Soil Biology and Biochemistry 119 (2018) 121–127

Contents lists available at ScienceDirect

Soil Biology and Biochemistry

journal homepage: www.elsevier.com/locate/soilbio

Selective ingestion contributes to the stoichiometric homeostasis in tissues of the endogeic earthworm *Aporrectodea turgida*

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ARTICLE INFO

Keywords:
Endogeic earthworms
Homeostasis
Nitrogen balance
Epidermal mucus
Gut transit time
Selective ingestion

ABSTRACT

Most detritivores maintain a stoichiometric homeostasis in their body tissue regardless of the chemical composition of the substrates they ingest, but the mechanisms for stoichiometric regulation in soil-inhabiting detritivores like earthworms are poorly understood. The objectives of this study were (1) to examine whether the endogeic earthworm *Aporrectodea turgida* (Eisen) exhibits a strict homeostasis in its tissue C:N ratio, (2) to determine if *A. turgida* controlled its tissue N concentration by changing the quantity of N excreted in epidermal mucus and (3) to consider how the gut transit time, gut load and cast production were related to selective ingestion, which is hypothesized to control the N stoichiometry in *A. turgida* tissues. Two laboratory experiments were designed to address these objectives. In the first experiment, we evaluated the C and N concentrations, and C:N ratio of *A. turgida* body tissue and epidermal mucus after the earthworm fed on soil mixed with ^{15}N-labeled plant litter (red clover leaves, wheat leaves, wheat stem) having variable N content and C:N ratios, as well as no litter, for 7 days. The second experiment measured the gut transit time, gut load and cast production of *A. turgida* fed soil marked with glass beads, either without litter or mixed with plant litter (soybean leaves with high N, wheat stems with low N). The endogeic earthworm *A. turgida* maintained strict homeostasis in their body tissue, with a C:N ratio of 3.9. The epidermal mucus of *A. turgida* also showed a strict homeostasis (C:N ratio = 4.6) and constant ^{15}N enrichment, regardless of the N content in plant litter. Therefore, N secretion through epidermal mucus cannot be a mechanism that regulates the N stoichiometry in the body tissue of *A. turgida*. The gut transit time of ingested substrates was the same, as both N-rich (i.e., soil-soybean mixture) and N-poor (i.e., soil-wheat mixture) substrates took 21 ± 1 h to pass from the mouth to the anus of *A. turgida*, however, there was significantly ($P < 0.05$) less material in the gut and less cast production from the N-rich than the N-poor substrate. We conclude that a selective ingestion process controls the intake of organic substrates and likely contributes to the conservation of N stoichiometry in A. *turgida* body tissues.

1. Introduction

Stoichiometric homeostasis, the degree to which organisms maintain a constant elemental composition in their body regardless of trophic resources, is a core concept in ecological stoichiometry (Sterner and Elser, 2002). In theory, organisms that maintain strict homeostasis will resist changes in their body composition by releasing elements in excess of their needs and retaining the most limiting elements from trophic resources. This may increase the recycling rate of the non-limiting elements, while depleting the limiting elements from the ecosystem (Vanni, 2002), altering nutrient availability and flows at an ecosystem-level (Sperfeld et al., 2017). For instance, changes in dominant species within a zooplankton community, from a homeostatic consumer with a low N:P ratio (i.e., having an elevated P body content, thus retaining P in their biomass and releasing N at higher rates) to a homeostatic consumer with a high N:P ratio (i.e., an elevated N body content), caused a shift in phytoplankton growth conditions from P limitation to N limitation in a freshwater ecosystem (Sterner et al., 1992). Primary production is generally limited by N availability in terrestrial ecosystems and particularly in agroecosystems, so populations of detrivorous organisms like earthworms that maintain strict homeostasis in their elemental N content could have important consequences for soil N cycling and crop production.

Earthworms are dominant detritivores in agroecosystems and strictly homeostatic in their C and N contents, as reported by Marichal et al. (2011) who found a constant C:N ratio of 4.1 in *Pontoscolex corethrurus*. Similarly, Scheu (1991) and Chen (2013) documented a C:N ratio of 3.8 and 3.7 in lumbricid earthworms of the endogeic functional

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https://doi.org/10.1016/j.soilbio.2018.01.014
Received 19 May 2017; Received in revised form 27 December 2017; Accepted 18 January 2018

group. The low C:N ratio in endogeic earthworm tissues is interpreted to mean they have a high biological N demand. However, earthworms often increase the amount of plant-available NH_4-N and NO_3-N in agroecosystems for the benefit of crops grown in pots and in field plots (Brown et al., 1999; Van Groenigen et al., 2014). From the perspective of stoichiometric homeostasis, it seems contradictory that strictly homeostatic, N-demanding earthworm populations could increase N availability and flows to agricultural crops. Considering their trophic position in the soil foodweb, this observation is logical because endogeic earthworms derive their nutrition from organic N compounds associated with the light fraction of organic matter (Abail et al., 2017) and release plant-available NH_4-N and NO_3-N into the environment through cast defecation, urine excretion and mucus secretion, as well as through mortality (Chertov et al., 2017; Whalen et al., 1999). In addition, the ecological engineering activities of earthworms are responsible for soil structure reorganization, organic substrate fragmentation and stimulation of soil microorganisms responsible for N mineralization and nitrification reactions, producing NH_4-N and NO_3-N (Bertrand et al., 2015; Blouin et al., 2013). Still, it is not known how endogeic earthworms regulate the stoichiometric homeostasis in C:N ratio of their tissues.

