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The fate of condensed tannins during litter consumption by soil animals

Mathieu Coulis [a], Stephan Hättenschwiler [a], Sylvie Rapior [a,b], Sylvain Coq [a,*]

[a] Centre of Evolutionary and Functional Ecology UMR 5175 (CEFE), CNRS, 1919, route de Mende, F-34293 Montpellier cedex 5, France
[b] Laboratory of Botany, Phytochemistry and Mycology, University of Montpellier 1, F-34293 Montpellier cedex 5, France

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ABSTRACT

Condensed tannins (CT) can strongly affect litter decomposition, but their fate during the decomposition process, in particular as influenced by detritivore consumption, is not well understood. We tested the hypothesis that litter CT are reduced by the gut passage of two functionally distinct detritivores of Mediterranean forests, the millipede *Glomeris marginata*, and the land snail *Pomatias elegans*, as a fixed proportion of initial litter CT, but more so in *Pomatias* since snails are known to have a more efficient enzymatic capacity. Contrary to our hypothesis, both detritivore species reduced litter CT to near zero in their faecal pellets irrespective of the wide range in initial leaf litter CT concentrations of 9–188 mg g^{-1} d m among three Mediterranean tree species (*Pistacia terebinthus*, *Quercus ilex*, *Alnus glutinosa*) and different decomposition stages of their litter. The almost complete disappearance of CT even from some litter types highly concentrated in CT, due to either degradation by gut microorganism or complexation of CT into insoluble high molecular weight structures, suggests a high "de-tanning" efficiency across functionally distinct detritivore species. The transformation of CT-rich litter into virtually CT-free faecal pellets by detritivores might be highly relevant for the subsequent decomposition process in ecosystems with a high macrofauna abundance and CT-rich plant species such as Mediterranean forests.

1. Introduction

Tannins are widely occurring plant polyphenols of varying structure and molecular weight, which differ from other plant phenolics in their ability to precipitate a range of organic molecules, including proteins (Spencer et al., 1988; Hartzfeld et al., 2002). Plant tannins are divided into the two major groups of condensed tannins and hydrolyzable tannins, which are both important for different biological processes, such as plant defence against herbivores (Heil et al., 2002) and pathogens (Bell, 1981; de Colmenares et al., 1998), protection against UV radiation (Close and McArthur, 2002) and oxidative stress (Dehon et al., 2001). Moreover, they may remain at relatively high concentrations in senescent plant material and enter the soil system in litter (Hättenschwiler and Vitousek, 2000; Lin et al., 2007). Within the soil, tannins may have allelopathic effects (Bais et al., 2004), and they can interfere with decomposition processes (Hättenschwiler and Vitousek, 2000; Kraus et al., 2003a). For example, tannins can complex leaf proteins (Bradley et al., 2000; Fierer et al., 2001) and exoenzymes from soil microorganisms (Goldstein and Swain, 1965; Scalbert, 1991), leading to decreased nitrogen availability and to lower enzyme activity, which both might contribute to nitrogen limitation of decomposition. These tannin effects may contribute to the negative correlation between litter polyphenol concentration and the decomposition rate of that litter (Nicolai, 1988; Valachovic et al., 2004; Schweitzer et al., 2005).

In some ecosystems, considerable amounts of leaf litter are consumed by the soil macrofauna. For example, in Mediterranean forests, the millipede *Glomeris marginata* can consume up to 50% of the total annual litter input of the dominant tree species *Quercus ilex* (David and Gillon, 2002). In other ecosystems, earthworms are reported to ingest nearly 90% of the total available litter pool (Coleman et al., 1983) and gastropods between 1.3 and 23% (Lavelle and Spain, 2001). Litter consumption by the soil macrofauna strongly modifies the decomposition process through direct effects on carbon and nitrogen mineralization (Verhoef and Brussaard, 1990; Schaefer, 1991), and through indirect effects by litter fragmentation and transformation into faecal pellets (Coleman et al., 1983; Scheu and Wolters, 1991). This litter transformation does not only change the physical properties of organic matter, but also its chemical composition (Rawlins et al., 2006). Such modifications in organic matter chemistry could include changes in tannin quantity and activity with potentially important consequences for further decomposition. However, such fauna effects on litter tannins are very poorly studied. To the best of our knowledge, the study by Zimmer et al. (2005) is the only one reporting on the degradation of polyphenols – with CT as one component among other phenolic compounds – by litter-feeding isopods.

