The effect of chronic treadmill exercise and acetaminophen on collagen and cross-linking in rat skeletal muscle and heart

Chad C. Carroll, Karl Martineau, Kathryn A. Arthur, Richard T. Huynh, Brent D. Volper, and Tom L. Broderick

Department of Physiology, Arizona College of Osteopathic Medicine, Midwestern University, Glendale, Arizona

Submitted 3 September 2014; accepted in final form 18 December 2014

Carroll CC, Martineau K, Arthur KA, Huynh RT, Volper BD, Broderick TL. The effect of chronic treadmill exercise and acetaminophen on collagen and cross-linking in rat skeletal muscle and heart. *Am J Physiol Regul Integr Comp Physiol* 308: R294–R299, 2015. First published December 24, 2014; doi:10.1152/ajpregu.00374.2014.—The purpose of this study was to determine whether exercise and/or acetaminophen (APAP) alter collagen and cross-linking in the rat gastrocnemius muscle, soleus muscle, and heart. Male Wistar rats (n = 50; 8 wk old) were divided into placebo (PLA) or APAP groups and sedentary (SED) or exercised (RUN) groups. APAP (200 mg/kg) was administered daily by oral gavage. Exercised groups ran on a treadmill 5 days/wk for 8 wk with progression to 60 min/day, 20 m/min, and 8° incline. Tissues were assayed for collagen (hydroxyproline) and hydroxylysylpyridinoline (HP) and lysylpyridinoline (LP) cross-links by HPLC. Collagen content (μg/mg dry weight) was greater in both the gastrocnemius (SED-PLA: 114 ± 16 vs. RUN-PLA: 244 ± 32; P < 0.001) and soleus (SED-PLA: 51 ± 7 vs. RUN-PLA: 99 ± 27; P = 0.005) of exercised animals. In contrast, collagen content was not significantly greater in exercised animals treated with APAP (SED-APAP: 113 ± 16 vs. RUN-APAP: 145 ± 21) and soleus (SED-APAP: 55 ± 8 vs. RUN-APAP: 57 ± 10). HP cross-linking (mmol/mol collagen) in the gastrocnemius (SED-PLA: 126 ± 28, RUN-PLA: 50 ± 7, SED-APAP: 41 ± 7, and RUN-APAP: 30 ± 4) and soleus muscles (SED-PLA: 547 ± 107, RUN-PLA: 318 ± 92, SED-APAP: 247 ± 64, and RUN-APAP: 120 ± 17) was lower in exercised rats compared with sedentary rats (P < 0.05). Cross-linking was further reduced in animals treated with APAP (P < 0.05). Neither heart collagen nor cross-linking was influenced by exercise or APAP (P > 0.05). Our findings suggest that exercise and APAP have tissue-specific effects on muscle collagen. Given the widespread use of APAP as an analgesic and antipyretic, further work in humans is warranted.

Acetaminophen; collagen; exercise; rat; skeletal muscle

Acetaminophen (APAP) is used for daily pain relief by millions of osteoarthritis patients and is commonly added to narcotic pain medications. Although traditionally thought to act only in the central nervous system, recent studies have demonstrated that APAP alters remodeling of peripheral tissues such as tendon and skeletal muscle (5, 6, 12, 34, 37). Specifically, when rats are treated with APAP, tendon collagen cross-linking is reduced (6). Collagen fibrils, cross-linked by hydroxylysylpyridinoline (HP) and lysylpyridinoline (LP), are the primary component of the extracellular matrix (ECM). The number of collagen fibrils and amount of cross-linking influence the tensile strength and stiffness of tissues (7–9, 29). An APAP-induced reduction in cross-linking may contribute to the reduction in tendon stiffness noted in humans chronically consuming APAP (5).