In other aquatic (Frost et al., 2005) and terrestrial invertebrates (Simpson et al., 1995), a number of physiological (e.g., differential digestion, assimilation, excretion) and behavioural (e.g., food selection) mechanisms can be involved in regulating N homeostasis. Physiological mechanisms that may regulate stoichiometric homeostasis occur in the digestion process, after organic substrates are ground in the earthworm gizzard and move into the proctodeum and foregut sections of the intestinal tract (Brown et al., 2000). Here, the secretion of intestinal mucus stimulates digestion by gut microbiota (Lavelle et al., 1995). Intestinal mucus production is negatively correlated with the quality of the ingested substrate (Barois, 1992; Trigo et al., 1999), indicating a differential digestion mechanism. Second, the earthworm may control the N assimilated through the gut wall, into the bloodstream and muscular tissue. Nitrogen assimilation efficiencies of 10–26% were reported for the endogeic *A. tuberculata*, and more N was assimilated when the organic substrate was mixed with glucose (Whalen and Parmelee, 1999). Finally, the earthworm could control the N losses from the body via urine excretion (quantity, urea and NH_4-N concentrations in urine) as well as epidermal mucus secretion, which varies depending on their body condition, handling, defensive action, and other factors (Laverack, 1963). Whalen et al. (2000) suggested that the amount of N excreted in urine-mucus mixture could be higher when earthworms are fed with N-rich materials than when they are provided with N-poor materials, while Needham (1957) noted that 50% of the total daily N loss from earthworm tissues occurs through the secretion of epidermal mucus. Thus, epidermal mucus could be a physiological mechanism to eliminate excess N from the body when earthworms feed on N-rich substrates.

Earthworm behaviour is another factor regulating N homeostasis. Earthworms ingest a variety of trophic resources: decaying plant litter, living and dead roots, animal dung and the microbiota associated with these organic substrates, along with adhering soil minerals. Curry and Schmidt (2007) documented the selective feeding behaviour of earthworms, and their ability to alter ingestion rates according to the quality of organic substrates, where high-quality substrates have a low C:N ratio (i.e., high N content) and a low-quality substrates have a high C:N ratio (i.e., low N content). Earthworms provided with low-quality substrates have greater ingestion rates and more cast production (Flegel and Schrader, 2000; Flegel et al., 1998), suggesting that the earthworm feeding strategy is to increase the amount of material passing through the gut, presumably so they can derive enough N from the low-quality substrate to meet their metabolic requirements. If earthworms rely on selective ingestion to regulate their N homeostasis, the hypothesis is that earthworms will ingest a larger quantity of N-poor substrates than N-rich substrates, and they will defecate more casts when feeding on N-poor substrates than N-rich substrates. Testing this hypothesis requires knowledge of the mass of substrates ingested and casts produced by earthworms during a period of time. Concurrent measurements of earthworm ingestion and casting rates can only be done in an artificial, soil-free environment (e.g., using the feeding system described by Whalen and Parmelee, 1999). An alternative approach is to evaluate the time for substrates to pass through the earthworm gut (i.e., the gut transit time), which varies from 1 h (Barley, 1959) to 24 h (Piearce, 1972) and is species-specific (Taylor and Taylor, 2014). Gut transit time is suggested to vary due to substrate quality, but no data on this topic was found in the literature, leading to the hypothesis that gut transit time is faster when earthworms consumes N-rich substrates than N-poor substrates.

The objectives of this study were (1) to examine whether the endogeic earthworm *A. Turgida* exhibits a strict homeostasis in their tissue C:N ratio, (2) to determine if *A. turgida* controlled their tissue N concentration by changing the quantity of N excreted in epidermal mucus, and (3) to measure the gut transit time, gut load and cast production as indicators of selective ingestion, which is hypothesized to control the N stoichiometry in *A. turgida* tissues. The objectives were evaluated in laboratory experiments where *A. turgida* were supplied with N-rich and N-poor substrates.

