Journal of Animal Science, 2022, **100**, 1–4
https://doi.org/10.1093/jas/skac120
Advance access publication 28 April 2022
News and Views

Contradictions on colostrum IgG levels and Brix values are real and can be explained. Response to letter by Lombard *et al.* (2022)

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Our conclusion that "˚Bx values do not reasonably indicate IgG concentration to serve as a measure of 'colostrum quality'" (Schalich *et al.* 2021), is based on irrefutable experimental evidence. Through detailing the component-by-component basis of Brix® refractometer readings (˚Bx), we revealed the impact of an independent variable, that effectively invalidated strong conclusions drawn in prior studies regarding the prediction of IgG concentration from ˚Bx values of colostrum. In this response to the letter by Lombard et al. (Lombard *et al.* 2022), we explain our findings and highlight why it challenges two key developments that are at the core of this management-centric concept termed "colostrum quality."

Colostrum ˚Bx-IgG correlation does not equal prediction

In colostrum, we uncovered that non-protein solutes (such as sugars and salts) are confounding variables responsible for a significant ~36% of the ˚Bx reading (Schalich *et al.* 2021). In accurately measuring IgG concentrations across the same ˚Bx value samples, we revealed the high magnitude of residuals (up to ±120 g/L IgG). It is well known that a regression model with high magnitude of the residuals will not offer a close fit for any predictions; thus, ˚Bx values would lack any predictive power for IgG concentrations. Not only did we find that this was true (Schalich *et al.* 2021), it was remarkably consistent with previous studies, in which the criterion of residuals was disregarded, over the emphasis on significant correlation.

To illustrate this clearly, we would like to examine the study (Quigley *et al.* 2013) indicated in the letter as evidence against our conclusion. First, only 13/183 samples in this study were ˚Bx <20, so the bulk of the regression estimate was indeed driven by higher ˚Bx value samples. Although the datapoints corresponding to ˚Bx values and IgG concentrations calculated by radial immunodiffusion (RID) were correlated ($r=0.75$; $p<0.01$), the residual magnitudes were high (up to ±40 g/L IgG) (Figure 1A). Therefore, if we consider the empirical data (retrospective prediction, using original data that was used to develop the model), it is entirely not clear how a cut-off value of ˚Bx 21 (recommended in this manuscript) is justified, as ˚Bx 20 was completely within the range of ˚Bx 21, and the overlap in range was 87% between ˚Bx 22 and ˚Bx 21. Even ˚Bx 16 was completely within the range of ˚Bx 21, with one of the two samples being ≥50 g/L of IgG. In fact, only 3/183 (1.6%) samples in ˚Bx <20 were below the IgG concentration range identified for samples that were ˚Bx ≥21. Such a problem associated with the magnitude of residuals (contributed by the non-protein solutes), is a common theme with differing severity in studies that have evaluated ˚Bx in straight colostrum [including those used in the meta-analysis of this correlation (Buczinski & Vandeweerd 2016)]; as residual magnitude is not indicated by the regression coefficient, it was clearly overlooked. However, the function of residual magnitude is crucial for prospective predictions (using new data, which nearly always performs worse than the fit to original data/retrospective predictions); thus, the significance of correlation is only part of the equation. To the best of our knowledge, validation by prospective predictions has not been performed in any of the studies promoting ˚Bx as an indicator of colostrum IgG concentrations.

Notably, the Quigley *et al.* (2013) study is one of the few that appears to be least perturbed by the magnitude of residuals, somewhat indicating that ˚Bx ≥24 should always have IgG concentrations above 50 g/L IgG, for the sample size examined. In another study (Elsohaby *et al.* 2017), which we believe is more rigorous as they compare both optical and digital ˚Bx side-by-side for 240 colostrum samples, extremely high residual magnitudes were recorded (up to ±110 g/L IgG). Although the correlation remained significant ($r=0.71$; $p<0.001$), a large subset of samples representing a wide range of ˚Bx values (from as low as 11 to as high as 32) contained samples that were below 50 g/L IgG (Figure 1B); this suggested that usefulness of ˚Bx values in prospective predictions is nonexistent. Therefore, the downstream predictive value of any measures or cutoffs using ˚Bx in straight colostrum, that forms the basis of "colostrum quality" is quite ambiguous.

Received February 19, 2022 Accepted April 4, 2022.

