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Digestive efficiency mediated by serum calcium predicts bone mineral density in the common marmoset (*Callithrix jacchus*)

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Abstract

Two health problems have plagued captive common marmoset (*Callithrix jacchus*) colonies for nearly as long as those colonies have existed: marmoset wasting syndrome and metabolic bone disease. While marmoset wasting syndrome is explicitly linked to nutrient malabsorption, we propose metabolic bone disease is also linked to nutrient malabsorption, although indirectly. If animals experience negative nutrient balance chronically, critical nutrients may be taken from mineral stores like the skeleton, thus leaving those stores depleted. We indirectly tested this prediction through an initial investigation of digestive efficiency, as measured by apparent energy digestibility, and serum parameters known to play a part in metabolic bone mineral density of captive common marmoset monkeys. In our initial study on 12 clinically healthy animals, we found a wide range of digestive efficiencies, and subjects with lower digestive efficiency had lower serum vitamin D despite having higher food intakes. A second experiment on 23 subjects including several with suspected bone disease was undertaken to measure digestive and serum parameters, with the addition of a measure of bone mineral density by dual-energy x-ray absorptiometry (DEXA). Bone mineral density was positively associated with apparent digestibility of energy, vitamin D, and serum calcium. Further, digestive efficiency was found to predict bone mineral density when mediated by serum calcium. These data indicate that a poor ability to digest and absorb nutrients leads to calcium and vitamin D insufficiency. Vitamin D absorption may be particularly critical for indoor-housed animals, as opposed to animals in a more natural setting, because vitamin D that would otherwise be synthesized via exposure to sunlight must be absorbed from their diet. If malabsorption persists, metabolic bone disease is a possible consequence in common marmosets. These findings support our hypothesis that both wasting syndrome and metabolic bone disease in captive common marmosets are consequences of inefficient nutrient absorption.

INTRODUCTION

Despite its long history of captive management, nutrition and dietary husbandry of common marmosets remains a major concern among colony managers in the United States (2004

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survey by the Marmoset Research Group of the Americas). Nutrient requirements for the common marmoset (*Callithrix jacchus*) are poorly characterized, and dietary husbandry of this species has been informed more by practical and anecdotal experience than it has been by nutritional science. Previous work has suggested that marmosets and other callitrichid primates have higher requirements for protein [Barnard and Knapka, 1993], energy [Barnard and Knapka, 1993; Barnard et al., 1988], and vitamin D [Yamaguchi et al., 1986] than other anthropoids. However, other nutritional investigations have generally found that nutrient requirements of callitrichids are not appreciably different from that of other anthropoid primates [Flurer and Zucker, 1985; Oftedal et al., 1997; Power et al., 1997; Ullrey et al., 1999]. Insufficient scientific knowledge of nutritional requirements likely contributes to some of the health problems that plague common marmoset captive colonies.

Two health problems that are particularly damaging to common marmoset colonies include marmoset wasting syndrome and metabolic bone disease. Marmoset wasting syndrome is characterized by frequent diarrhea, decreased muscle mass, alopecia, and inflammation of the intestinal tract [Layne and Power, 2003; Ludlage and Mansfield, 2003], likely resulting in varying degrees of nutrient malabsorption [Gore et al., 2001]. *C. jacchus* may be more susceptible to the intestinal inflammation that underlies marmoset wasting syndrome than other closely related primate species, however, susceptibility to marmoset wasting syndrome is not universal among captive common marmosets. Managers of marmoset colonies, using highly variable diets, consistently report having individual marmosets in their colony that suffer from some or all of the symptoms of marmoset wasting syndrome, while others in their colony do not exhibit symptoms [Ialeggio and Baker, 1995]. On necropsy, the affected marmosets are found to have extensive inflammatory disease of the upper intestinal tract. While this type of inflammation can sometimes have fatal consequences, more frequently intestinal inflammation persists in a subclinical state and is only apparent at necropsy [Logan and Khan, 1996]. We hypothesize that this chronic inflammation of the gut is can lead to the mineral depletion associated with metabolic bone disease.