In addition to the tendon, collagen is an essential component of many other tissues, such as skeletal muscle and heart tissue. In skeletal muscle, collagen forms the basis of the connective tissue sheaths, which envelop each layer of skeletal muscle. These connective tissue sheaths provide structural support but also facilitate the transfer of force generated in skeletal muscle fibers to tendon and bone. Exercise seems to be a potent stimulator of skeletal muscle and tendon collagen synthesis, an effect that appears to be coordinated (27). It is also well documented that chronic exercise training increases muscle strength and mass (34), as well as tendon stiffness and cross-sectional area (19). The effects of chronic exercise on skeletal muscle and tendon ECM are, however, less defined. We have recently demonstrated that rats exposed to chronic treadmill training have greater Achilles tendon collagen cross-linking but not collagen content (6). In rat skeletal muscle, Gosselin et al. (11) did not observe any differences in collagen or cross-linking content with chronic training. In contrast, Kovanen et al. (20) have found that rats trained throughout their life span tend to have greater amounts of soleus muscle collagen. Our data (6) and that of Gosselin et al. (28) suggest that acute increases in collagen synthesis may not always translate into chronic increases in tissue collagen. Additionally, changes in collagen cross-linking in the skeletal muscle and tendon with chronic training may not be coordinated (6, 11). Further evaluation of the potential coordinated or lack of coordinated adaptations of skeletal muscle and tendon ECM to chronic exercise training is warranted.

Although we have previously found that the effects of APAP on tendon collagen cross-linking occur regardless of exercise training, few studies have evaluated the influence of chronic exercise training and APAP on collagen and cross-linking in skeletal muscle or heart (11). APAP alters skeletal muscle prostaglandin production and receptor expression (35, 36), which may impact collagen formation (18). In addition to skeletal muscle, stiffness of the myocardium, which is influenced by collagen and cross-linking (2, 25), can alter force development via the Frank-Starling mechanism. Substantial evidence also highlights the fact that APAP is a selective inhibitor of cyclooxygenase-2 (16), which has led to some concern that APAP may hold similar risk of cardiovascular events that have been reported for other selective COX-2 inhibitors (15). Changes in heart ECM would certainly impact chamber force development. We are, however, not aware of any data evaluating the influence of chronic APAP consumption on components of heart ECM.

Given the vital role of collagen and previous findings that exercise training and/or APAP alters the functional and struc-
cultural properties of tendon (5, 6), we evaluated the influence of 8 wk of chronic treadmill exercise with daily APAP consumption on gastrocnemius, soleus, and total heart collagen and cross-linking in the same rats, in which we previously evaluated Achilles tendon ECM (6). On the basis of our work in the tendon (6), we hypothesized that chronic exercise training would increase collagen cross-linking and APAP would reduce collagen cross-linking regardless of exercise training.

**MATERIALS AND METHODS**

Details on the animals, exercise training protocol, and acetaminophen administration have been published previously (6). Eight-week-old male Wistar rats (n = 50) were obtained from Charles River Laboratories (Wilmington, MA). Animals were assigned to one of four treatment groups: 1) sedentary-placebo (SED-PLA), n = 15; 2) sedentary-acetaminophen (SED-APAP), n = 15; 3) exercise-placebo (RUN-PLA), n = 11; and 4) exercise-acetaminophen (RUN-APAP), n = 9. Rats were caged in pairs, allowed access to food and water ad libitum, and maintained on a 12:12-h light-dark cycle. The Midwest University Institutional Animal Care and Use Committee approved this study.

Rats in the exercise groups completed a progressive treadmill exercise program lasting 8 wk. Rats were exercised 5 days per week progressing to 60 min of exercise per day. Speed and elevation were progressed to 60 m/min and 8° grade, respectively. Liquid acetaminophen (100 mg/kg; Copenhagen Pharmaceutical, Madison, MD) was administered once daily via oral gavage (200 mg/kg). A dose of 200 mg/kg was chosen after considering the recommendations of the U.S. Department of Health and Human Services, which provides guidance on converting animal drug doses to human equivalent doses based on body surface area (http://www.fda.gov/downloads/Drugs/Guidances/UCM078932.pdf). Our dose was also chosen because of the known kinetics of this dose in rats (17) and the fact that such a dose had not been shown to induce liver damage in rats (32). Control animals received an oral gavage of saline of equivalent volume. Animals were weighed weekly for adjustment of acetaminophen dosing.

