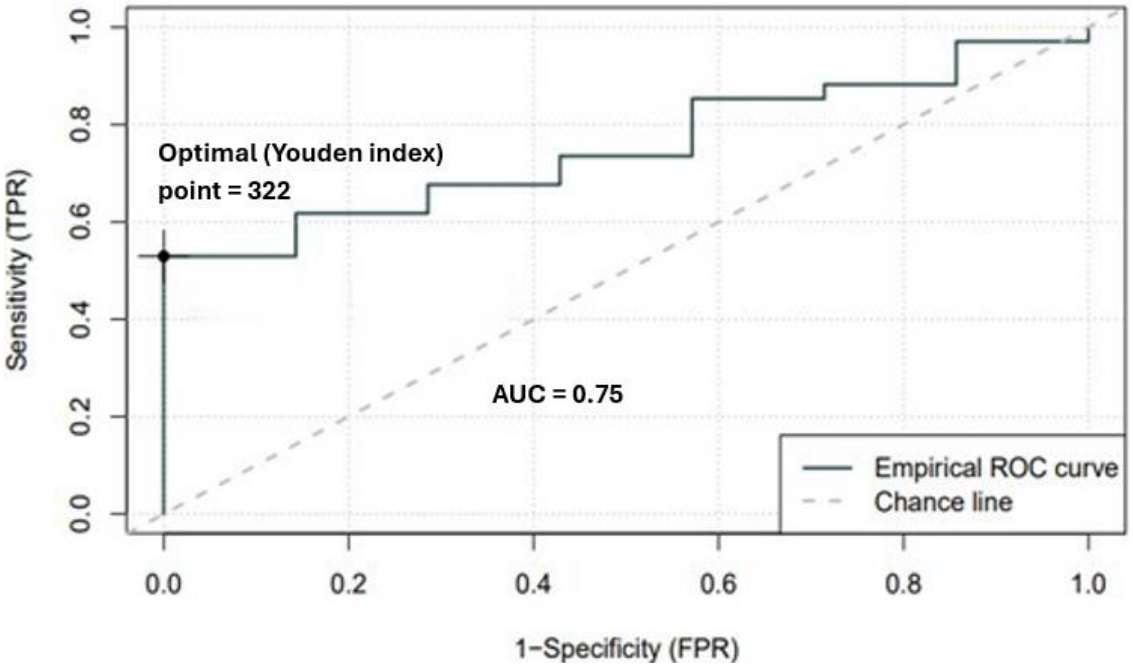
178 *Liver copper concentration and plasma copper concentration*

179 A positive correlation was observed between hepatic Cu concentration and plasma Cu concentration at
180 12.4 months (R^2_{adj} = 0.49, $P = 0.02$) of age. Two disease threshold values for plasma Cu concentrations
181 were assessed: 25 µmol/l [15] and 50 µmol/l [48], however, despite the positive correlation, the number
182 of heifers which fitted into diseased categories was too small to meet statistical power requirements.

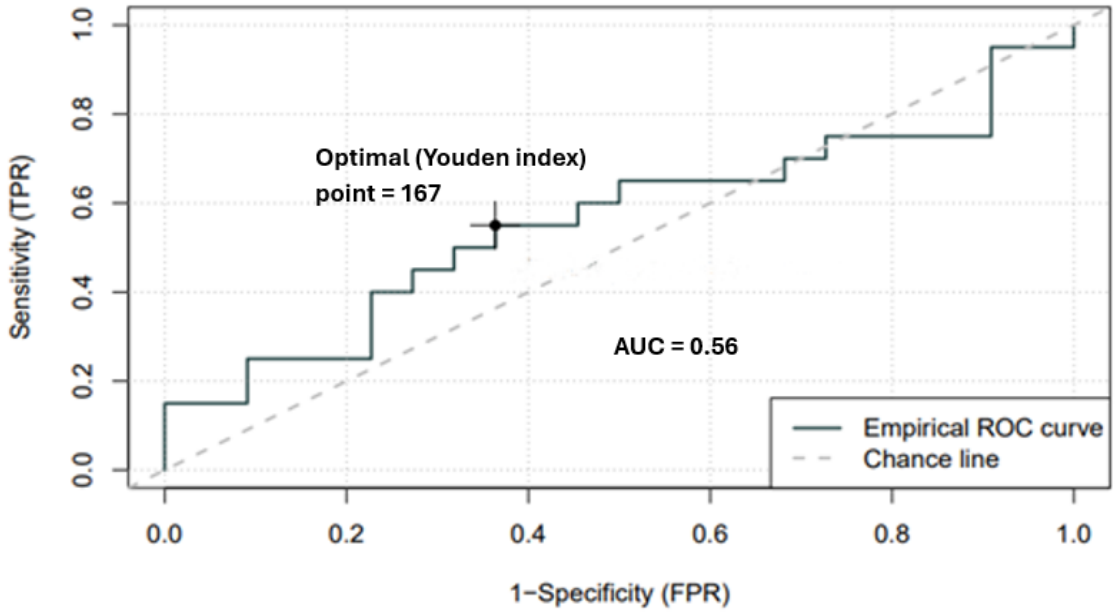
Liver copper concentration and fertility parameters