2. Materials and methods

2.1. Soil and earthworm collection

Soil and earthworms used in this study were collected from the top 15 cm of a cornfield at the Macdonald Campus Farm, Sainte Anne de Bellevue, Quebec, Canada (45°28′ N, 73°45’ W). The soil was a mixed, frigid Typic Endoquent, classified as a Chicot series sandy loam (609 g sand kg^{-1}, 246 g silt kg^{-1}, 145 g clay kg^{-1}) with 25.7 g organic C kg^{-1}, 2.9 g N kg^{-1}, and pH (H_2O) of 5.4. In these fields, *A. turgida* is the dominant endogeic species (Eriksen-Hamel et al., 2009) and it is often numerically dominant in cultivated agroecosystems of Quebec (Reynolds and Reynolds, 1992). Adult *A. turgida* were collected by hand sorting and kept at 20 °C for 3 to 8 wk in culture boxes containing the original field soil, moistened to 20% gravimetric moisture content. Earthworms were used for two separate laboratory experiments (described below). One day before each experiment began, earthworms were removed from the culture boxes, washed and placed on wet filter paper to void their guts at 16 °C. The mean individual fresh weight of earthworms after gut clearance was 628 mg (± 101 mg, n = 116).

2.2. Experimental setup: effect of plant litter on the C and N concentrations and C:N ratio in A. turgida tissue and mucus

The first laboratory experiment evaluated the C and N concentrations, and C:N ratio of *A. turgida* body tissue and epidermal mucus after feeding on ^{15}N-labeled plant litter with variable N content and C:N ratio. To obtain ^{15}N-labeled litter, wheat and red clover were grown from seeds in a greenhouse, and fertilized using a ^{15}N-enriched nutrient solution made up of 10% ^{15}N-KNO_3 (98 atom % ^{15}N) and 90% KNO_3 (0.367 atom % ^{15}N). After 7wk, plants were harvested, rinsed with distilled water, oven dried (40 °C for 3 d), and ground homogenously with a Wiley mill (< 1 mm mesh). Subsamples were analyzed for C and N content, and ^{15}N enrichment. The N content and C:N ratio varied depending on the litter type: red clover leaves had 47.3% C, 5.7% N, C:N = 8 and atom % ^{15}N = 5.2, while wheat leaves (46.6% C, 3.2% N, C:N = 15, atom % ^{15}N = 4.7) and wheat stems (44.0% C, 1.5% N, C:N = 29, atom % ^{15}N = 5.0) contained less N and a higher C:N ratio. The acid unhydrolyzable fraction (Van Soest et al., 1991), a proxy for lignin content, was similar among litter types: 50 g kg^{-1} in red clover leaves, 54 g kg^{-1} in wheat leaves and 58 g kg^{-1} in wheat stems.

The experiment was a completely randomized design with four treatments: red clover leaves, wheat leaves, wheat stems, and a control

treatment (without plant litter). The experimental unit was a mason jar (500 mL) filled with 150 g (dry weight basis) of soil. Jars with plant litter had 2 g of ground (< 1 mm) ^{15}N-labeled litter mixed in the upper layer (> 3 cm), representing the litter distribution in soils under perennial red clover and no-till/minimum till wheat production. Fourteen replicates of each treatment were prepared, for a total of 56 mason jars. Soil and soil-litter mixtures were moistened to 70% of water holding capacity (WHC), and preincubated at 16 °C in the dark for 2 d. After the preincubation, a single gut-cleared earthworm was added to each jar, which was covered with a 1 mm nylon mesh to allow for aeration while preventing earthworm escape. Then, the jars were placed randomly in an incubator at 16 °C in the dark for 7 d. Every 2 d, jars were weighed, distilled water was added to maintain the soil moisture level at 70% WHC and the jars were returned to a random location in the incubator.

After 7 d, earthworms were removed from microcosms, washed individually with double distilled water, and allowed to clear their guts (24 h) in the dark at 16 °C. After gut clearance and fresh weight measurement, half of these earthworms were euthanized by spraying with 70% ethanol and muscular tissue was collected from the anterior part of the earthworm body (first 20 segments), which was further dissected to remove the remaining part of the intestinal tract, then freeze-dried for 24 h, and subsequently ground with a mortar and pestle. Subsamples of the ground tissue were weighed into tin capsules for C, N and atom % ^{15}N analysis. The remaining earthworms were placed into individual petri dishes (60 mm × 15 mm) to stimulate the secretion of epidermal mucus. In addition, extra earthworms (n = 11) were selected at random from the culture boxes to assess the baseline levels of C, N and ^{15}N enrichment in muscular tissue and mucus. They also underwent 24 h gut clearance, were weighed (fresh weight) and then sacrificed (n = 6) for analysis of muscular tissue or transferred to individual petri dishes (n = 5) for epidermal mucus collection.

Secretion of epidermal mucus from earthworms was induced by electric stimulation based on the procedure used by Heredia et al. (2008) to collect mucus from *Eisenia fetida*. Briefly, each individual earthworm was placed in a small petri dish (60 mm × 15 mm) and an electrical current, generated by two electrodes connected to a 6 Volt battery, was applied to its body. Earthworms were subjected to this current intermittently for 60 s. A single stimulation lasted 4 ± 1 s. Following mucus secretion, earthworms were removed from the petri dish, rinsed in double distilled water, gently blotted dry with Kim Wipe tissue paper, and reweighed. Mucus was collected from petri dishes using a micropipette, transferred immediately into 8 × 5 mm tin capsules, frozen at -18 °C and then freeze-dried for 24 h.