Metabolic bone disease, a condition characterized by a reduction in the bone mineral density of the skeleton is frequently observed in captive *C. jacchus* colonies [Hatt and Sainsbury, 1998; Smith et al., 2009]. In the only case that has scientifically investigated this condition in *C. jacchus*, it was concluded that reduced bone mineral density was a consequence of lower than normal serum calcium and vitamin D₃ [Hatt and Sainsbury, 1998]. Given that chronic intestinal inflammation leads to nutrient deficiencies due to malabsorption, it is possible that metabolic bone disease is an indirect consequence of insufficient nutrient absorption [Semrad, 2000].

We propose that the two conditions described above – marmoset wasting syndrome and metabolic bone disease – are actually different sequelae of the same underlying pathology. The proposed sequence is as follows:

1. Some initial stimulus causes inflammation of the small intestine resulting in reduced ability to absorb nutrients. If the inflammation is severe, animals develop wasting syndrome. If the inflammation remains in a subclinical state, chronic nutrient malabsorption is possible.
2. Reduced ability to absorb macronutrients results in negative calorie balance, and reduced ability to absorb micronutrients such as vitamin D and calcium results in negative calcium balance.
3. Despite behavioral adaptations such as increased feeding behavior, prolonged negative calorie balance results in fat and muscle loss. Negative calcium balance results in activation of osteoclasts to free up calcium from the skeleton in order to compensate for the calcium deficiency.

4. If reduced digestive efficiency persists long enough, it results in a chronic state of negative calorie and calcium balance eventually causing continued weight loss via fat and muscle loss, as well as skeletal bone mineral density. This last step leaves the individual suffering from metabolic bone disease and highly vulnerable to skeletal fracture and reduced mobility.

Through a pair of studies, we investigated the relationship between digestive efficiency, peripheral concentrations of micronutrients – in particular serum calcium and vitamin D – and bone mineral density in captive adult common marmosets. In Study 1 we tested the hypothesis that variation in digestive ability, presumed to arise from variation in the health of the gastrointestinal tract, would be significantly correlated with micronutrient status in the blood. Specifically, we hypothesized that reduced digestive efficiency would be associated with lower calcium and vitamin D concentrations in the blood. In Study 2 we extended the design to include a measure of bone health by using dual-energy x-ray absorptiometry (DEXA) to quantify bone mineral density. This measurement was conducted in conjunction with digestion trials and measurements of serum Ca, P and 25 hydroxy-vitamin D (25-OH-D). In this study we tested the hypothesis that reduced digestive efficiency would be associated with both reduced calcium and vitamin D concentrations in the blood, and also reduced bone mineral density of the skeleton.

METHODS

Study 1 examined digestion efficiency, vitamin D status, and serum chemistry values for seemingly healthy, common marmosets ($n = 12$; see details below). These animals were fed a diet designed by M. Power in collaboration with Harlan Teklad (Madison, WI) to serve as a sole feed for marmosets. Study 2 ($n = 23$, 7 animals were common to the two studies) involved measuring the same parameters as those described in Study 1, with the addition of a measure of bone mineral density (see details below). Animals in Study 2 were fed an agar-gelled purified diet that is part of the colony base diet that all animals receive from infancy.

The animals used in both studies were singly housed adult common marmosets housed at the Southwest National Primate Research Center (SNPRC; San Antonio, TX). Animal cages were equipped with trays below them that facilitated collection of uneaten or discarded food as well as fecal samples. Animals ranged in age from 1.0 – 9.3 years, and in size from 227–474g. Common marmosets reach sexual maturity at 1.5 years, and reach their full adult size by three years of age [Abbott and Hearn, 1978]. Animals were balanced according to sex, both in Study 1 ($n = 5$ male, $n = 7$ female), and Study 2 ($n = 13$ male, $n = 10$ female). Animals were fed twice daily at 0730 and 1400 hours each day and the studies lasted for a period of nine days. Animals were fed one of two single-item homogenous diets. In Study 1 a grain-based gelatin-gelled diet was used, and in Study 2 a purified agar-gelled diet was used. Both diets are formulated to be a complete feed for common marmosets. Prior to beginning the first digestion trial (Study 1), animals were acclimated to the test diet for at least one week: in Study 2, the test diet was the same diet the animals had been fed on since weaning. Nutritional compositions for both diets can be found in Table 1. All procedures used in this study were approved by the Southwest National Primate Research Center Institutional Animal Care and Use Committee, adhered to the legal requirements of the United States, and adhered to the American Society of Primatologists (ASP) Principles for the Ethical Treatment of Non Human Primates.

