

# Ecologie chimique du milieu édaphique: interactions entre deux moisissures *Mucor* sp. et *Geotrichum candidum* et le ver rouge *Eisenia fetida* (Oligochaeta, Lumbricidae)

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## 1. Résumé du projet FRIA

Ce projet sera réalisé dans le but de comprendre les interactions existant entre les moisissures *Mucor* sp. et *Geotrichum candidum* d'une part, et le ver rouge *Eisenia fetida* d'autre part. En effet, la réalisation de mon travail de fin d'études a permis de mettre en évidence que le filtrat de culture de ces moisissures était significativement ( $p$ -value inférieure à 0,001) attractif pour *E. fetida*. La compréhension de cette attraction permettrait d'optimiser l'utilisation d' *E. fetida* dans les domaines de la lombriculture et de la bioremédiation.

La première étape sera de mettre au point deux olfactomètres, un olfactomètre à 4 voies et un olfactomètre compartimenté (Figure 1) et de les valider afin de déterminer leur efficacité à étudier le comportement d' *E. fetida* vis-à-vis des filtrats de culture de moisissures.



Figure 1: Olfactomètres à 4 voies (à gauche), et compartimenté (à droite)

La deuxième étape consistera à rechercher la molécule synthétisée par les moisissures qui est responsable de l'attraction d' *E. fetida*. Dans cette optique, trois pistes seront investiguées: celle des molécules organiques volatiles, celle des protéines, et celle des autres métabolites secondaires.

La troisième étape sera l'étude de la phylogénie de *Mucor* sp. et de *G. candidum* afin d'établir un arbre phylogénique des organismes qui produisent la même molécule attractive que les moisissures d'intérêt. Les microorganismes les plus apparentés seront testés en olfactométrie sur le comportement d' *E. fetida*.

L'étape suivante consistera à optimiser la production du métabolite d'intérêt.

Enfin, deux applications industrielles seront envisagées: la mise au point d'un système d'extraction d' *E. fetida* dans le cadre du lombricompostage, et d'un système de bioremédiation d'un sol contaminé.

## 2. Déroulement du doctorat depuis mai 2008

### 2.1. Interaction vers de terre – moisissures du sol

Pour rappel, *Eisenia fetida* est attiré par le jus de culture de *G. candidum*. Afin de comprendre cette attraction, les molécules volatiles du jus de culture de cette moisissure du sol ont été étudiées. Deux techniques de prélèvement ont été utilisées : prélèvement dynamique sur cartouche super Q, et prélèvement passif par SPME avec une fibre CAR/PDMS.

- Prélèvement dynamique

Après mise au point de la méthode de prélèvement et d'analyse en CPG, les molécules piégées sur la cartouche de Super Q sont éluées avec du dichlorométhane et sont identifiées en GC/MS sur colonne apolaire. Les 17 molécules détectées sont reprises dans le tableau 1.

Tableau 1: Identification des molécules volatiles de *G. candidum* par GC/MS après prélèvement sur cartouches Super Q

Molécules	Tps de rétention
3-hydroxy-2-butanone	3,28
Ethyl propionate	3,37
<b>3-méthylbutan-1-ol</b>	3,9
<b>2-méthylbutan-1-ol</b>	3,98
Molécule n.i.	4,44
Molécule n.i.	4,58
Molécule n.i.	5,11
Molécule n.i.	5,19
Molécule n.i.	5,83
Ethyl butanoate	6,06
Molécule n.i.	7,51
Ethyl isovalerate	7,56
Molécule n.i.	9
Molécule n.i.	10,9
Molécule n.i.	10,88
Molécule n.i.	12,58
Molécule n.i.	14,33

L'analyse des résultats montre que seulement 6 molécules ont pu être identifiées et que le 2-méthylbutan-1-ol et le 3-méthylbutan-1-ol sont présentes dans toutes les répétitions. Ces résultats peuvent s'expliquer par la présence seulement en trace des molécules.

- Prélèvement passif

Pour le 1<sup>er</sup> rapport, un essai préliminaire de prélèvement SPME avec analyse GC/MS avait été réalisé. Après affinement de la méthode de prélèvement et du programme de température GC/MS, plusieurs répétitions ont été réalisées avec une fibre CAR/PDMS et une colonne apolaire Factor Four sur le GC/MS. Les 18 molécules détectées sont présentées dans le tableau 2.