After the completion of the 8-wk treatment period, animals were euthanized. The soleus, gastrocnemius, and heart were carefully extracted. Muscle tissue was immediately frozen in liquid nitrogen and stored at −80°C. Before the analysis of collagen and cross-linking, ~10 mg of muscle tissue was freeze-dried for 36 h and then reweighed to obtain dry weight. All tissues were then hydrolyzed for 24 h at 100°C in 6 N HCl. Collagen concentration was determined by quantification of the collagen-specific amino acid hydroxyproline using HPLC and fluorometric detection, as previously described (3, 6, 30). Skeletal muscle HP and LP concentrations were evaluated using HPLC after CF1 cellulose partition chromatography, as previously described (13). Samples were then evaporated to dryness overnight at ambient temperature (Savant SPD131DDA SpeedVac Concentrator; Thermo Fisher Scientific, Waltham, MA). The evaporated sample was reconstituted with 100 µl of cross-link buffer [0.5% (vol/vol) heptafluorobutyric acid (HFBA) in 10% acetonitrile]. HP standards (PYD/ DPD HPLC Calibrator, 8004, Quidel, San Diego, CA) were prepared in cross-link buffer, diluted to 500 µl with 6 N HCl and then dried overnight with the samples. Samples and standards (PYD/DPD HPLC Calibrator, 8004; Quidel, San Diego, CA) were eluted with a Shimadzu RP 3-µm, 50 mm × 4.6 mm column (Shimadzu Scientific Instruments, Columbia MD) using an isocratic method [mobile phase A (0.13% HFBA) and mobile phase B (0.13% HFBA, 75% acetonitrile)]. Samples were eluted using 15% mobile phase B from 0–7 min, followed by 100% mobile phase B for a 3-min wash. The column was then reequilibrated with mobile phase B for 3 min before the next injection. A flow rate of 1.0 ml/min was used. Column temperature (Cryo-20A; Shimadzu Scientific Instruments, Columbia, MD) was held at a constant 40°C. Fluorescence was monitored at 295-nm excitation/395-nm emission, and peaks were integrated with chromatography software (LCSolution version 1.2).

**Statistics.** Tissue collagen and cross-link content were evaluated with a two-way ANOVA (exercise and drug). Collagen and cross-link content between tissues were compared using a one-way ANOVA. The Student-Newman-Keuls post hoc test was used to explore differences when a significant interaction was detected. Values were considered significant at an α level of P < 0.05. All data are expressed as means ± SE. All data were analyzed using SigmaPlot version 11 (Systat Software, Chicago, IL).

**RESULTS**

**Percent wet weight.** Gastrocnemius (SED-PLA: 75.1 ± 0.7, RUN-PLA: 72.3 ± 0.9, SED-APAP: 76.5 ± 0.7, RUN-APAP: 72.5 ± 1.0%; P < 0.001, main effect for exercise) and soleus (SED-PLA: 76.2 ± 0.8, RUN-PLA: 74.5 ± 0.8, SED-APAP: 77.6 ± 1.0, RUN-APAP: 73.7 ± 0.9%; P = 0.005, main effect for exercise) tissue wet weight was lower in exercised animals (Fig. 1, A and B). APAP treatment did not alter gastrocnemius or soleus tissue wet content (Fig. 1, A and B). Heart wet weight was not influenced by exercise or APAP treatment (Fig. 1C).

**Collagen content.** Collagen content was 72% greater in the gastrocnemius of exercise-trained animals given placebo compared with sedentary animals (Fig. 2A; SED-PLA: 114 ± 16, RUN-PLA: 244 ± 32 µg collagen/mg dry weight; P < 0.001). Collagen content was 63% greater in the soleus of exercise-trained animals given placebo compared with sedentary animals (Fig. 2B; SED-PLA: 51 ± 7, RUN-PLA: 99 ± 27 µg...
collagen/mg dry weight; *P = 0.007). In contrast, collagen content was not greater in the gastrocnemius (SED-APAP: 113 ± 16, RUN-APAP: 145 ± 21 µg collagen/mg dry weight; P > 0.05) or soleus (SED-APAP: 55 ± 8, RUN-APAP: 57 ± 10 µg collagen/mg dry weight; P > 0.05) of exercised APAP-treated animals compared with sedentary APAP-treated animals (Fig. 2, A and B). Heart collagen content (SED-PLA: 28 ± 2, RUN-PLA: 26 ± 1, SED-APAP: 26 ± 1, RUN-APAP: 27 ± 2 µg collagen/mg dry weight) was not influenced by exercise or APAP treatment (Fig. 2E).