All diet offered was weighed prior to feeding, and all refusals and feces were collected, dried and weighed. Refusals and feces were collected twice per day, just before the next feeding. This information was used to determine the amount of diet consumed, and ultimately, to calculate dry matter intake and apparent digestibility of energy. Additionally, a sample of diet was reserved for nutritional analysis from each feeding period. All food and

fecal samples were frozen until assayed for dry matter, gross energy, crude protein, calcium and phosphorus (assays described below).

In the first study animals were hand restrained and a blood sample obtained from the femoral vein. In the second study subjects were bled immediately after the DEXA measurement was taken (See Bone mineral density, below). Both blood sampling techniques were conducted once per animal (after ~12-hr fasting) during the digestion trial, and yielded approximately 1 ml of blood. Samples were centrifuged to extract serum for clinical blood chemistry and to measure 25-hydroxy-vitamin D concentrations, which is the best indicator of vitamin D status [Power et al., 1997; Ullrey et al., 1999]. Serum calcium concentration was one of the parameters of the clinical blood chemistry assay. Concentrations of 25-hydroxy-vitamin D were determined at the Wisconsin National Primate Research Center with commercially available radioimmunoassay kits (DiaSorin, Stillwater, MN). This assay is specifically designed for marmosets and has standards ranging from 11.4 to 227 ng/ml, with a minimum detectable concentration of 0.589 ng/ml [Ersfeld et al., 2004].

Nutritional assays of food and feces were completed at the Nutrition Laboratory of the Smithsonian National Zoological Park (Washington, DC). Diet and fecal samples were weighed before and after oven drying at 100°C to determine dry matter. Gross energy was determined using an adiabatic bomb calorimeter, and is expressed in kcal/g. Dry matter intake was calculated as the difference between the dry weight of food offered and the dry weight of uneaten food. This calculation was conducted separately for every animal, for every feeding period and an overall average dry matter intake was calculated for each animal. Dry matter intake is a measure that can be modified by the individual animal through changes in behavior (i.e. eat more or less). Gross energy intake was calculated by multiplying the dry matter intake by the average energy content of food for each feeding period. Apparent digestibility of energy was calculated as the difference between the gross energy intake and the energy in fecal samples, divided by the gross energy intake. Apparent digestibility of energy is a measure that can not be modified by behavioral changes in the animal, and is used here as an estimate of digestive efficiency.

Bone mineral density was measured by DEXA. Subjects were fasted the morning of the procedure, anesthetized with ketamine (Ketaset, ketamine HCl 15–20mg/kg) and butorphanol (Torbugesic, 5–10mg/kg, Fort Dodge, IA) prior to scanning via intramuscular injection. Once anesthesia had taken effect, subjects were weighed and scanned with DEXA (System ID: GE Lunar DPX serial 5603; DPX collimator assembly model 3008 serial C660; X-ray tube housing assembly model 2299 serial DPXB824; tube insert model BX-1L-0.5 serial 6303; DPX X-ray Bone Densitometer with Smart Scan v. 4.7e) following manufacturer protocols.

Values for measured parameters are given as mean $\pm$ SEM. Pearson correlation analysis was used to analyze associations between physiological variables (e.g. apparent digestibility of energy vs. vitamin D level). General linear models were used to determine whether any physiological measures predicted bone mineral density. In these models, apparent digestibility of energy, serum calcium, and serum vitamin D were used as predictors to model bone mineral density, and animal ID was used as a random factor. Lastly, mediation analysis was used to investigate whether any measures played a role in mediating the effects of another variable as predictors of bone mineral density. Mediation analysis generates a model that allows the investigator to determine whether two variables (e.g. X and Y) that appear to be related through correlation analysis are actually related only through a third *mediator* variable (e.g. Z). A variable is a mediator when the following criteria are met: 1) X is correlated with Y; 2) X is correlated with Z; 3) Z is correlated with Y; and, critically, 4) when the variance in X that is explained by Z is removed, X is no longer correlated with Y.