2.3. Experimental setup: effect of plant litter on the gut transit time of substrates ingested by A. turgida

The second laboratory experiment evaluated the gut transit time of *A. turgida* fed a soil and a soil-litter mixture made from plant materials having contrasting N content, namely soybean leaves with a high N content (47.2% C, 3.7% N, C:N = 12) and wheat stems with a low N content (45.8% C, 0.8% N, C:N = 54). Soybean leaves had a smaller acid unhydrolyzable fraction of 41 g kg^{-1} than wheat stems, which contained 110 g kg^{-1}. Soybean leaves and wheat stems were obtained from plants grown in pots in the greenhouse for 7 and 12 wks, respectively. Plant materials were prepared by rinsing, drying, grinding (< 1 mm), and sieving sequentially through < 1 mm and < 0.5 mm mesh sieves, so the litter used in the experiment was between 0.5 and 1 mm in size. The decision to use litter of 0.5–1 mm size was based on: (1) the observation that a related endogeic species *A. caliginosa* (Savigny), derives its nutrition from litter particles between 0.25 and 0.5 mm (Abail et al., 2017), and (2) logistic considerations of how to measure gut transit of litter with a visible tracer– glass beads – of comparable size. The gut transit time of *A. turgida* is not reported in the literature but *A. caliginosa* is reported to have a gut transit time from 9 h (Taylor and Taylor, 2014) to 24 h (Piearce, 1972), so earthworms were given access to the soil-litter mixture for 8, 16 and 24 h (i.e., feeding period). We used a soil marked with glass beads to evaluate the gut transit time, as the appearance of glass beads in earthworm faeces was a surrogate for the passage of the ingested soil-litter mixture through the earthworm gut.

Marked soil was prepared by homogenously mixing 3 g of glass beads (0.5–1 mm) with 27 g of soil that was physically treated to remove the fraction size of 0.5–1 mm. The physical treatment involved two steps: (1) passing 5 kg of air-dried and pre-sieved soil (< 2 mm) through a 0.5 mm sieve, and (2) wet-sieving the soil (0.5–2 mm in size) to remove the soil fraction of 0.5–1 mm size and floatable organic matter. Afterwards, all soil that passed a < 0.5 mm sieve during the dry- and wet-sieving procedures, plus the wet-sieved soil of 1–2 mm size were combined, saturated with distilled water, and oven-dried (100 °C for 2 d).

The experiment was conducted in polyethylene containers (100 cm^3) using a factorial design with 3 litter treatments (soybean, wheat, and no litter) and 3 feeding periods (8, 16 and 24 h), for a total of 9 factorial combinations. Eight replicate containers of each factorial treatment were prepared, but some earthworms went into hibernation, possibly triggered by seasonal changes in their hormonal levels or metabolic functions that coincided with the timing of this experiment, which occurred in December. All hibernating earthworms were removed from the experiment, leaving a number of replicates between 4 and 8, resulting in a total of 55 containers with active earthworms. Every replicate container had 30 g of marked soil that was mixed with no litter, soybean leaves or wheat stems. The soil and soil-litter mixtures were moistened to 70% WHC, and preincubated at 16 °C in the dark for 2 d. After the preincubation, a single gut-cleared earthworm of *A. turgida* was added to each container, sprayed with 1–2 mL of distilled water, the top covered with a 1 mm nylon mesh screen, and the containers were distributed in a completely randomized design in an incubator at 16 °C in the dark. After 8, 16, and 24 h, containers were taken from the incubator, earthworms were removed from the containers, gently rinsed with double distilled water, weighed, and then put into individual petri dishes (100 mm × 15 mm) to clear their guts. The soil and soil-litter mixtures from containers with active (non-aestivating) earthworms were gently spread in a thin layer (< 2 cm high) on a plastic sheet, then casts were carefully removed from the soil, dried at 105°C for 24 h and weighed. Petri dishes, used for gut clearance, were examined every hour, and the time when an earthworm defecated material containing glass beads in the faeces was noted. The gut clearance period lasted for 24 h for all treatments, then earthworms were removed from the petri dishes, gently rinsed with double distilled water and weighed. The casts collected from the petri dishes were also dried (105 °C for 24 h) and weighed to determine the gut load.

2.4. Analytical procedures and calculations

2.4.1. C and N concentrations, and ^{15}N enrichment

The C and N concentrations in plant material and earthworm tissue were determined with a Thermo FinniganFlash 1112 EACN analyzer (Carlo Erba, Milan, Italy). The ^{15}N in labeled plants, earthworm tissues and mucus, as well as the C and N concentration in the freeze-dried mucus samples (estimated to be < 20 mg dry weight) were analyzed using a PDZ Europa ANCA-GSL elemental analyzer interfaced to a PDZ Europa 20-20 isotope ratio mass spectrometer (Sercon Ltd. Cheshire, UK) at the UC Davis Stable Isotope Facility.