There was no association between hepatic Cu concentration at 12.4 months and the number of days to first observed oestrus (R^2_{adj} = 0.001, p = 0.33). However, conception rates to either first service, or first and second service were both lowered by higher hepatic Cu concentrations at 12.4 months of age with $R^2_{McFadden}$ values of 0.35 and 0.31, respectively. This led to the production of ROC curves with conception rates and hepatic Cu values which in turn allowed Youden's *J* statistics of 167 mg/kg of DM (Figure 1b) and 260 mg/kg of DM (Figure 1c) to be generated for first, or first and second services respectively.

(a)



(b)



(c)

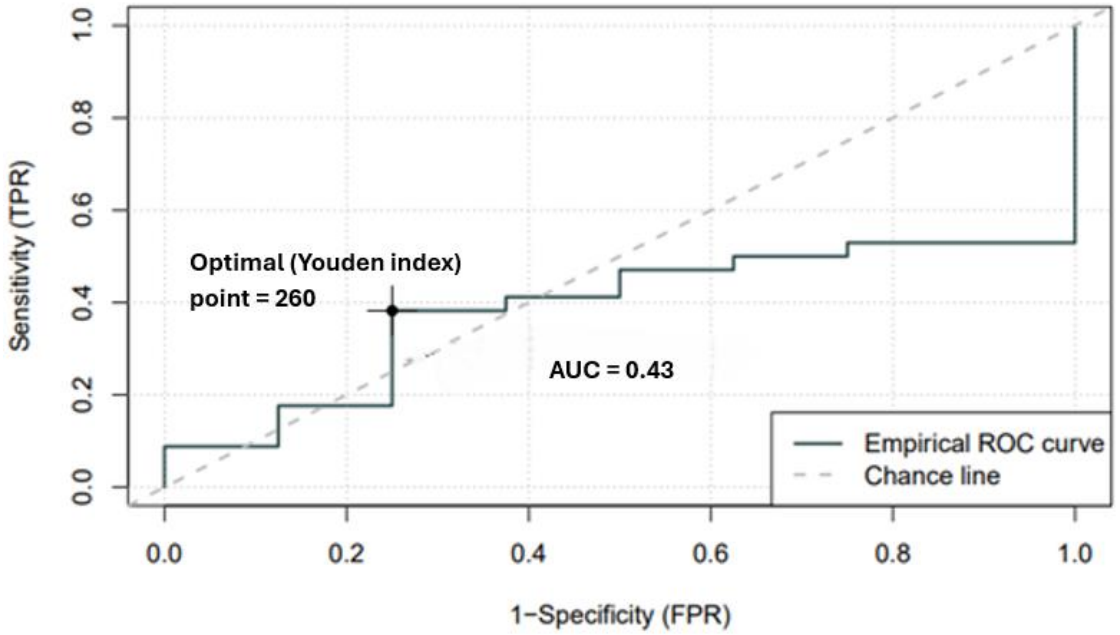


Figure 1 Receiver operating characteristics (ROC) curves displaying sensitivity and specificity for determining optimal hepatic Cu concentrations utilising GLDH (glutamate dehydrogenase; a), conception rate to first service (b), and conception rate to first and second service (c) for replacement Holstein Friesian heifers.

Discussion

Copper toxicity, hepatic copper concentrations and glutamate dehydrogenase

Despite multiple historic case reports of Cu poisoning in dairy cattle [49–51], and a decline in annual UK cases from a mean of 34 between 2000 and 2004 [52] to 10 between 2020 and 2024, the disorder is still the most commonly diagnosed mineral toxicity within cattle [25]. The liver plays a critical role in this discourse, not only is it the primary organ responsible for Cu storage, but it also maintains Cu homeostasis within the animal [53]. Sites of Cu storage within each hepatocyte include the nucleus, cytosol, and the large granule fraction which accounts for the greatest proportion of the element [54]. The development of Cu toxicity within the animal is generally considered to be a two-stage process [48]. The initial pre-haemolytic stage consists of Cu accumulating sub-clinically in the liver, accompanied by an increase in blood enzymes indicative of liver breakdown, as exemplified by GLDH [28,55]. Literature to date has not attempted to determine the precise liver Cu concentration above which an increase in GLDH can be expected. However, Suttle [15] reported that biochemical changes could be observed within a marginal hepatic toxicity range with a lower limit of 350 mg/kg of DM, which would broadly agree with the current study's threshold hepatic Cu value of 322 mg/kg of DM, above which serum GLDH activity becomes abnormal. Indeed, a linear relationship is hypothesised to exist between the pattern of Cu storage within the hepatocyte and overall hepatic Cu concentration throughout both the healthy and pre-haemolytic phases [54]. However, it should also be taken into