In other words, when the mediator is included in the model, the original two variables that were once associated with each other are no longer directly associated, but are instead indirectly associated through the mediator. The output for these analyses are reported as unstandardized regression coefficients (*bs*), that are accompanied by *p* values, which evaluate whether the coefficients are significantly different from zero (i.e. no relationship).

RESULTS

Correlation analyses were conducted to evaluate relationships among physiological measures from marmosets in Study 1, and several significant relationships emerged. The first of these was a positive relationship between apparent digestibility of energy, a measure of digestive efficiency, and the serum concentration of 25-hydroxy-vitamin D ($r_{10} = 0.794$, $p = 0.002$; Fig. 1), suggesting that individual ability to digest food was a critical factor in determining vitamin D levels in the blood. In addition, apparent digestibility of energy was also related, although negatively, to dry matter intake ($r_{10} = -0.647$, $p = 0.023$), suggesting that the more efficient an individual digestive systems is, the less that individual needs to consume in order to obtain the same nutrition. Lastly, dry matter intake was negatively related to the serum concentration of calcium ($r_{10} = -0.771$, $p = 0.003$), suggesting that those individuals with the lowest values of calcium in the blood were the same animals that were eating the most food.

Digestive Efficiency and Blood Chemistry

Correlation analyses were conducted for Study 2 in the same way as for Study 1. As in Study 1, a positive relationship emerged between marmoset apparent digestibility of energy and serum 25-hydroxy-vitamin D concentrations ($r_{21} = 0.630$, $p = 0.001$). Animals in Study 2 also showed a positive relationship between apparent digestibility of energy and serum calcium ($r_{21} = 0.558$, $p = 0.009$; Fig. 2). Positive relationships were also seen between serum calcium and 25- hydroxy-vitamin D ($r_{21} = 0.522$, $p = 0.015$), and serum calcium and body weight ($r_{21} = 0.534$, $p = 0.013$).

Bone Mineral Density

Correlation analyses were used to investigate the relationship between measures of digestive efficiency and our measure of bone mineral density. These analyses revealed that bone mineral density was positively associated with apparent digestibility of energy ($r_{21} = 0.584$, $p = 0.003$; Fig. 3A), 25-hydroxy-vitamin D ($r_{21} = 0.489$, $p = 0.018$), serum calcium ($r_{21} = 0.706$, $p < 0.001$), and animal body weight ($r_{21} = 0.804$, $p < 0.001$).

To investigate these relationships further, general linear models were used to determine whether any of the above variables that were correlated with bone mineral density were significant predictors of bone mineral density. Specifically, apparent digestibility of energy, 25-hydroxy-vitamin D, serum calcium, and animal body weight were included as predictors of bone mineral density, with animal ID as a random variable. Results of this analysis showed that only serum calcium significantly predicted bone mineral density ($F_{1,11} = 7.69$, $p = 0.018$; for all other variables $p > 0.25$).

Because certain variables were correlated with, but did not predict, bone mineral density, we used mediation analyses to investigate whether any variables predicted bone mineral density when mediated by serum calcium. We hypothesized that apparent digestibility of energy might affect serum calcium, which might, in turn, affect bone mineral density. This hypothesis was supported by mediation analysis. When apparent digestibility is the only predictor, it shows a trend towards predicting bone mineral density ($b_1 = 2.02$, $p = 0.057$). When serum calcium is included as a mediator, apparent digestibility of energy significantly

predicts serum calcium ($b_1 = 2.93$, $p = 0.009$), serum calcium predicts bone mineral density ($b_1 = 3.41$, $p = 0.003$), and the relationship between apparent digestibility of energy and bone mineral density is eliminated ($b_1 = 0.19$, $p = 0.849$). This finding lends additional support to our hypothesis that digestive efficiency influences skeletal health indirectly, through mediation by calcium concentrations in the blood. That is, when serum calcium is low, a relationship emerges between digestive efficiency and bone mineral density that is not present when serum calcium is high (Fig. 3B). We also hypothesized that vitamin D might mediate the relationship between apparent digestibility of energy and bone mineral density [Kocian, 1977; Luisier et al., 1977], however mediation analyses did not support this hypothesis. Similarly, vitamin D did not mediate the relationship between serum calcium and bone mineral density.