The C and N concentrations in plant material, earthworm tissue and epidermal mucus were expressed as mg nutrient g^{-1} dry weight of the biological material. The mass of freeze-dried mucus was not measured, but estimated to be 20% fresh weight, the same as earthworm dry weight (Whalen et al., 2000). The^{15}N enrichment was expressed in atom percent excess, determined by subtracting the atom % ^{15}N in the background sample (i.e., earthworms taken from the culture box) from the atom % ^{15}N in the enriched sample (i.e., earthworms that consumed

^{15}N-labeled plant material).

2.4.2. Earthworm weight change and quantity of mucus

Earthworm weight gain or loss was evaluated as the percentage change in an individual's weight over the duration of the experiment relative to its initial weight. All biomass values were reported as fresh weight, which refers to the dry-blotted biomass. Mucus secretion was estimated from the difference in earthworm biomass before and after the electric stimulation, and expressed relative to its weight before stimulation (mg g^{-1} earthworm fresh weight).

2.4.3. Estimation of food retention and gut transit time

The food retention time was the period of time that elapsed between the placement of earthworms on petri dishes and the egestion of faeces containing a glass bead. The gut transit time was the sum of the feeding period (8, 16 or 24 h) plus the food retention time. No earthworms produced casts during a feeding period of 8 h, but 47% and 83% of the active earthworms produced casts during the 16 and 24 h feeding periods, respectively (data not shown). Our method of assessing the gut transit time depends on detection of the first egested faeces that contains a glass bead in the individual petri dishes during the 24 h of gut clearance, thus gut transit time was calculated for replicates from the 8 h feeding period.

2.4.4. Estimation of gut load and cast production

The gut load was calculated by dividing the total amount of faeces egested (mg per faeces dry mass) in the individual petri dishes during the 24 h of gut clearance by the weight of the earthworm (g fresh weight). The cast production was evaluated for the earthworms that were provided with food substrates for 24 h. It was calculated by dividing the total amount of faeces egested in the polyethylene containers during the 24 h feeding period by the weight of the earthworm. The cast production was then expressed as mg dry weight of faeces g^{-1} earthworm fresh weight d^{-1}.

2.5. Statistical analysis

Data were first tested for normality with the Shapiro-Wilk test and homogeneity of variances using Levene's test prior to analysis of variance (ANOVA) with SPSS software (IBM SPSS Statistics 20.0). In the first experiment, the effect of plant litter treatment (no litter, red clover leaves, wheat leaves, and wheat stems) on the weight change of *A. turgida,* the C and N concentrations and ^{15}N enrichment of their muscular tissue and epidermal mucus was determined by a one-way ANOVA. Within each plant litter treatment, the C:N ratio of the muscular tissue and the epidermal mucus was compared with a t-test.

The main and interactive effects of litter treatment (no litter, soybean, and wheat) and the feeding period (8, 16 and 24 h) on the food retention time and the gut load in *A. turgida* were evaluated by two-way ANOVA using SPSS software. The effect of litter treatment on the gut transit time and the earthworm weight change were evaluated by one-way ANOVA. When the litter treatment or feeding period effects were significant ($P < 0.05$), mean values were compared with Fisher's LSD post hoc test. Cast production data was not normally distributed, so differences between litter treatments were evaluated with a Kruskall-Wallis test, followed by a Mann-Whitney U test with a Bonferroni adjustment.

3. Results

3.1. Effect of plant litter on the C and N concentrations and ^{15}N enrichment of the muscular tissue and epidermal mucus of A. turgida

All earthworms (n = 56) survived the 7 d incubation in soil only and soil-litter mixtures, although they lost weight when no litter was added and gained weight when ground (< 1 mm) plant litter was added (Table 1). Earthworms in soil-wheat mixtures had 37–45% more body mass than earthworms provided with soil-red clover mixture, probably due to greater assimilable energy content in the wheat leaves and stems than in clover leaves. The C and N concentrations, and C:N ratio in the muscular tissues of *A. turgida* were not affected by the litter treatment (Table 1), although litter contained from 1.5 to 5.7% N and had a C:N ratio between 8 and 29. The wet mass of mucus secreted by *A. turgida* was on average 96 ± 5 mg, accounting for 20 ± 1% of its fresh body weight. Mucus production did not significantly differ among litter treatments ($P = 0.568$), although earthworms fed on soil only tended to have lower mucus production rate. The C and N concentrations, and C:N ratio of the epidermal mucus were similar among litter treatments, and they were significantly ($P < 0.05$) higher than the muscular tissue (Table 1). Similarly, the atom % ^{15}N enrichment in muscular tissue and in epidermal mucus was not affected by litter treatments (Fig. 1), although the atom % ^{15}N enrichment in epidermal mucus was nearly two-fold greater than the atom % ^{15}N enrichment in muscular tissue ($P < 0.05$, t-test), indicating a faster turnover of N in the mucus than in the tissue.