* Corresponding author.
E-mail address: sylvain.coq@cefe.cnrs.fr (S. Coq).

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In the present study, we addressed the question how two functionally different litter-feeding animals influence the fate of condensed tannins (CT) in litter of different Mediterranean tree species varying widely in CT concentrations. We have chosen *G. marginata* (Diplopoda) and *Pomatias elegans* (Gasteropoda) as two typical litter-feeding animals in Mediterranean forests. These two common calcicole organisms are abundant in Mediterranean ecosystems of Southern Europe, but they have a contrasted physiology. Gastropods are generally known for the production of a wide spectrum of different enzymes (Newell, 1967; O'Reilly-Wapstra et al., 2004) associated with high assimilation efficiencies (Brussaard et al., 1997). In contrast, diplopods are commonly assumed to have a rather limited enzymatic capability and accordingly, assimilation efficiencies remain quite low (David and Gillon, 2002). *P. elegans* is thus expected to have a stronger effect on litter CT than *G. marginata*. Litter from the three Mediterranean tree species *Q. ilex*, *Pistacia terebinthus*, and *Alnus glutinosa* were used in our experiment. These species span a wide range of litter quality traits (De Oliveira et al., u*npublished data*), including CT concentration. The evergreen *Q. ilex* is the dominant tree species on calcareous soil in southern France, *P. terebinthus* is a common broadleaf deciduous small tree, and *A. glutinosa* a characteristic riparian N-fixing deciduous tree species. These species can co-occur naturally along the many seasonally dry streams in Mediterranean forests.

We hypothesized that (1) the concentration of condensed tannins decrease in faecal pellets compared to the litter material consumed by animals, and that the degree of this decrease depends on the litter species, (2) the reduction of CT concentration during gut passage differs between animal species, with a greater reduction in *P. elegans* than in *G. marginata* faeces, because of a greater enzymatic capacity of *P. elegans*.

2. Material and methods

2.1. Plant and animal material

Freshly fallen leaf litter was collected using several 2 × 2 m litter traps that were suspended (1.5 m above the ground) underneath multiple individual trees (20–40 individuals per species). Litter traps were emptied weekly in autumn 2006, except for *Q. ilex*, which was collected from late May to early July 2007, during peak leaf litter fall of this evergreen species. *Q. ilex* and *P. terebinthus* litter was collected in forests 30 km north-west of Montpellier, France (43°39′N, 3°40′E, altitude 280 m), and *A. glutinosa* litter was collected in a forest near Calmont, 60 km south of Toulouse, France (43°29′N, 1°63′E, altitude 210 m). Leaf litter was dried 48 h at <40 °C and only clean and intact leaves were selected for our experiment. Because the macrofauna usually feed preferentially on older, partially decomposed leaf litter material, we decided to use both, freshly fallen and partially decomposed leaf litter of all three species. This allowed to test whether the fauna effect on CT depends on the decomposition stage of litter material, and also to include an even larger range in initial CT concentration that is lower in partially decomposed litter. After each species-specific litter pool was well mixed, we split each litter pool in two equal parts. One part was stored as is, and the second part was placed on the forest floor (previously freed of naturally occurring litter) in a *Q. ilex* forest of the experimental field site of the CEFE–CNRS (Montpellier, France) within large 0.5 × 1 cm mesh width litterbags. The more rapidly decomposing *A. glutinosa* litter was exposed for 42 days (mid December 2006 to end of January 2007), and the more recalcitrant *Q. ilex* and *P. terebinthus* litter were exposed for 65 days (mid November 2006 to end of January 2007), in order to create a partially decomposed litter material (11–12% litter mass loss for all three species). Following exposure, bags were oven-dried at <40 °C for 48 h and intact leaves were selected for the experiment.

G. marginata was collected in forests in the same area as we collected leaf litter of *Q. ilex* and *P. terebinthus* (see above) in March 2008. Only mature or pre-mature animals with 12 tergites were collected. In contrast of the younger stages, they occur in the litter and soil throughout the year and account for most of the population biomass (Mole and Waterman, 1987; David and Gillon, 2002). *P. elegans* was collected on the experimental field site of the CEFE–CNRS (Montpellier, France) in February 2008. Similar sized, presumably adult individuals had been selected for the experiment. Prior to the experiment, animals were kept in large transparent plastic boxes (each species separate) with natural soil substrate and a mix of tree litter from the experimental field site. Litter was regularly moistened and boxes were placed in a shaded green house that provided animals with a natural range of temperatures (2°–26 °C), and which was used thereafter for the experiment.