**Tableau 2: Identification des molécules volatiles de *G. candidum* par GC/MS après prélèvement par SPME**

<b>Molécules</b>	<b>Tr</b>
Acetic acid, ethyl ester	2,37
<b>2-méthylpropan-1-ol</b>	2,52
Ethyl propionate	3,38
<b>3-méthylbutan-1-ol</b>	4,08
<b>2-méthylbutan-1-ol</b>	
<b>Propanoic acid, 2-méthyl, ethyl ester</b>	4,28
Molécule n.i.	4,33
Molécule n.i.	4,53
<b>Butanoic acid, ethyl ester</b>	5,09
<b>2-Butenoic acid, ethyl ester</b>	6,07
<b>Butanoic acid, 2-méthyl, ethyl ester</b>	6,16
<b>Butanoic acid, 3-méthyl, ethyl ester</b>	6,28
<b>Ethyl pentanoate</b>	7,54
<b>2-butenoic acid, 3-méthyl, ethyl ester</b>	8,22
<b>2-butenoic acid, 2-méthyl, ethyl ester</b>	8,64
3-octanone	10,03
Hexanoic acid, ethyl ester	10,39
<b>2-hexenoic acid, ethyl ester</b>	11,77

Les molécules en caractère gras sont celles qui étaient également présentes dans l'essai préliminaire.

Deux témoins (milieu de culture sans moisissure et vial sans échantillon) ont été réalisés et permettent d'affirmer que ces molécules volatiles sont bien produites par la moisissure.

- Analyse comportementale du ver de terre

Les molécules identifiées sont ensuite testées dans l'olfactomètre à 4 voies afin de déterminer la ou lesquelles sont impliquées dans l'attraction des vers de terre.

Jusqu'à présent, 3 molécules ont été testées (10 répétitions) :

- 2-méthylbutan-1-ol : pas d'attraction
- 3-méthylbutan-1-ol: pas d'attraction
- 3-octanone: pas d'attraction

## 2.2. Application industrielle

Une des applications industrielles proposée dans la réalisation de cette thèse de doctorat est la mise au point d'un système d'extraction d' *E. fetida* dans le cadre du lombricompostage.

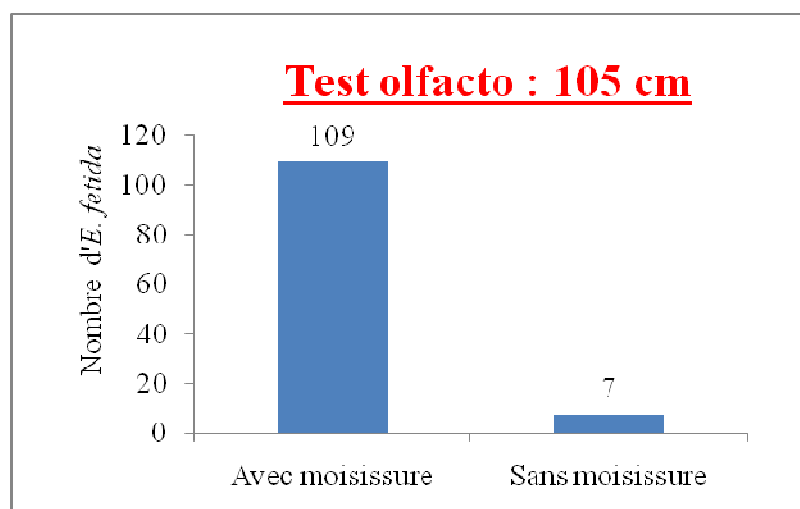
Des expériences ont été réalisées sur la distance à laquelle les vers pouvaient être attirés et sur l'influence de la densité des vers sur l'attraction du jus de culture de *G. candidum*.

- Distance



**Figure 2 : Olfactomètre vertical**

Un olfactomètre vertical (Figure 2) a été mis au point afin de déterminer si les vers étaient capables de monter dans le tube quand le jus de culture était présent et jusqu'à quelle distance. Trois distances ont été testées: 25cm, 42cm et 105cm. Le comportement des vers a été étudié en présence (25ml de jus de culture dans le haut de l'olfactomètre) et en absence de jus de culture afin de pouvoir faire une comparaison. Après 24h, le nombre de vers dans les 5cm de la partie supérieure de l'olfactomètre est déterminé. Pour la distance de 105cm, les vers sont comptés après 65h. 18 répétitions ont été réalisées pour chaque traitement (avec ou sans moisissures). Les résultats obtenus pour la distance de 105cm sont représentés par la figure 3.



**Figure 3: Attraction de 10 vers dans un olfactomètre vertical de 105 cm en présence et en absence de *G. candidum***

L'analyse des résultats montre qu'*E. fetida*, un ver de terre épigé se déplaçant naturellement horizontalement, est capable de modifier son comportement face à une source de nourriture attractive et ce sur une distance de 1m. En absence de source de nourriture, le ver n'atteint pas la partie supérieure de l'olfactomètre.