Comparison of Animals in both Studies 1 and 2

In this study seven animals were assessed in both studies. Of these animals, six had consistent apparent digestibility of energy and serum 25-hydroxy-vitamin D across the two studies (three consistently below average and three consistently above average) and one animal changed from below average digestibility in Study 1 to above average digestibility in Study 2. Interestingly, the 25-hydroxy-vitamin D values for this animal showed a corresponding increase across the two trials (Table 2).

DISCUSSION

In these two studies, we investigated the links between our measures of digestive efficiency, serum micronutrients, and bone mineral density. Our first hypothesis was that marmosets with reduced digestive efficiency would also have reduced calcium and vitamin D concentrations. The results of these studies support this hypothesis. Specifically, both Study 1 and Study 2 showed that animals with lower digestive efficiency also exhibited lower 25-hydroxy-vitamin D concentrations in the blood serum. Our second hypothesis took the association between digestive efficiency and serum micronutrient concentrations one step further and proposed that marmosets with higher digestive efficiency would also have higher bone mineral density. The results of Study 2 revealed an association between digestive efficiency and serum calcium concentrations. Further, Study 2 showed that both serum calcium and vitamin D were associated with bone mineral density, and that serum calcium significantly predicted bone mineral density. Taken together, these findings support the hypothesis that reduced digestive efficiency results in reduced concentrations of certain micronutrients in the blood (e.g. calcium, vitamin D), and that reduced micronutrient concentrations in the blood are associated with physical consequences (e.g. reduced bone mineral density). Lastly, mediation analysis showed that digestive efficiency significantly predicted bone mineral density when mediated by serum calcium concentration. Together, these results suggest that metabolic bone disease is a condition that is caused, in part, by reduced digestive efficiency resulting in animals' inability to absorb critical micronutrients.

Importantly, this work provided preliminary evidence that serum calcium concentration was associated with animal body weight. Specifically, those animals with the lowest serum calcium concentrations also showed the lowest body weight. This suggests that reduced micronutrient absorption may be tied to marmoset wasting syndrome, a condition characterized by rapid weight loss. Longitudinal studies investigating serum calcium concentrations and body weight over time would be ideal to determine whether there is a causal relationship between these two variables. An additional finding involved negative associations between serum calcium and dry matter intake. This finding suggests that animals in poor nutritional status make a behavioral adjustment to increase their food intake, perhaps in an attempt to remedy their nutritional deficit. However, in many cases in this study, this behavioral adjustment did not fully remedy their nutritional deficit due to

malabsorption. Individual animals that showed lower apparent digestibility of energy may have been marginal or deficient in other nutrients that we did not measure, such as the other fat soluble vitamins.

The findings of Study 2 have several critical implications. First, this sample was composed primarily of seemingly healthy common marmosets fed single-item homogenous diets. Almost all of the subjects in this study would have been considered healthy based on a physical examination. The digestive deficits and any nutritional deficits were subclinical, and none of the animals were considered to be suffering from marmoset wasting syndrome or metabolic bone disease. There were no diarrheas or obvious weight loss. However, individual marmosets exhibited a wide range of digestive abilities, as has been reported elsewhere [McWhorter and Karasov, 2007; Power and Myers, 2009]. The results of this study suggest that variation in apparent digestibility of energy could have significant health consequences. The variability in digestive efficiency present in the current sample suggests that successful captive common marmoset husbandry may be more complicated than previously thought. If seemingly healthy animals exhibit highly variable digestive abilities, then commercially available diets may be sufficient for some animals and entirely insufficient for others. As a result, some individuals may eventually exhibit marmoset wasting syndrome and/or metabolic bone disease. Therefore, dietary supplements may be necessary for certain individuals. Second, investigators conducting research on skeletal health should consider measuring digestive efficiency in addition to measures of skeletal health as this may represent a potential source of variation in their outcome measures. One last implication of this study is that direct monitoring of bone mineral density may not be necessary for successful colony husbandry. Indeed, direct monitoring of bone mineral density may not be a feasible option for many captive colony managers due to lack of necessary equipment or financial resources. However, given our findings that serum calcium predicts bone mineral density, indirect monitoring of skeletal health may be possible by evaluating serum calcium concentrations in blood samples. This is not only a less expensive method, but might also allow managers to identify at risk animals before nutrient deficiency persists long enough to result in reduced skeletal health. In these cases preventative measures like supplemental calcium and vitamin D could be used to potentially help those animals with lower digestive efficiency avoid metabolic bone disease. For example, six of the seven animals that were used in both studies had consistent apparent digestibility of energy and serum 25-hydroxy-vitamin D. Only one animal changed from below average digestibility in Study 1 to above average digestibility in Study 2, and the 25-hydroxy-vitamin D values for this animal showed a corresponding increase across the two trials.