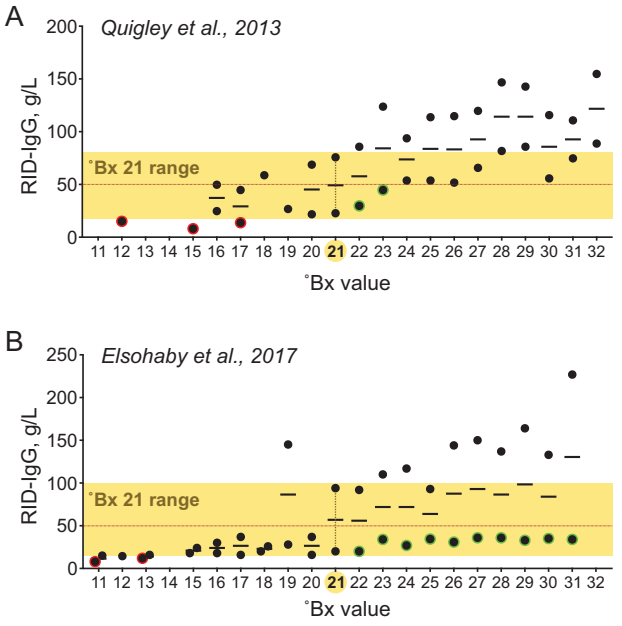

Figure 1. Prediction of IgG concentrations from °Bx values is ambiguous and inconsistent between studies. When we examine two studies correlating °Bx values and IgG concentrations: (A) Quigley *et al.*, 2013 (r=0.75; p<0.01; n=183), and (B) Elsohaby *et al.*, 2017 (r=0.72; p<0.001; n=240), fitting the original empirical data to the model (retrospective prediction) reveals that most IgG concentrations are within the range set by the "cut-off" °Bx 21. The graphs indicate the upper and lower limit of the range of IgG concentrations and the median for each °Bx value. The IgG concentration range associated with °Bx 21 is shaded across the entire °Bx value range. In (A), the IgG concentration range for °Bx 21 was not distinctive, and is calculated to recognize only with a ~54.5% probability, the desired level of ≥50 g/L IgG concentration; this was almost identical in °Bx 20, but could not be examined for °Bx 19 and °Bx 18 due to lack of sample size. IgG concentrations for samples that tested °Bx 16 were still completely within the range identified for °Bx 21. Only 3 samples (emphasized with red) showed IgG concentrations lower than the range for °Bx 21. At sample sizes included for °Bx ≥24, all IgG concentration measurements were above 50 g/L IgG, and appeared to be least perturbed by the magnitude of residuals. This was not the case in (B), where a large subset of samples representing a wide range of °Bx values (from a low 11 to as high as 32) contained samples that were below 50 g/L IgG (emphasized with green for °Bx >21). Similar to (A), IgG concentration range for °Bx 21 in (B) was not distinctive, and is calculated to recognize only with a ~57% probability, the desired level of ≥50 g/L IgG concentration. Higher °Bx did not amount to higher IgG concentrations, as all samples in °Bx as high as 25 were still completely within the IgG concentration range identified for °Bx 21. These severe limitations integral to these models in (A) and (B), and the inconsistencies between them highlight their dubious value in prospective predictions with new °Bx data.

°Bx values can accurately estimate IgG in whey:

Early studies that associated °Bx values to colostrum IgG concentrations were not performed on straight colostrum (Harker 1978; Molla 1980). In these studies, colostrum samples were clotted with rennin and centrifuged to extract whey. Refractometry and protein/IgG estimations were performed using dilutions of the extracted whey. These processing steps removed any excess of non-protein solutes, and therefore eliminated the problem of high residual magnitude. We believe that this precise approach is still a valid method to indirectly estimating colostrum IgG protein levels using °Bx values. The shift to straight colostrum, subsequently introduced to simplify sample processing for the method is at the root of the high residual magnitude problem.

Findings regarding high residual magnitudes in straight colostrum was not only consistent in our results (Schalich *et al.* 2021), but was more prominent in that the regression estimate itself did not show a clear trend and was not significant. We agree that there could be added differences due to the analytical method used. But as our study did not compare quantitative Western blots with RID or enzyme-linked immunosorbent assay (ELISA), we cannot comment on how they might correlate. Quantitative Western blots are proven to have clear benefits as the protein (IgG) is separated by electrophoresis from other colostrum components, processed in a fully denatured form (separating heavy and light chains), and the quantitation is performed by specific visualization of the IgG heavy chain; this dispels any concerns raised regarding the variables known/indicated to affect other assays. RID is certainly a popular standard, and we avouch that "it may be less precise than other methods of IgG analysis", but we do not concur that it sets the benchmark for the reasons already described (Schalich *et al.* 2021). We agree that increasing sample size and including low °Bx colostrum samples would have added to the exactitude of our results, but we don't believe that it could have weakened the conclusion as implied. Moreover, when we discovered that the total IgG in colostrum is only 25% of the mammary IgG secreted post-partum (Schalich *et al.* 2021), we deliberated whether the concept of "colostrum quality" itself might need reevaluation.