Ces résultats sont particulièrement intéressants pour la mise en place du système d'extraction des vers de terre hors du lombricompost. On pourrait imaginer qu'après lombricompostage, des tubes contenant la moisissure soient placés à la surface du lombricompost afin que les vers montent dans ces tubes et puissent ainsi être récupérés.

- Densité des vers

Six densités de vers (5, 10, 20, 40, 80 et 160 vers) ont été étudiées dans les olfactomètres à 4 voies afin de déterminer l'impact de la densité sur l'attraction des vers par le jus de culture de *G. candidum*. Les vers sont placés au centre de l'olfactomètre après que 25ml de jus de culture aient été placés dans un des bras de l'olfactomètre. Le reste du système est rempli avec du terreau. Après 24h, le nombre de vers dans chaque bras est déterminé. Quelle que soit la densité, l'attraction des vers de terre par le jus de culture de *G. candidum* est vérifiée (test  $\chi^2$  d'ajustement,  $p = 0,000$ ). L'impact de la densité sur cette attraction est alors évalué par une analyse de la variance à un facteur. Le pourcentage moyen de vers présent dans le bras de l'olfactomètre contenant la moisissure est déterminé pour chaque densité (Tableau 3).

**Tableau 3: Pourcentage moyen de vers ayant choisi le bras de l'olfactomètre traité avec *G. candidum* en fonction de la densité de vers de départ**

Densité des vers	Pourcentage des vers dans le bras traité (%)
5	72
10	61
20	53,1
40	64,5
80	56,3
160	75,1

L'analyse statistique montre qu'il y a une différence significative du pourcentage de vers dans le bras traité en fonction de la densité. Une structuration de moyenne met en évidence que les densités de 5, 10, 40 et 160 vers donnent les plus grands pourcentages de vers dans le bras de l'olfactomètre avec moisissure.

### 2.3. Agrégation des vers de terre

Comme mentionné dans mon premier rapport, le comportement agrégatif des vers est devenu mon deuxième axe de recherche sur *Eisenia fetida*.

Dans cette optique, plusieurs expériences ont été réalisées afin de déterminer si les vers de terre *E. fetida* avaient bien un comportement grégaire et quels mécanismes (piste ou contact) pouvaient être à l'origine de ce comportement. Pour répondre à ces deux questions, des expériences ont été menées dans deux olfactomètres à deux voies. Les résultats ont fait l'objet d'une publication "A new case of consensus decision: collective movement in soil" soumise dans Plos One. En voici l'abstract :

“Collective movements are reported for many species from microorganisms to humans. But except for a few soil inhabiting species, intra-specific interactions are poorly studied. Virtually nothing is known about social behavior in earthworms that represent the dominant biomass of the soil. This study, the first one on Annelids, highlights a consensual decision phenomenon based only on contact between followers.

Using olfactometer set-up and modeling, we show that earthworms *Eisenia fetida* influence each other by contact to select a common direction during their migration. This coordinated movement would allow earthworms to locate food sources more efficiently and leads to aggregation. The resulting local higher densities enhancing individual survival and favoring the cooperation may be at the origin of Allee effects reported for these species.”

La publication est jointe en annexe.

### 3. Formation doctorale

- Séminaire sur la spectrométrie de masse organisé par Interscience: "Mass spectrometry ". Le 10 juin 2008 de 9h30 à 16h à Louvain-la-Neuve. **1 crédit**
- Conférence de Fiona L. Goggin: "Plant-aphid interaction: molecular and ecological perspectives". Le 25 août 2008 de 11h à 12h à Gembloux. **1 crédit**
- Intervention d'une heure dans le cours de Zoologie appliquée (Zg 202) concernant "La faune du sol" (+ préparation du cours). Le 25 septembre 2008 à Gembloux de 8h à 9h. **1 crédit.**
- Participation à " La nuit des chercheurs" à Liège: présentation d'un poster sur le lombricompostage ménager et préparation d'un vivarium explicatif. Le 26 septembre 2008 de 16h30 à 22h. **2 crédits.**