In conclusion, it appears that among common marmosets, a species highly susceptible to both marmoset wasting syndrome and metabolic bone disease in captivity, a possible common underlying mechanism exists. We found significant relationships between blood calcium and animal body weight, indicative of a relationship between reduced digestive efficiency and marmoset wasting syndrome. Further, reduced digestive efficiency was associated with, and predicted, reduced bone mineral density when mediated by serum calcium. These results represent preliminary evidence in support of our hypotheses, however, additional work is necessary to confirm these findings.

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Figure 1. Digestive efficiency and serum vitamin D concentrations from Study 1. There was a positive relationship between marmoset apparent digestibility of energy and serum vitamin D concentrations.

Figure 2. Digestive efficiency and serum calcium concentrations from Study 2. There was a positive relationship between marmoset apparent digestibility of energy and serum calcium concentrations.

Figure 3.

Digestive efficiency and bone mineral density from Study 2. There was a positive relationship between marmoset apparent digestibility of energy and bone mineral density (A). Apparent digestibility of energy was predictive of bone mineral density when mediated by serum calcium concentrations. Animals with below average serum calcium concentrations (filled circles) showed a stronger relationship between apparent digestibility of energy and bone mineral density than animals with above average calcium concentrations (open circles) (B).

Table 1

Nutritional compositions of diets used in Studies 1 and 2.

	Study 1: Grain-based diet	Study 2: Agar-gelled diet
<i>Protein (% weight)</i>	22.2	14.0
<i>Carbohydrate (% weight)</i>	52.5	64.3
<i>Fat (% weight)</i>	5.9	5.6
<i>Fiber (% weight)</i>	1.9	5.0
<i>Calcium (%)</i>	1.26	1.20
<i>Phosphorous (%)</i>	0.79	0.81
<i>Potassium (%)</i>	0.97	1.00
<i>Sodium (%)</i>	0.33	0.30
<i>Chlorine (%)</i>	0.51	0.46
<i>Magnesium (%)</i>	0.16	0.15
<i>Copper (mg/kg)</i>	21.9	15.0
<i>Iron (mg/kg)</i>	103.1	180.6
<i>Zinc (mg/kg)</i>	130.0	54.9
<i>Manganese (mg/kg)</i>	111.4	30.3
<i>Vitamin A (IU/kg)</i>	15380	15000
<i>Vitamin B-6 (IU/kg)</i>	8.00	12.35
<i>Vitamin B-12 (IU/kg)</i>	0.08	0.05
<i>Vitamin C (mg/kg)</i>	500	525
<i>Vitamin D (IU/kg)</i>	8000	9000

Table 2

Seven marmosets were in both studies and had data on both apparent digestibility of energy and vitamin D. Animals with low apparent digestibility of energy had low vitamin D values. Five of the animals were consistent in their values of ADE and vitamin D between studies; three with both low apparent digestibility of energy and low vitamin D for both trials and two with both high apparent digestibility of energy and high vitamin D. One animal had low apparent digestibility of energy and low vitamin D in Study 1, but high apparent digestibility of energy and high vitamin D in Study 2.

Animal ID	Apparent Digestibility of Energy		Vitamin D (ng/ml)	
	Study 1	Study2	Study 1	Study 2
8	0.932	0.920	293.0	193.0
11	0.896	0.860	274.5	154.0
7	0.880	0.876	149.0	73.0
14	0.849	0.838	67.5	12.0
12	0.841	0.785	13.5	2.0
3	0.827	0.816	23	10.0
10	0.778	0.886	12.5	182.0
Sample Average	0.858 ± 0.02	0.854 ± 0.02	119.0 ± 46.2	89.4 ± 32.3

Note: Values shown in bold are above sample average.

* Values shown are sample mean ± SEM.