2.2. Experimental setup

Litter and animals were added to transparent, non-hermetically closed plastic boxes (175 × 115 × 65 mm, LAB no. 4, Caubères, Yebles, France) in March 2008 over a ten day period. We used a total of 54 boxes for 18 treatment combinations (3 tree litter species × 2 decomposition stages × 3 animal treatments), i.e. a replication of 3 per treatment combination. The three animal treatments consisted of the two different animal species and a control without any animals. A total of 1.5 g of litter material was added to each box (non-limiting over the course of the experiment). Animals were weighed individually after a starvation period of 72 h and after cleaning off occasionally attached litter or soil particles to their bodies. A total of ten individuals were placed in each box according to treatment assignment, which corresponds to a mean animal fresh weight of 1.4 ± 0.2 g and 2.7 ± 0.4 g for *G. marginata* and *P. elegans* (excluding the average shell weight of 150 mg per individual), respectively. Litter within the boxes was moistened daily by spraying mist. Faecal pellets were collected on a daily basis and immediately dried at 40 °C in order to stop any microbial activities. In addition to the starvation period, the faecal pellets collected during the first day were not considered for analyses, in order to make sure that none of the faecal material was influenced by litter consumed before the experiment started. We continued the faecal pellet collection for a total of nine days, when the experiment was stopped.

2.3. Condensed tannins measurements

Subsamples of the six different initial litter types (freshly fallen and partially decomposed material of the three species), the faecal pellets collected during the experiment (all pellets pooled within each box), and the remaining litter in the control treatment without animals were ground with a Cyclotec 1093 mill (Foss Tecator, Höganäs, Sweden) by passing through a 1 mm screen, and analyzed for CT. Two complementary methods of butanol-HCl (Porter et al., 1986; Waterman and Mole, 1994) and vanillin-HCl assay (Price et al., 1978) were used to determine tannin concentrations in the experimental materials. After cleavage, the butanol method detects the monomers (anthocyanidins) released from the CT polymers, and is thus specific to CT, whereas the vanillin method also detects flavonols in addition to anthocyanidins.

We used a sonicator during 30 min for CT extractions, and absolute methanol as the solvent for both the vanillin-HCl assay, and the butanol-HCl assay.

The choice of the appropriate standard for tannin analysis is critical (Hagerman and Butler, 1989). We used the monomers of catechin and cyanidin as standards for the vanillin and butanol method, respectively. However, monomers have contrasted reactivity, which can lead either to underestimation or to overestimation of CT in the extract.

The commercially available Quebracho tannin is often used as a polymerised standard of CT, but the disproportionate abundance of the 5-desoxy-flavan-3-ols, as a rather uncommon monomer, influences the reactivity of Quebracho CT, and thus, the calibration curve fitted on this standard. Instead of using the problematic Quebracho tannins, we used purified grape seed CT, which has a more common structure (see Prieur et al., 1994; Souquet et al., 1996).

2.4. Statistical analysis

We tested for differences in CT concentration in initial litter material using two-factorial analysis of variance with tree species and decomposition stage (freshly fallen vs. partially decomposed material) as fixed factors. We adjusted a linear model to the data set of final material CT concentration, with CT concentration as the dependent variable and litter species, decomposition stage and consumption by organisms as explanatory factors for the three-factorial ANOVA procedure in R. Equality of variances and normality of residues were tested by Harrison–McCabe test. In view of the very low CT concentrations in faecal pellets near zero, we additionally tested whether or not CT concentrations in faeces were different from 0 using Student *t*-tests. We used the R software, version 2.7.2 (R Development Core Team, 2006) for all statistical analyses.