- Présentation de l'intro TP sur les Annélides aux 1<sup>er</sup> Bacheliers (1h + préparation du cours) et encadrement de 12h de travaux pratiques sur la dissection et les coupes du ver de terre. Le 7 novembre et du 17 au 19 novembre 2008. **3 crédits**
- Présentation de l'état d'avancement de mes résultats au Conseil d'administration de la société Ouroboros. Le 14 octobre 2008 à Gembloux. **3 crédits**.
- Participation au 15<sup>e</sup> Congrès Benelux de Zoologie à l'Université de Liège. Présentation orale "Social behaviour in *Eisenia fetida* (Oligochaeta, Lumbricidae). Le 30 et 31 octobre 2008. **5 crédits**.
- Encadrement didactique d'enfants de l'atelier "Art des Sciences". Le 10 décembre 2008 de 13h30 à 17h à Gembloux. **1 crédit**.
- Présence à la séance publique de la Classe des Sciences au Palais des Académies à Bruxelles. Le 13 décembre 2008 de 15h à 16h30. **1 crédit**.
- Acceptation de l'article "Mise en relation de la diversité des vers de terre et des caractéristiques du sol de Thua Thien Hue (Centre Vietnam)" dans la revue Open Access Tropical Conservation Science. **8 crédits**.
- Chaire Francqui au titre belge 2008-2009. Professeur Jean-Louis Deneubourg. **2 crédits**
  - Leçon inaugurale: "Peut-on vivre en société avec des robots?: auto-organisation dans les systèmes naturels et artificiels". Le 11 mars de 16h à 18h.
  - Leçon 1: "Décisions collectives dans les sociétés d'insectes". Le 13 mars de 9h à 12h.
  - Leçon 2: "Communications et systèmes animaux robots". Le 20 mars de 9h à 12h.
  - Leçon 3: "Un pas plus loin que l'agrégation". Le 6 mai de 16h à 18h.
- Conférence d'Anne-François Schmid: "L'éthique appliquée. Une réponse aux enjeux contemporains. Nouvelles méthodes en éthique technologique" au Palais des Académies à Bruxelles. Le 26 mars de 16h à 18h. **1 crédit**.
- Participation au Workshop de clôture du projet PIC "Improving food crop productivity in the coastal sandy area of the Thua Thien Hue province Central Vietnam". Présentation orale: " Diversité des vers de terre dans les sols de la zone côtière sableuse de la province de Thua Thien Hue (Vietnam)". Le 8, 9 et 10 avril 2009 à Hué (Vietnam). **5 crédits**.

- Article "Valorisation of a water hyacinth in vermicomposting using an epigeic earthworm *Perionyx excavatus* in Central Vietnam" soumis dans la revue Bioresource Technology. (En attente)
- Article "A new case of consensus decision: collective movement in soil" soumis dans la revue open access Plos One. (En attente)



## Récapitulatif des activités de la formation doctorale depuis le début de la thèse

<b>Formation doctorale</b>	<b>Crédits</b>
<b>Formation transversale</b>	
Encadrement d'un stagiaire/mémorant Wladimir Fanali	5
Cours de méthodologie documentaire	1
Représentation de la fonction de bioingénieur au Forum aux professions	1
Reviewer d'un article pour BASE	2
Prix AIGx	1
Nuit des chercheurs à l'Ulg (Poster et vivarium)	2
Intervention dans le cours de Zoologie appliquée Zg 202	1
Intro TP sur les Annélides + 12h de travaux pratiques	3
Encadrement didactique de l'atelier Art et Sciences	1
Séance publique de la Classe des Sciences	1
Conférence sur "l'éthique de l'ingénieur" au Palais des Académies	1
<b>Total</b>	<b>19</b>
<b>Formation thématique</b>	
Chaire Francqui au titre belge 2007-2008, Willy Verstraete	2
Séminaire de Damien Charabidze	1
Séminaire de Michel Cusson	1
Séminaire sur la spectrométrie de masse (Interscience)	1
Séminaire de Fiona Goggin	1
Chaire Francqui au titre belge 2008-2009, Jean-Louis Deneubourg	2
<b>Total</b>	<b>8</b>
<b>Production scientifique</b>	
Présentation des résultats de mon doctorat à Ouroboros	3
15e Congrès Benelux international de Zoologie - présentation orale	5
Article dans Tropical Conservation Science	8
Workshop de clôture d'un projet PIC au Vietnam - présentation orale	5
<b>Total</b>	<b>21</b>

**Total**

**48**

Le nombre crédits attribués aux différentes activités l'a été conformément aux recommandations du FNRS "Valorisation des activités liées au doctorat et à la formation doctorale ED/CA/07/3 et du règlement interne de la faculté.

## **Annexe**

### **A new case of consensual decision: collective movement in soil**

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## Abstract

Collective movements are reported for many species from microorganisms to humans. But except for a few soil inhabiting species, intra-specific interactions are poorly studied. Virtually nothing is known about social behavior in earthworms that represent the dominant biomass of the soil. This study, the first one on Annelids, highlights a consensual decision phenomenon based only on contact between followers.

Using olfactometer set-up and modeling, we show that earthworms *Eisenia fetida* influence each other by contact to select a common direction during their migration. This coordinated movement would allow earthworms to locate food sources more efficiently and leads to aggregation. The resulting local higher densities enhancing individual survival and favoring the cooperation may be at the origin of Allee effects reported for these species.