3.2. Effect of plant litter on the food retention and gut transit time

Food retention time in *A. turgida* was not affected by the litter treatment or the length of the feeding period (Table 2). Organic substrates took the same amount of time to travel from the mouth to the anus of *A. turgida,* although the litter contained from 0.8 to 3.7% N, with C:N ratios of 12–54, and feeding periods varied from 8 to 24 h. The average gut transit time for *A. turgida* was 21 ± 1 h (Table 3).

3.3. Effect of plant litter on the gut load and cast production

The gut load of *A. turgida* varied significantly due to the litter treatment ($P < 0.05$) and the length of the feeding period ($P < 0.05$), but there was no interaction between these treatments (Table 2). The average gut load recorded for earthworms fed for 8 h was two times lower than that obtained for earthworms fed for 16 and 24 h (Table 2). For each feeding period, earthworms had significantly ($P < 0.001$) lower gut load when fed on soil-soybean mixture than soil-wheat mixture or soil only (Table 2).

The cast production after 24 h was also affected significantly ($P = 0.001$) by the litter treatment. Earthworms provided with soil-soybean mixture had the lowest cast production, with more casts produced when they fed on soil-wheat mixture, and the greatest cast production was from earthworms fed with soil only (Table 3).

4. Discussion

4.1. The muscular tissue and epidermal mucus of A. turgida maintain strict homeostasis in their C and N concentrations

As expected, the stoichiometric composition of C and N in the muscular tissue of *A. turgida* was not influenced by the litter treatment (with or without addition of plant litter), nor by the C:N ratio of the plant litter. This confirms that earthworms are strictly homeostatic with respect to their C and N concentrations and have a constant C:N ratio in body tissue, consistent with the reports of Marichal et al. (2011) and Chen (2013). The C:N ratio of about 3.9 in the endogeic *A. turgida* is close to the C:N ratio of 3.7 reportedby Chen (2013) for the endogeic earthworm *A. tuberculata*, the C:N ratio of 4.1 obtained by Marichal et al. (2011) for the tropical endogeic earthworm *P. corethrurus*, and the C:N ratio of 3.8 for earthworms reported by Pokarzhevskii et al. (2003).

In addition, *A. turgida* maintained strict homeostasis in the C and N concentration of their epidermal mucus, although the mucus had a slightly higher C:N ratio than the body tissue because it is relatively richer in C and N (Table 1). Mucus is composed primarily of glycoproteins and peptides with C:N ratios of 6.9 and 3.3, respectively

Table 1
The weight change (%), mucus production (mg wet mucus g^{-1} earthworm fresh weight), C concentration, N concentration, and C:N ratio in the muscular tissue and epidermal mucus of *Aporrectodea turgida* provided with various soil-litter mixtures (treatment). Mean values (± standard error) in each column followed by different letter are significantly different (LSD test, P < 0.05). In each row, the significant difference in C concentration, N concentration and C:N ratio between muscular tissue and epidermal mucus from earthworms given the same treatment is indicated at the * P < 0.05,**P < 0.01, or ***P < 0.001 level (t-test).

Treatment	Weight change (%, n = 14)	Mucus (mg g^{-1}fw, n = 7)	Muscular tissue			Epidermal mucus		
			C (mg g^{-1}, n = 7)	N (mg g^{-1}, n = 7)	C:N ratio (n = 7)	C (mg g^{-1}, n = 7)	N (mg g^{-1}, n = 7)	C:N ratio (n = 7)
No litter	−4.33 ± 1.66c	181 ± 12.1	4.46 ± 0.07	1.16 ± 0.02	3.85 ± 0.03	7.29 ± 0.84**	1.59 ± 0.20*	4.65 ± 0.20**
Red clover leaves	11.6 ± 2.08b	200 ± 12.9	4.58 ± 0.03	1.17 ± 0.01	3.90 ± 0.04	7.40 ± 0.89*	1.65 ± 0.20*	4.51 ± 0.06***
Wheat leaves	17.8 ± 1.97a	207 ± 11.6	4.54 ± 0.04	1.15 ± 0.02	3.95 ± 0.05	7.68 ± 0.63***	1.64 ± 0.13**	4.69 ± 0.10***
Wheat stems	21.7 ± 1.49a	194 ± 14.6	4.42 ± 0.10	1.11 ± 0.03	3.98 ± 0.05	7.28 ± 0.71***	1.56 ± 0.14*	4.67 ± 0.11***