Narrow focus on first feeding

In 1976, the precise serum IgG and IgM levels that can be considered as failure of passive transfer was uncovered by studying calves that died from infections (McGuire *et al.* 1976). In this study in which calves were allowed to suckle and stay with the dam for 24 hours, 19 random animals were sampled to get mean values; post-mortem diagnosis and measurements were performed on another 19 calves that died. It was concluded that acquisition of ≥10 mg/ml of IgG levels in serum by 48 hours might represent successful transfer of passive immunity (TPI). From this point forward, several studies calculated the risk of mortality linked to TPI, assessed primarily by the serum total protein levels estimated between day 2 and 7 of age in calves (Nocek *et al.* 1984; Tyler *et al.* 1998). A consensus emerged that for optimal survival, calves needed to achieve >5.5 g/dL of serum protein. However, all these studies used calves that directly suckled for 12-24 hours. In one controlled study, the total grams of IgG that needed to be provided in colostrum was analyzed (Besser *et al.* 1991), and it was concluded that failure of passive transfer (FPT; ≤10 mg/ml of IgG levels in serum by 48 hours) was infrequent in calves receiving ≥100 g of IgG (using an esophageal tube feeder). Irrespective, there was variability noted in intestinal IgG absorption in calves at all feeding levels. The upper 200 g limit of IgG, beyond which the trend showed no corresponding increase in serum IgG, subsequently formed the basis of colostrum feeding today; it was formulated that a single 4 liter feeding of colostrum with an IgG concentration ≥50 g/L as being sufficient for TPI. This remains in mainstream practice at the present time (Godden 2008).

As a single feeding was quite convenient for calf management, it was widely promoted and quickly adopted. Therefore, the target of 50 g/L IgG concentration in a colostrum single feeding became crucial, with detecting and ensuring this target "colostrum quality" becoming a focus of numerous

research works. Although the possibility that IgG absorption, albeit at a lower efficiency, can continue to occur in the calf was long known (Kruse 1970), improving serum IgG levels (to ≥10 mg/ml) through multiple feedings during the neonatal period was not carefully investigated/recommended. Contributing to the uncertainties was also the fact that colostrum IgG uptake by calves can be quite variable in efficiency (Besser *et al.* 1991). Recent research indicates that multiple feedings during the first 24 hours can not only increase serum IgG levels (Hare *et al.* 2020), but also promote overall health and growth in calves (Conneely *et al.* 2014). This is consistent with our finding that the mammary gland continues to secrete IgG in transition milk. Therefore, as others are also recently noting there is benefit to a "multiple feeding model" in which the calf can receive subsequent feedings of either additional colostrum or transition milk to increase TPI, local enteric protection and overall fitness. This might also moderate the idiosyncrasies presented by calves with regard to IgG uptake. We believe that this approach has potential to ultimately mitigate the overwhelming emphasis on the first feeding and putative thresholds of "colostrum quality" that we struggle to uphold.

On oxytocin use:

We noted the comment on our use of oxytocin for the different milkings, and the possible impact on IgG levels. But what has been shown is that the modest effect of oxytocin in elevating colostrum IgG levels is almost identical in side-by-side comparisons to that when a calf was present during milking (Sutter *et al.* 2019). So, we do not believe that our oxytocin use is creating artificially high IgG levels beyond the realm of normal lactation physiology. Possible shift in other milk components have negligible impact on our results and interpretations. Nevertheless, we did not provide oxytocin via the milk vein, as performed in the study indicated (Gorewit & Sagi 1984). Another study providing intramuscular oxytocin, as we have done, does not report any such aberrant outcomes to milk composition (Nostrand *et al.* 1991). We used oxytocin to record accurate yield/volumes; moving forward, we concur that oxytocin use could be avoided to preclude any undetermined effects.

In summary, °Bx-based predictions of IgG concentrations are not accurate to reliably select or eliminate colostrum based on a putative 50 g/L IgG threshold. Inconsistencies between studies (as illustrated in Figure 1) is more than sufficient evidence that prospective predictions for °Bx will be extremely poor. Moreover, biological considerations force reevaluation of the need for the concept of "colostrum quality," which is coupled with an emphasis on single feeding. We do acknowledge, however, that efforts and progress made till date have been on a positive trajectory in TPI management (Urie *et al.* 2018). There is now tremendous awareness regarding adherence to timing and quantity of the first colostrum feeding. Still, it should be noted that morbidity remained high at 38% and mortality at 5% (at mean age 24.4 days) in this same study (Urie *et al.* 2018). Morbidity was addressed with antibiotics in 76.8% of the cases, which is another cause for concern for both animal health and the global rise of antimicrobial resistance. These not only indicate that there is room for improvement, but also highlight the need for research on temporal relationships between mammary synthesis and calf needs in the neonatal period, rather than just rely on reducing mortality/morbidity with the first feeding. Moreover, focus on IgG as a singular factor does not necessarily translate to all aspects of TPI. The incidence of ill calves with digestive (56%) and respiratory (33.4%) problems (Urie *et al.* 2018) points to an association with failed mucosal immunity provided by IgA. Although IgA is considered a minor component in colostrum, it is absolutely essential for presenting an immune barrier at the mucosal interfaces. Human colostrum IgA absorbed in the gut has been shown to be actively presented at mucosal interfaces in infants (Fitzsimmons *et al.* 1994). In calves, it has been noted that colostrum IgA absorption occurs at a much slower rate compared to IgG (Stott *et al.* 1979), and therefore, its benefits might only be seen in a multiple feeding model. It is becoming increasingly obvious that there are open questions to be answered regarding colostrum/transition milk management and the health of newborn calves. We certainly hope that our work will continue to provide synergy in problem-solving towards realizing benefits in practice.

Disclosures

The authors declare that there is no conflict of interest.

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