3. Results

3.1. Initial litter tannin concentration

Initial litter condensed tannin concentration varied significantly among the three litter species studied, and was also significantly different between freshly fallen and partially decomposed leaf litter (Table 1). Using purified grape seed CT as the common standard allows a direct comparison of the data obtained with the butanol assay and those obtained with the vanillin assay. Across the entire data set, butanol and vanillin determined litter CT correlated well ($r^2 = 0.87, n = 84$). Particularly, species rankings along the CT gradient did not change between the two different methods (Table 1), suggesting a quite robust species comparison. However, occasionally CT concentrations differed considerably in the same sample depending on the assay. For example, there was more than twice as much "vanillin-CT" than "butanol-CT" in freshly fallen leaf litter of *P. terebinthus* (Table 1). Because the butanol assay detects condensed tannins more specifically than the vanillin assay, and because data from the two methods correlated strongly, we refer exclusively to butanol-HCl determined data in the following.

Across species CT concentrations were on average 70% lower in partially decomposed litter than in freshly fallen leaf litter (Table 1). However, CT loss during an initial decomposition period differed among species (significant litter species × decomposition stage interaction, $F = 78.1$, $P << 0.001$, Table 1). *P. terebinthus* and *A. glutinosa* had 83% and 75% lower CT concentrations in partially decomposed litter compared to freshly fallen leaf litter. In contrast, the difference was smaller for *Q. ilex* with only 51% lower concentrations in partially decomposed leaf litter. Consequently, while freshly fallen *Q. ilex* litter had roughly half the CT concentration compared to freshly fallen *P. terebinthus* litter, partially decomposed *Q. ilex* litter had higher concentrations than partially decomposed *P. terebinthus* litter. The strong differences in CT among species and between freshly fallen and partially decomposed litter created an important gradient in initial litter CT, with a 20-fold difference between the CT-richest litter type (freshly fallen *P. terebinthus*) and the CT lowest litter type (partially decomposed *A. glutinosa*).

Table 1
Concentrations of condensed tannins (mg grape seed CT g^{-1}) in freshly fallen and partially decomposed leaf litter of different Mediterranean tree species (Mean ± SE).

Litter species	Butanol-HCl		Vanillin-HCl	
	Freshly fallen	Partially decomposed	Freshly fallen	Partially decomposed
Pistacia terebinthus	187.7 ± 7.0	32.6 ± 0.6	428.6 ± 13.8	38.1 ± 3.3
Quercus ilex	94.9 ± 1.8	46.1 ± 2.0	121.5 ± 4.6	40.0 ± 1.9
Alnus glutinosa	37.6 ± 1.4	9.2 ± 0.4	25.2 ± 1.2	5.6 ± 0.3

3.2. The fate of condensed tannins

In freshly fallen leaf litter, CT decreased on average by 65% over the 10-day exposure when no animals were present (Table 2). This decrease was particularly strong in *A. glutinosa*. In partially decomposed litter, the average CT decrease was 28% when no animals were present, and species varied less in their relative CT loss from partially decomposed litter compared to freshly fallen litter (Table 2). Similar to initial litter material, litter species, decomposition stage and their interaction significantly affected CT concentration in litter at the end of the experiment (Table 2).

The passage of leaf litter material through the guts of *G. marginata* and *P. elegans* strongly reduced CT concentrations in faeces to near zero (Table 2). There was no significant difference in CT concentration between *G. marginata* and *P. elegans* faeces (Tukey post hoc contrasts in the full model). We therefore simplified the statistical model by pooling across the treatments *G. marginata* and *P. elegans* presence, thus, reducing the factor "animals" to just two levels with and without fauna. All the interaction terms with the factor "animals" were significant (Table 3), indicating that the alteration of CT concentrations in animal faeces compared to litter material that remained untouched by litter-feeding animals, differed among litter species and decomposition stage. It appears that these interactions were mainly driven by *P. elegans,* because three out of the four faeces CT concentrations that differed significantly from zero were from *P. elegans* faeces (Table 2). *P. elegans* faeces produced from partially decomposed litter had on average 75% lower CT concentrations than the initial litter material at the beginning of the experiment, compared to 98% lower CT concentrations in faeces produced from freshly fallen litter. Also, CT concentrations in *P. elegans* faeces produced from *A. glutinosa* litter differed only slightly from remaining *A. glutinosa* litter with no animals.

4. Discussion

4.1. Interspecific variation in tannin concentration

The concentration of condensed tannins in leaf litter differed strongly among the three different Mediterranean tree species included in our study. Interspecific differences in leaf tannins determined with colorimetric assays using calibration curves based on a common standard must be interpreted with caution. These methods are not always very specific in measuring tannins, and the

Table 2
Average concentrations of condensed tannins (mg grape seed CT g^{-1}) in remaining litter without animal presence and in faeces of *G. marginata* and *P. elegans* at the end of the experiment (determined with the butanol-HCl assay). The relative change in CT concentration compared to initial litter material (% loss of initial) is given in brackets. For faecal pellets, Student *t*-tests were performed to test if CT concentrations differ from zero ($P < 0.05^*$, $P < 0.01^{**}$).