## Introduction

Unlike inter-specific interactions [1,2,3,4], intra-specific interactions in soil are poorly studied [5,6]. For example, little information about the chemical and social behavioral ecology of earthworms is available, therefore earthworms represent up to 70% of soil biomass [7]. The spatial heterogeneity of the population depends on food distribution, abiotic parameters but also intra-specific interactions including inter-attraction that is likely to generate an Allee effect [8] and collective movement [9].

In organisms living in groups [9], collective movements are a widespread phenomenon, being the keystone of other social activities such as aggregation [10,11,12,13,14]. Collective movements involve many associated questions about the decision to take: to move or to stop? Which direction to choose? How the individuals are distributed among the alternative? ...

The mechanisms underlying such group decisions remain poorly understood. Two extreme cases are identified: the leadership and consensual mechanisms [9,15]. In case of leadership one individual decides for the entire group. In case of consensual mechanisms the collective decision results from the mutual influence between individuals.

In consensual decision, the diversity of collective decision largely depends on the mechanisms and on the species. In some cases, all the individuals having previously moved and chosen influence the follower decision (trail recruitment in ants or silk following in spiders) [16,17].

In some other cases, short range interactions govern the individual behaviour: an individual only influences its close neighbours or followers (tandem recruitment in ants, social caterpillars and lobsters) [16,18,19]. Moreover the response of individuals to the signal emitted by their conspecific could also implicate: A non-linear response to the signal favoring a strong asymmetry between the alternatives [20]

In many species such as social caterpillars, the mutual influence by physical contact between close neighbors seems involved in collective decision-making, this influence being associated

to other signal like trail or silk [19]. However such social cues are poorly studied.

Earthworms evade unfavorable environmental conditions like flooded soil and low oxygen tension or to seek new habitat [21]. Sometimes, the number of earthworms migrating is especially significant, suggesting that social cues may be responsible for collective movement [11,22]. For example, noxious stimulation of earthworm elicits secretion acting by contact as an alarm pheromone [23,24]. Here, we investigated whether chemical and physical cue are involved in collective movement in earthworms.

## Results and discussion

To determine whether interaction exists between individuals of *Eisenia fetida*, their behavior was observed in a 2-arm olfactometer (Figure 1a) filled with moist (20% water) compost and the same food source was present at the both extremities. *Eisenia fetida* earthworms were released in the centre of the olfactometer, where they were free to enter the arms up to their extremities. One day after being released, the number of earthworms was recorded in each arm.

### *Zirbes\_fig 1*

Figure 2a shows the distribution of the total number of earthworms leaving the central chamber of the olfactometer. This distribution was compared to a binomial theoretical distribution which should be observed when no interaction exists between individuals. Among the 1200 tested earthworms, 1005 left the central chamber. The theoretical binomial distribution was then generated with an estimated probability of leaving of 0.84 (1005/1200) that an individual leaves the central chamber. In this case, experimental distribution was not different from the theoretical distribution (Chi-square Goodness-of-fit test,  $\chi^2_4 = 4.09$ ,  $p = 0.394$ ).

The second analysis has concluded that the distribution of the choice of one arm (right or left) was significantly different (Deviance test,  $\chi^2_{29} = 71.05$ ,  $p < 0.001$ ) from the binomial distribution with a trend toward statistical overdispersion. These results strongly imply dependence between the choices of a single individual earthworm (Figure 2b).

These results show that a single earthworm moving out of the central chamber influences the directional choice of other earthworms, but not their propensity to leave the central chamber. A similar behavior was observed with another kind of worm, namely the nematode *C. elegans* [25].

To understand mechanisms of these earthworms' interactions, two hypotheses were tested: (i) chemical trail following and (ii) contact between animals. Both these social behaviors are commonly spread in animal kingdom [26]. In this experiment, a second set-up was used (Figure 1b) which required earthworms to select between two Plexiglas branches of this behavioral set-up from a starting point to reach the food source. This set-up was filled with moist (20% water) compost. To determine whether earthworms follow a conspecific trail, two behavioral trial tests were used. First, one *Eisenia fetida* (N = 45) earthworm was placed at the starting point and was free to choose a direction (right or left). Upon arriving at the food source, the earthworm was removed from the set-up, and a new individual was then placed at starting point and its direction was recorded. In these conditions: 46.7% of the time, earthworms chose the same direction as the previous one and 53.3% of time not. The results are not significantly different from 50% - 50% (Chi-square Goodness-of-fit test,  $\chi^2_1 = 0.2$ ,  $p = 0.655$ ). The second trial test was conducted in the same conditions as previously. However, five *E. fetida* earthworms (N = 10) were forced to move in the same direction (right or left) and the direction of the sixth earthworm was recorded. In this case, five earthworms have

followed their predecessors and five earthworms not. These results indicate that the earthworms did not follow a conspecific trail.