consideration that a lack of association between hepatic Cu status and serum GLDH has been reported in dairy cull cows, with a greater range in hepatic Cu to those utilised in the current study [56]. There are multiple possible explanations for these contrasting findings, it maybe that under instances of chronic Cu challenge to the liver, cuproptosis replaces the cell necrosis observed under an acute challenge [57,58]. This programmed cell death may then exhibit a liver function profile which appears clinically normal [59,60]. Another explanation may reside in the study population utilised by Strickland et al. [56], who hypothesised that any association may have been masked by the metabolic stress of early lactation cows in their study.

The second stage of Cu toxicity involves a haemolytic crisis characterised by extensive liver damage and the mass release of Cu into circulation leading to increased kidney Cu levels [48]. Much debate surrounds the chain of events associated with this haemolytic phase. Lopez-Alonso et al. [54] associated its occurrence with an increase in hepatic Cu storage within the lysosomal fraction at approximately 1500 mg/kg of DM. Suttle [15] appears to broadly agree with this by capping the marginal toxicity band at a threshold of 1500 mg/kg of DM. However, Hunter et al. [47] observed cases of fatal Cu toxicity without haemolytic crisis, suggesting that alternative biochemical pathways may be involved. Livesey et al. [14] defined chronic Cu poisoning as the occurrence of liver and kidney degeneration upon histopathological examination, in addition to a hepatic Cu concentration above a threshold value of 508 mg/kg of DM, and a kidney Cu concentration above 41.3 mg/kg of DM. The reasons for this varying case presentation surrounding Cu induced fatality are unclear but may result from differences in breed type and/or stage of production [61,62]. It is well understood for example, that Jersey cattle have a greater susceptibility to Cu toxicity than other dairy breeds [61]. Additionally, lactating cattle may have a reduced hepatic Cu tolerance due to the stress associated with milk production [62], negative energy balance [63,64] and greater liver triglyceride levels [65]. However, it should be noted that the maximum threshold values of 167 and 260 mg/kg of DM identified for optimal conception rates in the present study, are well below those associated with the various defined stages of Cu toxicity within the literature [15,47,54].

Plasma copper concentration as a biomarker for hepatic Cu concentration

Plasma Cu concentrations for all heifers in the current study population were well above the 9 μmol/L considered to denote an adequate Cu status [14]. Much debate surrounds the plasma Cu toxicity threshold, with Suttle [15] associating toxicity with a plasma Cu concentration of 25 μmol/L or higher, whereas Laven and Livesey [48] suggested a diagnosis of Cu poisoning in live animals above 50 μmol/L with appropriate associated clinical signs including increased kidney and blood plasma Cu concentration. Indeed, a positive relationship between plasma Cu and hepatic Cu concentration was observed in the current study. However, due to insufficient numbers of heifers meeting toxicity thresholds, this study was unable to generate a suitable ROC curve which associated hepatic Cu

accumulation with toxicity risk. These findings align with those of others who question the reliability of plasma Cu as a biomarker for an elevated hepatic Cu status [66,67]. Instead, plasma Cu concentration may only become diagnostically relevant in animals with a low Cu status [27] or advanced stages of Cu accumulation, such as when hepatic Cu levels exceed 1500 mg/kg of DM at which point haemolysis occurs, thereby releasing Cu into the bloodstream immediately prior to death [54]. Ceruloplasmin is a Cu containing metalloenzyme which is primarily responsible for Cu transport around the body [68] but can also act as an acute phase protein during the inflammatory response [69]. Serum activities of this enzyme have been used previously as an indicator of Cu status in ruminant animals during cases of molybdenum exposure or Cu deficiency [70,71]. However, there is evidence to suggest that enzymatic activity does not consistently reflect hepatic Cu status changes in lactating dairy cattle [72] with some authors also suggesting little advantage of ceruloplasmin activity over plasma Cu due to a high degree of correlation between them [73]. In either case, no conclusion can be drawn on the validity of ceruloplasmin activity in the current study, as the variable was not monitored.