Collagen cross-link content. In the gastrocnemius HP (SED-PLA: 126 ± 28, RUN-PLA: 50 ± 7, SED-APAP: 41 ± 7, and RUN-APAP: 30 ± 4 mmol HP/mol collagen) and LP (SED-PLA: 15 ± 2, RUN-PLA: 6 ± 1, SED-APAP: 13 ± 2, and RUN-APAP: 8 ± 1 mmol LP/mol collagen) cross-linking were lower in exercised rats compared with sedentary rats (Fig. 3A; HP: *P < 0.05, LP: *P < 0.001, main effect for exercise). A similar effect was noted in the soleus muscle [Fig. 3B; HP: *P < 0.05, LP: *P = 0.005, main effect for exercise, HP (SED-PLA: 547 ± 107, RUN-PLA: 318 ± 92, SED-APAP: 247 ± 64, and RUN-APAP: 120 ± 17) and LP (SED-PLA: 42 ± 7, RUN-PLA: 21 ± 5, SED-APAP: 45 ± 10, and RUN-APAP: 22 ± 3)]. Additionally, gastrocnemius and soleus HP content was lower in APAP-treated rats compared with placebo-treated animals (Fig. 3, A and B; *P = 0.005, main effect for drug). LP content in either muscle was not altered by APAP treatment (Fig. 3, A and B; *P > 0.05). In heart tissue, HP (SED-PLA: 281 ± 38, RUN-PLA: 285 ± 15, SED-APAP: 276 ± 17, and RUN-APAP: 250 ± 33) and LP (SED-PLA: 53 ± 6, RUN-PLA: 53 ± 4, SED-APAP: 55 ± 4, and RUN-APAP: 51 ± 6) collagen cross-linking was not altered by either exercise training or APAP consumption (Fig. 3C; *P > 0.05).

**DISCUSSION**

To our knowledge, this is the first investigation evaluating the effects of APAP and chronic exercise on collagen and cross-linking in skeletal muscle and heart. Overall, we demonstrate that chronic treadmill exercise and APAP have effects on collagen that are tissue-specific. We also find that the previously reported coordinated increase in collagen synthesis in tendon and skeletal muscle after acute exercise (27) may not translate into coordinated adaptations in collagen content after chronic training. Exercise training led to an increase in skeletal muscle collagen in the gastrocnemius and soleus—in contrast to the tendon (6)—an effect that was not seen in APAP-treated animals. Similar to our previous work on the Achilles tendon (6), the skeletal muscle of APAP-treated animals was found to have lower collagen cross-linking. Interestingly, it appears that skeletal muscle collagen cross-link formation was not coordinated with collagen formation leading to a lower ratio of cross-linking to collagen in trained skeletal muscle of placebo-treated animals compared with nonexercised controls. In contrast to the tendon, skeletal muscle water content was not lowered by APAP consumption but was slightly lower in exercise-trained animals. Neither exercise nor APAP consumption had an impact on collagen or cross-link formation in the heart.

![Fig. 2](http://ajpregu.physiology.org/)

**Fig. 2.** A: gastrocnemius collagen content. *P < 0.001, SED-PLA vs. RUN-PLA. †P < 0.004, RUN-PLA vs. RUN-APAP. B: soleus collagen content. *P < 0.007, SED-PLA vs. RUN-PLA. †P < 0.05, RUN-PLA vs. RUN-APAP. C: heart collagen content. Data are presented as means ± SE.

![Fig. 3](http://ajpregu.physiology.org/)