Litter species	Decomposition stage	No animals	*G. marginata*	*P. elegans*
P. terebinthus	Freshly fallen	93.1 (50)	ND	−2.9(101)
	Part. decomposed	23.1 (29)	2.9 (91)	5.4 (83)
Q. ilex	Freshly fallen	40.1 (58)	2.2 (98)*	−0.5(101)
	Part. decomposed	36.5 (21)	1.5 (97)	9.1 (80)*
A. glutinosa	Freshly fallen	5.3 (86)	ND	2.8 (93)**
	Part. decomposed	6.1 (34)	−0.3 (103)	3.4 (63)*

Table 3
Three-factorial analysis of variance to test for effects of litter species, decomposition stage, and animal consumption, and their interactions on CT concentration (butanol-HCl assay).

Source variance	df	Sum of squares	Mean square	*F*-value	*P*-value
Litter species (L)	2	0.003	0.001	25.1	<0.001
Decomp. stage (D)	1	0.001	0.001	8.7	0.006
Animals (A)	1	0.011	0.011	168.0	<0.001
L × D	2	0.002	0.001	11.6	<0.001
L × A	2	0.005	0.002	37.9	<0.001
D × A	1	0.002	0.002	31.7	<0.001
L × D × A	2	0.003	0.002	24.0	<0.001
Residuals	36	0.002	0.00007		

tannin standard used may vary in its structure from the tannins of the studied species which influences the yield of the colour reaction (Kraus et al., 2003b). Also, litter CT may comprise a range of size and solubilities from monomers to structures that are insoluble due to either high molecular weight or cross-linkages to other biopolymers (Makkar et al., 1999). We think that our species comparisons are fairly robust for several reasons. First, we used the CT specific butanol-HCl assay. It has recently been shown that CT concentrations in litter from 16 different tropical tree species measured with the butanol assay correlated well with detailed HPLC based measurements (Coq et al. *unpublished data*). Second, our data correlated well between the butanol and the vanillin assays which is considered to indicate reliable tannin measurements (Yu and Dahlgren, 2000), and most importantly, species rankings in CT concentrations did not change between the two different methods. Third, instead of using assay specific monomers as standards, we used the purified grape seed CT with a rather common structure, as an appropriate standard for CT determination (Souquet et al., 1996).

Partially decomposed litter had substantially lower CT concentrations compared to freshly fallen litter across all species, which could be due to leaching (Schofield et al., 1998), microbial degradation (Zimmer, 1999), or complexation into insoluble compounds of higher molecular weight (Nierop and Verstraten, 2006). In *P. terebinthus* and *A. glutinosa* litter, CT concentration decreased more rapidly during this initial decomposition phase than in *Q. ilex* litter. In the case of *P. terebinthus* this faster initial CT loss could be due to a higher proportion of more easily leachable smaller CT and other small phenolic compounds like flavonols, as indicated by the distinctly higher yield with the vanillin assay that is more sensitive for these compounds than the butanol assay (Hartzfeld et al., 2002). In fact, *Pistacia* sp. leaves are known for their high concentrations and wide range of flavonoids (Kawashty et al., 2000; Duru et al., 2003). *Q. ilex* on the other hand might have lost less CT to leaching because the thick cuticle and wax layers on the leaf surface effectively inhibit leaching losses. In line with the supposedly rapid leaching and/or microbial degradation of CT during the initial phase of decomposition, we reported a substantial loss of an average 65% of CT from freshly fallen leaf litter during the 10-day microcosm exposure, and a much lower average loss of 28% from partially decomposed litter. This result suggests that CT loss is a non-linear process and supports the earlier studies by Schofield et al. (1998) and (Nierop and Verstraten, 2006), who both found a fast decrease of CT during the first month of decomposition. Spanning a longer period of decomposition in spruce forests, (Lorenz et al., 2000) reported a CT loss of 70% during the first six months of decomposition, and a quite stable CT concentration thereafter for the following six months.