To establish whether or not contact between *E. fetida* earthworms influences *E. fetida* interaction, two earthworms (N = 45) were simultaneously placed at the starting point (Figure 1b). In this experiment seventy percent moved together while thirty percent of the pairs did not select the same direction. The directional selection of earthworm pairs indicated a highly significant difference (Chi-square Goodness-of-fit test,  $\chi^2_1 = 7.53$ ,  $p = 0.006$ ) between experimental and theoretical distributions, if contact had no impact on behavior (50% of pairs of earthworms do not select the same direction and 50% of pairs move together) (Figure 2c). The contact between earthworms strongly influences the directional choice of the follower. Indeed, the probability of following the predecessor (Q) is estimated to be 0.7.

#### *Zirbes\_fig 2*

A simple model shows that such probability of following generates the earthworm distribution among both branches when groups of 40 individuals were tested in the 2-arms olfactometer. Forty earthworms were given the choice of leaving the central chamber and choosing between two identical branches. The individual probability of leaving the central chamber (P) is equal to 0.84. The first individual probability of choosing the left ( $P_L$ ) or right branch ( $P_R$ ) is equal to 0.5. For the others,  $P_L = Q$  ( $P_R = Q$ ) if its predecessor chooses the left (right) branch, Q being the probability of following its predecessor and choosing the same direction. In case of social attraction, the probability of following its predecessor (Q) is  $> 0.5$  (Figure 2a). The number of earthworms selecting the specific choicer branch was counted at the end of the simulation. The mean distribution of 30 experiments average was calculated (Figure 3a). The probability to obtain an equivalent average number of earthworms on the choicer branch to experimental data (mean = 20.27) was maximal with the Q value resulting from the pair experiment (Q =

0.7). With weaker social interaction, ( $Q < 0.6$ ) this probability was  $< 0.05$  and without social interaction the probability was slight ( $= 0.002$ ). Our simulation has shown that contact explains cooperation observed between *E. fetida* earthworms. We have also showed that the average fraction of earthworms on the choice branch abruptly decreases as the number of earthworms increases for each Q value (Figure 3b) (See supporting information for equation development)

*Zirbes\_fig 3*

We have demonstrated the *E. fetida* earthworms' cooperative capacity to select direction through contact among individuals. To our knowledge this is the first example of collective movement in animals based only on contact between followers and also the first one of collective movement in Annelids. It is well known that earthworms use tactile receptors on the surface body for thigmotactic responses [7]. Moreover, earthworms have chemoreceptors principally on the prostomium or on the buccal epithelium [22]. Contact due to surface secretion of earthworms has a quieting influence on other earthworms of the same species [27]. Collective movement mediated by chemical and/or physical contact has been observed in a large diversity of invertebrates [19,28]. *Eisenia fetida* seems to use the same strategy to cooperate with conspecifics.

This collective movement could allow earthworms to locate scarce or distant food sources more efficiently [25,29]. Indeed, Darwin in 1881 has shown that earthworms could distinguish readily between different food substances [30].

The collective movement could also be a mechanism leading to aggregation. The aggregation could enhance chemical defenses [6]. For the earthworm *Eisenia fetida*, the defensive line comprises cells floating in coelomic fluid and humoral effector proteins secreted into the coelomic fluid. This body fluid is known to contain a variety of cytolytic and bacterial



activities to combat potential soil pathogens [31]. *E. fetida* are also the prey of terrestrial flatworms. *E. fetida* secrete yellow coelomic fluid in response to flatworm, *B. adventitium* attack producing an aversive response by the flatworm thus increasing the survival rate of this earthworm specie [32]. Aggregations of earthworm could produce greater amounts of defensive compounds thus providing better resistance to soil pathogens and predators. Such cooperative defense behavior would be particularly vital when only some earthworms are under high predation pressure. Moreover, positive interactions exist between hatchlings and conspecific adults during the development [33,34]

These results strongly suggest a positive relationship between individual fitness and density and may explain that an Allee effect is present in earthworms [8].

## Materials and Methods

### *Eisenia fetida* rearing

Earthworms, *E. fetida* (see supporting information) used in this study come from vermicomposting Ouroboros s.a. (Belgium). They are reared in PVC box (42 cm long, 30 cm wide and 10 cm high) filled with universal compost. This compost is changed every two months and cocoons and hatchling earthworms are sorted out in order to be released in a new box with fresh compost. During these experiments, only mature earthworms (with clitellum) were used.

### *Binary choice experiments*

Binary choice experiments remain one of the easiest ways to study the collective decision-making and to analyze the relation between the individual behavior, and the collective decision-making. The distribution of the number of individuals among both alternative may be different according the species and situation: it varies from an equi-distribution to a strong difference between the number of individuals choosing both directions.