**Fig. 3.** A: gastrocnemius hydroxyllysylpyridinoline (HP) and lysylpyridinoline (LP) cross-linking normalized to collagen. *P < 0.05, main effect for exercise. †P < 0.001, main effect placebo vs. APAP. B: soleus HP and LP cross-linking normalized to collagen. *P < 0.05, main effect for exercise. †P = 0.005, main effect placebo vs. APAP. C: heart HP and LP cross-linking normalized to collagen. Data are presented as means ± SE.
The effects of exercise and APAP on collagen were most prominent in skeletal muscle with no difference between the gastrocnemius and soleus. Collagen content was nearly twofold greater in the soleus and gastrocnemius of trained, placebo-treated animals compared with controls. An increase in collagen content is consistent with previous animal studies demonstrating an increase in skeletal muscle collagen biosynthesis enzymes (21) and collagen content (10, 23) after chronic training. An increase in collagen content with exercise training is, however, not a consistent finding (11, 43). Zimmerman et al. (43) and Gosselin et al. (11) did not observe an increase in either soleus or gastrocnemius collagen, but these differences may be related to differences in treadmill exercise protocols or rat strain (Wistar vs. Fischer 344) studied. The large increase in skeletal muscle content is in stark contrast to the Achilles tendon, in which collagen content was not greater in exercise-trained animals (6). Given the large and coordinated increase in skeletal muscle and tendon collagen synthesis seen in humans (27) after acute exercise, we were surprised by the different response to chronic training. Direct comparisons to Miller et al. (27) are somewhat limited because of species differences (rat vs. human) and tissues evaluated (quadriceps and patellar tendon vs. calf and Achilles tendon). The length of exercise training may also have a large impact on the observed changes in skeletal muscle collagen. Shorter durations (4 wk) of training do not alter soleus collagen content (22), whereas studies of longer duration (23), including the current investigation, have observed an increase in skeletal muscle collagen. Interestingly, Michna (26) found that collagen fibril diameter initially increased after 3 wk of training followed by a decrease, and then the diameter increased again at 7 wk. Assuming that changes in collagen content contributed to the changes in fibril diameter, these findings support our conclusion that timing and duration of training are important factors to consider when assessing the impact of exercise training on skeletal muscle collagen. Intriguingly, collagen content was not higher in exercised animals treated with APAP compared with their nonexercise counterparts. Chronic training has also been shown to increase skeletal muscle stiffness in some models (10). Increased muscle stiffness is directly correlated to collagen content (10), suggesting that the loss of collagen observed in APAP-treated trained rats may have led to a reduction in skeletal muscle stiffness, which, if true, would be consistent with the effects of chronic APAP on tendon stiffness in humans (5). The interpretation of our collagen findings is limited due to the fact that we did not evaluate the relative proportions of the two most common collagen isoforms, collagen I and the less mature type III collagen. Work in rats indicates that muscle contraction increases gene expression of both type III and type I collagen (14). In humans, exercise has been shown to increase total collagen synthesis (27). While type I collagen is far more prominent in tissues, type III is upregulated in states of tissue remodeling. Clearly, collagen synthesis was likely upregulated with exercise in our study. The abundance of type I collagen vs. other collagen isoforms combined with the large increase in collagen noted in exercised animals would suggest that the majority of new collagen was mature type I collagen.

The extent of collagen cross-linking was lower in exercise-trained animals relative to sedentary controls. In contrast, Gosselin et al. (11) did not demonstrate any effect of exercise on rat skeletal muscle collagen cross-linking content after 10 wk of training. This suggests that, as with collagen, the length of training (8 wk vs. 10 wk) may be an important factor to consider when evaluating the effects of chronic training on collagen cross-linking. The age of the rats being studied may also impact the interpretation of the effects of exercise on collagen cross-linking. Zimmerman et al. (43) did not observe an effect of chronic training on skeletal muscle cross-linking in young (10 wk old) rats, but cross-linking was dramatically reduced in middle-aged (12 mo old) and old rats (20 mo old). The rats used in the current study were studied at 18 wk, suggesting that rodent age may be a critical factor when evaluating the effect of exercise training on skeletal muscle extracellular matrix adaptations. Regardless, it seems likely that cross-link formation did not maintain pace with the large increase in tissue collagen. The lower cross-linking per collagen fibril may impact skeletal muscle stiffness and, thus, power output (4). As with the Achilles tendon (6), the effect of APAP on rat skeletal muscle was independent of any exercise stimulus; e.g., cross-link formation was lower in both trained and untrained APAP-treated tendons. This finding suggests a common mechanism by which APAP inhibits cross-linking.