Liver Cu status and fertility

Fertility is critically important for dairy cattle, as cows must produce a calf to initiate each lactation throughout the production cycle [74]. A key component of optimal fertility and long-term productivity is ensuring that heifers calve at an age which optimises that animals' lifetime production [75]. Calving early at 18 to 22 months has been shown to decrease milk yield by 593 kg per lactation, similarly, increasing age at first calving beyond 26 months has been reported to decrease the total number of lactations and associated productive days [76]. Several studies have explored the impact of Cu intake on fertility parameters in dairy cattle, yielding mixed results. Hamali et al. [77] reported improved fertility as identified by a reduced interval between calving and observed oestrus, when multiparous Holstein dairy cows were supplemented with 2.5 gram slow-release Cu capsules. In contrast, Hawkins [78] observed a decrease in 21-day submission and conception rates when 200 mg of Cu as Ca-Cu-EDTA was injected 10 days before the PSM in seven New-Zealand dairy herds. It should, however, be taken into consideration that indicators of animal Cu status and basal dietary Cu concentration were not monitored in either study [77,78]. In contrast, McCaughern et al. [28] was the first to report a 17.5% reduction in the conception rate of replacement Holstein-Friesian heifers fed above their Cu requirements, with mean hepatic Cu concentrations that would be considered normal, with no clinical signs of Cu toxicity [47].

Findings from the present study further support the potential of animal Cu status to impact fertility, with conception rate to first service declining at hepatic Cu concentrations above 167 mg/kg of DM, whilst conception rates to first and second service declined above 260 mg/kg of DM. This is particularly pertinent, as extrapolation from Kendall et al. [13] in relation to the latter threshold would place 96.7% of UK Holstein-Friesian cull cows at a hepatic Cu status that has the potential to alter their fertility [13]

although, it is important to note that Kendall et al. [13] did not include replacement Holstein-Friesian heifers in their survey. The exact mechanisms by which Cu status influences fertility in dairy cattle remains unclear, McCaughern et al. [28] proposed a hypothesis surrounding the liver, whereby elevated hepatic Cu concentrations drive an increase in circulating concentrations of insulin-like growth factor-1 [79,80], with the potential concurrent reduction in progesterone lowering conception rates [81,82]. Literature is lacking regarding the direct influence of Cu status on the bovine reproductive tract; however, evidence has emerged from the human population linking increased serum Cu levels with polycystic ovary syndrome [83,84]. It is hypothesised that an increased Cu content within the follicular fluid of these patients affects follicle maturation and embryo development [85]. There are multiple pathways by which increased serum Cu concentrations may contribute to the condition including the generation of oxidative stress [86], and promotion of inflammatory cytokines [87]. Additional variables pertaining to fertility were non monitored in the current study but warrant exploration in future research.

Conclusion

Relationships exist between hepatic Cu concentrations, blood GLDH activity, and measures of fertility in replacement Holstein-Friesian dairy heifers. Alterations in liver function as indicated by a diseased level of GLDH occurs at hepatic Cu concentrations above 322 mg/kg of DM. A hepatic Cu concentration above 167 mg/kg of DM lowers the probability of conception to first service in Holstein-Friesian heifers, whilst hepatic Cu concentrations above 260 mg/kg of DM lowers conception probability to first and second service. It should be considered that all liver Cu thresholds pertaining to fertility in this study, are well below current published values relating to Cu toxicity and would indicate that performance can be altered at concentrations considered to be normal. Indeed, these findings may warrant a discussion of the definitions surrounding Cu toxicity, as the thresholds identified in this study may be better associated with Cu status optimization for maximal animal performance, as opposed to toxicity. The reasons for these relationships between liver Cu concentrations and measures of fertility are unclear and warrant further investigation, but findings highlight the need to consider dietary Cu supply for optimal animal performance.

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Statements and declarations

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Competing interests

The authors declare they have no conflicts of interest.

Author contributions

Study conception, design, and planning were undertaken by Amy Marsh, James McCaughern, Liam Sinclair and Alexander Mackenzie. Analysis of the data was performed by Amy Marsh and Joe Roberts, with all authors contributing to the interpretation of research findings. All authors also participated in drafting and approval of the submitted manuscript.

Data availability

The data that supports the findings of this study are available from the corresponding author upon reasonable request.

Ethics approval

The analysis of this secondary data is in keeping with the principles of replacement, reduction and refinement as defined under the UK Animals (Scientific Procedures) Act 1986 (amended 2012). All further data analyses presented in this manuscript received local ethical approval from the Harper Adams University Research Ethics Committee (18101200PHD-P0583).