### *Olfactometer assays*

The behavior of earthworms was tested in a belowground 2-arms olfactometer consisting of a central PVC chamber (20cm x 20 cm x 20cm) with two equally distributed side arms (9 cm in diameter, 18 cm deep) (Figure 1a). These arms connected to the central chamber where food sources could be placed. For each experiment, the entire system was filled with moist (20% water) universal compost DCM ®. 40 earthworms were released in the centre of the central chamber. One day after release, the arms of each olfactometer were disassembled from the central chamber, the compost in each arm was placed in container and earthworms were

counted. Thirty repetitions were conducted and the olfactometer was washed with water between each repetition.

#### *Contact/ trail assays*

A diamond-shaped set-up (15 cm long, 10 cm wide, 5 cm high) with start and end point (3cm long, 1,5 cm wide, 5 cm high) (Figure 1b) was used to study contact and trail following for *E. fetida* mature earthworms. This set-up is filled with 56.25 cm<sup>3</sup> of universal compost DCM®. This experimental set-up was emptied and washed with clear water between each repetition.

In trail-following assay, two experiments were performed. In first case, one earthworm was placed on start point set-up and it is free to choose a branch; it is then removed when it reaches the end point with food source. After that, a second earthworm was released on start point and its choice was observed. This experiment was repeated 45 times. The two possibilities for earthworms were to move in the same direction or not. In the second case, five earthworms (one after the other) were force to move on the same branch set-up and the choice of the sixth one was recorded. The experiment was repeated 10 times.

In contact trial tests, two earthworms were placed at the start point of the set-up and both selected direction were observed and recorded when they reach end point filled with food resources. This experimentation was repeated 45 times. Two results could be observed: (1) both earthworms on the same direction (right or left), and (2) one earthworm on the left and one on the right.

#### *Statistical analyses*

A Chi-square Goodness-of-fit test ( $N = 30$ ,  $\alpha = 5\%$ ) was used to compare distribution of the number of earthworms in both arms versus the central chamber of the 2-arms olfactometer and a binomial distribution. Among the 1200 tested earthworms (30 earthworms x 40

replicates), 1005 left the central chamber. This binomial distribution was generated with an estimated probability of 0.84 (1005/1200), based on the proportion of choices observed on the whole experiment, that earthworms are independents. The results were not biased by experimental set-up: at the end of 30 repetitions, the same number of earthworms was not significantly different in both arms of the olfactometer (right = 521, left = 484).

Statistical differences in direction selection by earthworms were determined by fitting a constant probability generalized linear model on the data. The generalized linear model was used to take into account the non-constant number of earthworms in both arms for the thirty repetitions of the test. Under the assumption of independence of choices of the individual worms, the residual deviance of this model should follow a Chi-square distribution with 29 degrees of freedom [35]. An overdispersion test was then realized to check this assumption (R 2.7.1).

For trail and contact assays, a Chi-square Goodness-of-fit test ( $N = 45$ ,  $\alpha = 5\%$ , 1 degree of freedom) was used to compare theoretical distribution (Respectively, 50% - 50% for each possibilities) and observed distribution.

### *Simulation*

At the start of a simulation, all the individuals ( $N$ ) are in the central chamber. For each simulation,  $N$  earthworms were given the choice of leaving the central chamber and choosing between two branches. The individual probability of leaving the central chamber ( $P$ ) is equal to 0.84. The individual decision of leaving the chamber was checked and depends on the comparison between  $P$  and a random number sampled from a uniform distribution between 0 and 1. If its value is  $\leq P$ , the individual leaves the chamber and its selection of direction is tested.  $P_L$  and  $P_R$  are respectively the probability of choosing the left or right branch. If the value of a second random number is  $\leq P_L$  the individual chooses the left branch. If not, it takes

the opposite direction. For the first individual living the chamber,  $P_L = P_R = 0.5$  (the two branches of the experimental set-up are identical).

For the others,  $P_L = Q$  ( $P_R = Q$ ) if its predecessor chooses the left (right) branch,  $Q$  being the probability of following its predecessor and choosing the same direction.  $Q = 0.5$  corresponds to a random choice (no social influence). In case of social interaction,  $Q > 0.5$  corresponds to a social attraction and  $Q < 0.5$  corresponds to the situation where the earthworm prefers to take the opposite direction to its predecessor.

The percentage of earthworms that chose the winner branch was counted at the end of the simulation. For both hypotheses (with or without social interaction), the possible outcome of the average from 30 experiments and their distribution was calculated 1000 times (Figure 3a).