The mechanism(s) contributing to the effect of APAP on skeletal muscle collagen and cross-linking requires additional studies. It is possible that APAP is inhibiting the activity of lysyl oxidase (LOX), although regulation of LOX activity is not well studied in whole tendon in vivo experiments. Several recent reports do clearly demonstrate that APAP can alter...
phosphorylation of several signaling pathways (39–42), including MAPK and Akt, which may have a role in the regulation of collagen (18) and LOX-mediated cross-link formation (38). Additionally, in the same rats used in this study, we have found that APAP has a strong influence on anabolic signaling (p70s6k) and the αβ1 integrin mechanotransduction pathway in skeletal muscle (unpublished observations). Activation of mechanotransduction pathways by APAP could contribute to changes in skeletal muscle collagen and cross-linking by influencing collagen production and fibrogenesis (24). Trappe et al. (36a) has reported a training-induced increase in IL-6 gene expression, which was blunted in humans consuming APAP (36a). IL-6 is a potent stimulator of collagen synthesis, at least in the tendon (1); thus, chronic suppression of IL-6 may have contributed to the lower collagen content noted in the current investigation. Also intriguing is the fact that, in contrast to cross-linking, which seems to be reduced by APAP regardless of exercise, the effects of APAP on muscle collagen are dependent on exercise training, e.g., APAP had no impact on total collagen in nonexercised rats. Further investigation of the mechanisms contributing to the effects of exercise and APAP on skeletal muscle collagen formation is warranted, especially given the strong impact of collagen on skeletal muscle function.

We were surprised by the lack of change in heart collagen and cross-linking with exercise and APAP consumption, especially given our findings in skeletal muscle and tendon (6). In contrast to skeletal muscle, the heart contracts on a consistent basis, which could influence how APAP is metabolized in heart tissue. We have also previously demonstrated that the delivery of APAP is not consistent between various tissues, e.g., skeletal muscle and tendon (12). Although such a difference in APAP kinetics would be difficult to observe in the heart, in vivo, it could explain the lack of any effect of APAP on heart tissue. The lack of change in heart collagen with exercise training is consistent with previous exercise studies (31) in dogs and rats (33). Total heart protein content did increase with training in these animals (6), but collagen does not appear to contribute to this increase in protein content. Myocardial stiffness correlates well with collagen and cross-linking (2), suggesting that APAP did not alter the mechanical properties of the heart. While these findings are reassuring, we analyzed only total heart levels and did not isolate ventricular tissue, which would have provided a more comprehensive view of the potential effects of exercise and APAP on the myocardium. Additionally, as with our skeletal muscle analysis, results from our work are limited to only total collagen and cross-linking. There are several collagen isoforms, which could change and impact tissue mechanical properties without influencing total collagen content. There are also many other ECM proteins, such as proteoglycans, which impact tissue function. Further research should attempt to identify any potential effect on right and left ventricle collagen content specifically.

When comparing our previously published tendon data to that of the current investigation, Achilles tendon collagen content was greater than all other tissues studied (P < 0.001; Fig. 4A). In contrast, tendon collagen cross-linking was similar to that observed in the gastrocnemius and heart (Fig. 4A). Soleus collagen cross-linking was four times greater than the gastrocnemius (Fig. 4B). Soleus collagen cross-linking was also greater than that observed in heart tissue and the Achilles tendon (Fig. 4B).

Perspectives and Significance

APAP is widely used throughout the world as an analgesic and antipyretic. Generally viewed to have no impact on tissues outside the central nervous system, our findings add to the growing body of work demonstrating the strong impact of APAP on peripheral tissues. In humans, APAP use during chronic resistance training leads to an increase in skeletal muscle mass (34), but our findings in rats imply that collagen formation may be limited by APAP. Lower collagen content could impact tissue stiffness and functional performance. Similar to tendon, APAP also reduces skeletal muscle collagen cross-linking, which could affect tissue stiffness. Lastly, our findings suggest that tendon and skeletal muscle ECM adaptations to chronic training may not be as coordinated as the response to acute exercise. Length of training and animal age, however, may be important factors to consider when interpreting the effect of chronic exercise on skeletal muscle ECM adaptations. Clinical practice may benefit from more detailed studies evaluating the mechanism(s) by which APAP impacts tissue ECM.

GRANTS

Funding for this study was provided by Midwestern University Intramural Funds to C. C. Carroll.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

Author contributions: C.C.C. conception and design of research; C.C.C., K.M., K.A.C., R.T.H., B.D.V., and T.L.B. performed experiments; C.C.C., K.M., K.A.C., R.T.H., B.D.V., and T.L.B. analyzed data; C.C.C. interpreted results of experiments; C.C.C. prepared figures; C.C.C. drafted manuscript; C.C.C., K.M., K.A.C., R.T.H., B.D.V., and T.L.B. edited and revised manuscript; C.C.C., K.M., K.A.C., R.T.H., B.D.V., and T.L.B. approved final version of manuscript.

REFERENCES