The quantity and quality of litter CT apparently differed among the three studied species that was further accentuated by different dynamics in CT leaching and/or degradation losses. The dynamics of CT losses depend on both, CT composition and structure, and on the general physical and chemical characteristics of the litter species.

4.2. Fauna effects on the fate of condensed tannins

We initially hypothesized that condensed tannin concentrations decrease in faeces compared to the litter animals fed upon, and that the degree of this decrease depends on the litter species, because tannins with different structure could potentially be affected differently by gut passage. Our results confirm that CT decrease in faeces due to animal consumption. However, this decrease did not differ among litter species, but soil fauna rather led to a drastic decrease of CT concentration of consumed litter in all studied litter types. Irrespective of the 20-fold difference in initial CT concentration in the different litter types we used, essentially no CT could be detected in faeces. Faeces from only four of the total 12 treatments contained very low CT concentrations that differed significantly from zero. This strong decrease of CT in faeces was observed in both functionally different animal species we used in our test. It is striking, that two taxonomically and functionally different animals produced the same result across a large gradient in litter tannin concentration. Hence, our second hypothesis that *P. elegans* is more efficient in reducing CT in consumed litter than *G. marginata* was not confirmed.

There might be several explanations for this general disappearance of CT in faeces irrespective of differences in initial litter quality and irrespective of litter-feeding animals. Even though the composition of organic matter of faeces of most the litter-feeding macrofauna is not very different from that of the initial litter (Gillon and David, 2001), its structure is fundamentally changed (Tajovsky et al., 1991) and facilitates leaching of soluble compounds (Kaneko, 1999) and microbial access (Hanlon, 1981). Therefore, leaching and/or microbial assimilation of CT may be more rapid in faeces than in intact leaf litter. However, complete leaching and/or microbial assimilation is unlikely in our study, since faecal pellets were collected and dried on a daily basis. Alternatively, CT might actually have been degraded during gut passage, or they changed their physicochemical state, by for example complexation into heavier molecular weight structures. The potential of the macrofauna to degrade tannins either by enzymes produced by themselves or by gut microorganisms is poorly studied. Microbial symbionts in the digestive tract have been reported for both diplopods (Konig, 2006) and gastropods (Walker et al., 1999). More specifically, among the several bacteria genera found in the gut of *G. marginata* (Byzov et al., 1993), *Klebsiella* is actually reported to be able to degrade tannic acid (Deschamps et al., 1980), *Enterobacter* has the capacity to degrade CT (Bhat et al., 1998), and the actinomycete *Debaryomyces hansenii* apparently produces tannin degrading enzymes (Deschamps, 1989). No data of this kind are available for *P. elegans*.

The second possible fate of CT during gut passage mentioned above, is that they were just not any longer detectable as CT because of oxidation or complexation with other molecules such as other tannins, proteins or glycoproteins (Hagerman et al., 1998). Yu and Dahlgren, 2000 found a positive correlation between insoluble tannins and nitrogen content of conifer foliage, suggesting that complexation of CT with proteins could decrease CT solubility and consequently their detection with the common methods. Other studies emphasized that a large amount of CT could be remaining in the solid state (Makkar et al., 1999; Yu and Dahlgren, 2000; Nierop and Verstraten, 2006) suggesting CT complexation. Both, enzymatic breakdown and complexation of CT during gut passage might have contributed to the apparent disappearance of CT in faeces observed in our study. The relative importance of these two pathways might actually differ between the studied animal species and between litter types, wherefore we cannot entirely refute our second hypothesis without further studies to unravel the underlying mechanisms of CT disappearance.

The observed animal effects on litter CT might have important ecological consequences. In Mediterranean forest ecosystems, *G. marginata* can be highly abundant with up to 188 individuals per

meter square (David, 1999), and representing up to 80% of the total soil macroarthropod biomass (Bertrand et al., 1987; David, 1999). *G. marginata* is estimated to consume up to 50% of the total annual *Q. ilex* litter fall in *Q. ilex* dominated forests. Our results suggest that the transformation of such significant amounts of leaf litter into faecal pellets fundamentally changes either the quantity or quality or both of CT at the litter–soil interface. Consequently, the strong tannin control on litter decomposition rates and nutrient dynamics observed when macrofauna is excluded or not abundant (e.g. Northup et al., 1995; Driebe and Whitham, 2000), is likely modified in ecosystems where a large quantity of litter is consumed by soil animals.

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