The simulation was also used to estimate the mean number of earthworms selecting the winner branch as a function of the number of earthworms choosing one direction and of the value of  $Q$  (Figure 3b).

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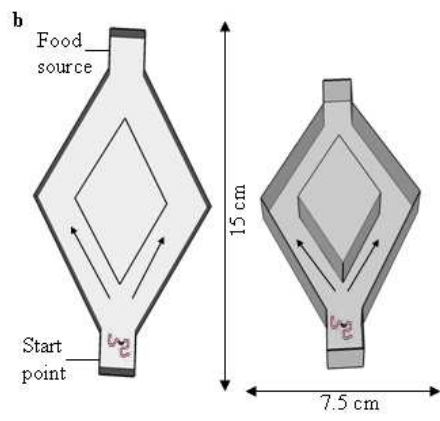
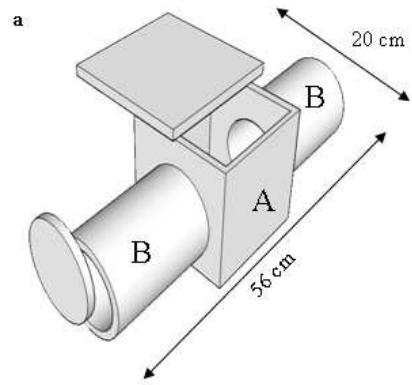
**Authors' contributions** L.Z. conceptually designed and coordinated all experimental works, conducted most measurement, and wrote the manuscript. E.H. has supervised the study. J.L.D. and Y.B. made all modeling, simulation and statistical analysis.

**Figure 1** Experimental set-up. **a**, 2-arms olfactometer to earthworms' cooperation assays. A = central chamber, B = arms of the olfactometer. **b**, behavioral set-up to trail following and contact bioassays. Start point = point where earthworms were placed at the beginning of experimentations, food source = destination of earthworms.

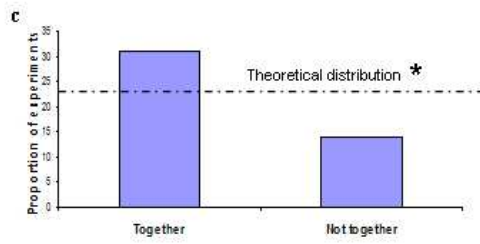
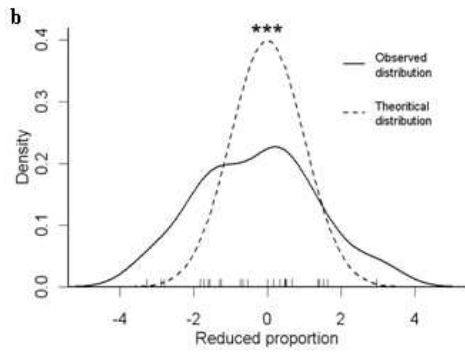
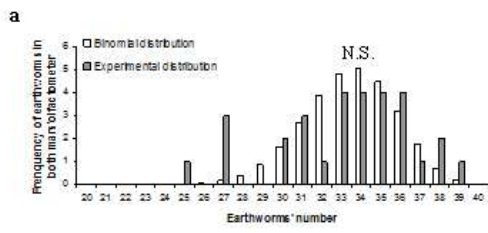
**Figure 2** Experimental results. **a**, Observed (grey) and theoretical (white) distributions of *Eisenia fetida* earthworms in both olfactometer arms. N.S. (Not Significant) indicates no significant difference between observed and expected distribution. **b**, Theoretical (discontinuous stroke) and experimental distributions (continuous stroke) of the proportion of earthworms on the left olfactometer's arm. Variable "proportion of earthworms on the left" was reduced. This reduced distribution tends to a reduced normal distribution by Moivre's theorem. So this figure supersedes the density of reduced observed earthworms' proportions in left arm and reduced normal distribution. Bars at the bottom of chart represent reduced observed proportions that we used to calculate the density of probability. Triple asterisk indicate significant difference between observed and expected distributions at  $p < 0,001$  **c**, Observed distributions of the proportion of earthworm which select a direction together or not in behavioral bioassays when two earthworms were in contact to start experiment. Single asterisk indicates significant difference between observed and theoretical distributions at  $p < 0.05$ .

**Figure 3** Simulation results. **a**, Analytical results for parameter values  $P = 0.84$ ,  $Q = 0,5$  (blue) and  $Q = 0,7$  (green). These distributions of the earthworm number in the winner of the 2-arms olfactometer are generated after 10.000 runs of 30 manipulations. **b**, The curves show the average number of earthworms in winner branch for different earthworms density and for different probability to follow its predecessor.

Zirbes\_fig 1



# Zirbes\_fig 2



Zirbes\_fig 3