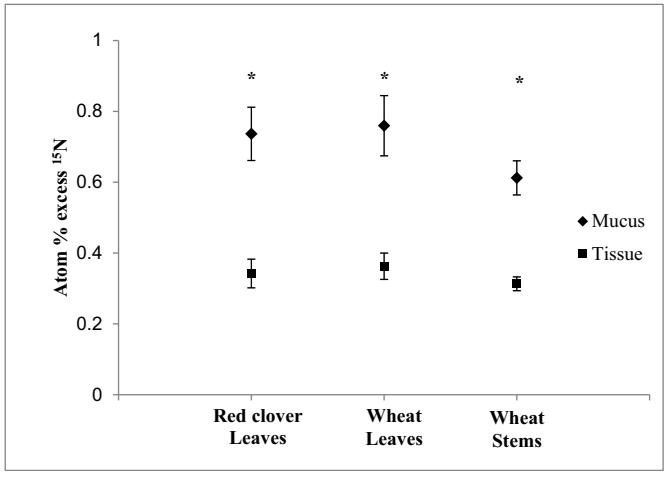

Fig. 1. Enrichment of earthworm muscular tissue and epidermal mucus with ^{15}N after 7 days in soil with no litter or in soil mixed with ^{15}N-labeled red clover leaves, wheat leaves and wheat stems. Significant difference (P < 0.05, t-test) in the^{15}N enrichment of muscular tissue and epidermal mucus of earthworms consuming each plant litter is indicated with an asterisk (*).

Table 2
Food retention time (h) and gut load (mg dry weight of faeces g^{-1} of earthworm fresh weight) measured in *Aporrectodea turgida* after 8, 16 and 24 h of feeding on soil only, soil-soybean mixture, or soil-wheat mixture. Within each time interval, mean values (± standard error, with the number of observations in brackets (n)) having the same letters are not statistically different (P < 0.05, LSD test).

Time (h)	Litter treatment	Food retention time (h)	Gut load (mg dw g-1fw)
8	Soil only	15.0 ± 2.37 (6)	32.7 ± 4.67 (6) a
	Soil-soybean mixture	9.40 ± 1.75 (5)	12.2 ± 2.95 (5) b
	Soil-wheat mixture	15.2 ± 2.12 (6)	34.9 ± 7.17 (6) a
16	Soil only	9.50 ± 3.44 (6)	74.3 ± 9.20 (6) A
	Soil-soybean mixture	12.5 ± 4.17 (4)	32.9 ± 6.78 (4) B
	Soil-wheat mixture	10.5 ± 3.97 (4)	51.1 ± 10.4 (4) A
24	Soil only	11.6 ± 1.21 (8)	61.6 ± 8.62 (8) A′
	Soil-soybean mixture	13.0 ± 2.08 (5)	30.4 ± 3.76 (5) B′
	Soil-wheat mixture	11.9 ± 0.95 (8)	54.2 ± 4.24 (8) A′
ANOVA		P value	
Litter		0.518	< 0.001***
Time		0.917	< 0.001***
Litter x time		0.407	0.473

Table 3
Gut transit time (hours) after 8 h, cast production (mg dry weight of faeces g^{-1} earthworm fresh weight d^{-1}) and percent weight change (%) after 24 h for *Aporrectodea turgida* feeding on soil only, soil-soybean mixture, or soil-wheat mixture. Mean values (± standard error, with the number of observations given in brackets (n)) having the same letters are not statistically different (P < 0.016, post-hoc U test with a Bonferroni adjustment).

Treatment	Gut transit time (h)	Cast (mg dw g^{-1}fw)	Weight change (%)
Soil only	23.0 ± 2.37 (6)	492.0 ± 71.52 (6) a	2.83 ± 0.65 (8)
Soil-soybean mixture	17.4 ± 1.75 (5)	104.0 ± 25.75 (6) c	2.81 ± 1.57 (7)
Soil-wheat mixture	23.2 ± 2.12 (6)	243.1 ± 23.96 (7) b	3.41 ± 1.18 (8)
	P value		
ANOVA	0.148		0.917
Kruskal-Wallis		0.001**	

(Cortez and Bouché, 1987). The C:N ratio of epidermal mucus in this study was about 4.6, which is slightly higher than the C:N ratio of 3.8 obtained by Scheu (1991) for the endogeic species *Octolasion lacteum*. Schmidt et al. (1999) observed that *Lumbricus festivus* had similar C:N ratio in the epidermal mucus of fasting (C:N ratio = 4.2) and feeding (C:N ratio = 4.1) individuals, although laboratory animals tended to have a lower C:N ratio than field-collected individuals (C:N ratio = 5.9). Using data from the study of Schmidt et al. (1999), we calculated 6.4 mg C g^{-1} mucus dry weight and 1.1 mg N g^{-1} mucus dry weight for field-collected individuals of *Lumbricus festivus*. These values are consistent with results obtained in this study. Overall, it appears that the C and N concentrations in the epidermal mucus of earthworms is maintained by homeostatic regulation.

4.2. Epidermal mucus and gut transit time have no effect, but selective feeding regulates the N homeostasis in A. turgida

Epidermal mucus was hypothesized to be a pathway for secretion of excess N from the earthworm body, but there is no evidence to support this assertion. Since the N balance was maintained in the epidermal mucus and the^{15}N enrichment in epidermal mucus did not differ when the earthworm ingested various litter, the epidermal mucus could not be a pathway of N excess disposal when an earthworm fed on N-rich substrates. This is the first study to provide such evidence, which could be an argument that urine excretion is solely responsible for the variation in the concentration of N excreted in the mucus-urine mixture observed in previous studies (Salmon, 2001; Whalen et al., 2000). Further investigations are needed to confirm this hypothesis.

The second hypothesis is that earthworms would modulate the gut transit time of the ingested substrate, such that a hard-to-decompose material would pass more slowly and a readily-degradable substrate would pass more quickly. This is not correct, since the N-rich (i.e., soil-soybean mixture) and N-poor (i.e., soil-wheat mixture) substrates, as well as the soil without litter, required the same amount of time to pass

from the mouth to the anus of *A. turgida*. Hartenstien et al. (1981) also found no significant difference between the gut transit time of a mineral soil and a fibrous organic material for the epigeic species *E. fetida*. However, a question could be asked about how earthworms maintained the same gut transit time and could have eliminated such high amounts of casts when fed on soil only and N-poor substrates. This is possibly associated with a differential digestion and assimilation process occurring in the earthworm gut related to N cycling by gut-inhabiting and ingested microbiota as well as intestinal mucus N, which may be resorbed into tissues or eliminated from the body with casts. It is well established that earthworms secrete higher amounts of intestinal mucus when feeding on poor than good quality substrates, leading to higher stimulation of the gut-inhabiting and ingested microbiota, accelerating thus the digestion and assimilation process of ingested materials (Barois, 1992; Lavelle et al., 1995; Trigo et al., 1999). An alternative explanation is that the content of organic carbon in the ingested substrate may also affect its digestibility. This lead us to suggest that a better characterization of substrate quality should be based on the total amount of assimilable energy provided by the ingested substrate rather than its content on single elements such as nitrogen or carbon. The gut transit time of 13.4–21.4 h in this study is similar to the estimates of 12–24 h gut transit time by Piearce (1972) and 20 h reported by Barley (1959) for the endogeic *A. caliginosa*. The measurements relied on an inert tracer (silica glass beads) that are similar to sand particles, thus avoiding any potential adverse effect associated with the use of a chemical dye (Taylor and Taylor, 2014) or a possible inference between the substrate and the natural tracer (i.e., fungal spores). The method relies on visual observations to determine the time when individual earthworms start ingesting organic substrates, which makes the estimation of gut transit time prone to human error. The method could be improved by using shorter feeding period (less than 8 h) and more replicates to overcome the inherent variability in feeding pattern between individual earthworms.

The gut load and cast production data showed that earthworms ingested significantly ($P < 0.05$) less of the soil-soybean mixture (i.e., N-rich substrate) than the soil-wheat mixture and soil only (i.e., N-poor substrates). This implies that *A. turgida* engaged in selective ingestion process, probably based on the N content of litter mixed with soil. This observation is consistent with the often-reported fact that endogeic species ingest more soil when provided with a material of low nutritional quality, leading to higher cast production (Buck et al., 1999; Flegel and Schrader, 2000; Flegel et al., 1998). However, this is the first report, as far as we are aware, that explains the feeding and casting habits of endogeic earthworms in the context of their strict homeostasis.

5. Conclusion

This report confirms that the endogeic earthworm *A. turgida* maintains a strict homeostasis in its tissue C:N ratio and its epidermal mucus C:N ratio. The evidence presented here indicates that earthworm behaviour, based on a selective ingestion process, controls the intake of organic substrates with varying N concentration. We posit that selective ingestion contributes to the N stoichiometry in *A. turgida* body tissues, although we cannot neglect to consider the *in vivo* digestion and N assimilation processes attributed to the mutualistic interaction between earthworms, gut-inhabiting microbiota and ingested microbiota, as well as physiological processes that remove N from the body like urine excretion and intestinal mucus elimination with casts. Future work in this area of soil biology should focus on the physiological and behavioural mechanisms that earthworms rely upon to regulate their N homeostasis, in realistic soil environments, perhaps using ^{15}N stable isotopes to trace the amount of N that is ingested, retained within earthworms and released into the soil-microbial-plant system as a function of substrate quality. Finally, characterizing substrate quality on the basis of litter N content is too simplistic because this definition does not consider the digestibility of the organic material, i.e., the rate at which the substrate is transformed physically and biochemically into monomeric compounds that are then assimilated and metabolized in cells within earthworm tissues. Assimilable energy in the form of carbohydrates, fats and proteins released from decomposing plant litter, or as by-products of the gut-associated microbiota, could be a suitable indicator of substrate quality in future studies of earthworm stoichiometry.

Acknowledgments

Financial support for this project was provided by the Natural Sciences and Engineering Research Council of Canada (NSERC) through Grant # 2383823-10 and Discovery Grant # RGPIN-2017-05391. ZA was supported by a postgraduate scholarship from the IDB Merit Scholarship Program for High Technology. We would like to thank Hicham Benslim, Hélène Lalande and Khosro Mousavi for their technical assistance, and two anonymous reviewers for helpful suggestions on an earlier version of this manuscript.

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