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# Growth, reproduction and recruitment of *Eisenia andrei* in natural substrates: A functional approach

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*A mis padres,*
*por estar siempre ahí*
*the wind beneath my wings*

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## <u>Abbreviation list</u>

**AP**          Asymptotic Point
**C/N**         Carbon to Nitrogen ratio
**CC**          Coconut Coir
**CI**          Confidence Intervals
**DF**          Degrees of Freedom
**DW**          Dry Weight
**EP**          *Eucalyptus globulus* Plantation
**F**           Fibre
**FM**          Field Method
**FQR**         *Quercus robur* Forest
**GS**          Geometric Surface
**GV**          Geometric Volume
**HM**          Horse Manure
**ISR**         Initial Size Range
**L**           Length
**LM**          Laboratory Method
**LM$_{nh}$**   Laboratory Method Newly Hatched worms
**LM$_p$**      Laboratory Method Population (excluding hatchlings)
**LW**          Live Weight
**LWC**         Live Weight at clitellum appearance
**ΔLW**         Biomass Variation
**MBC**         Mean Biomass per Cocoon
**MET**         Mean Eclosion Time
**MIB**         Mean Individual Biomass
**MIC**         Mean Individuals per Cocoon
**MIS**         Mean Initial Size
**MLW**         Maximal Live Weight
**MMS**         Mean Maximal Size
**MVC**         Mean Variation Coefficient
**NFO**         Non-Fibre Organic
**OM**          Organic Matter
**P$_{O2}$**    Oxygen Partial Pressure
**PS**          Photographic Surface
*r*             radius in chapter 2
**r**           Growth Rate in the rest of chapters
**RH**          Relative Humidity
**S/V**         Surface to Volume ratio
**SC**          Scrub
**SCH**         Soluble Carbohydrates
**SD**          Standard Deviation
**t**           Time
**TMS**         Time Maximal Size
**TPC**         Total Polyphenolic Content
**VC**          Variation coefficient
**VO$_2$**      Oxygen Consumption

# <u>Content</u>

# Summary

This thesis deals with two factors that alter the physiological performance of the epigeic earthworm *Eisenia andrei*: isolation and population density; and diet. As regards the first, two culture methods were employed, grouped and isolated, and a technique, tagging with fluorescent elastomers was standardized (Chapter 3) to allow individual identification within the group-cultured organisms. Isolation and density effects were ascertain by differences in growth and metabolism of *E. andrei* between both culture methods. To assess the influence of the diet on life cycle traits of *E. andrei*, two different kinds of substrates, apart from horse manure, were employed: cut-grass composted for different periods and soil (litter + soil) collected from three different vegetations units (*Eucalyptus globulus* plantation, Forest dominated by *Quercus robur* and scrub dominated by *Crateagus monogyna*).

A photogrammetric method that allows non-invasive biomass measurements compared with gravimetric methods, suitable for every stages of the vital cycle, has been developed and is explained in the second chapter of the present work. This technique has been employed in the following chapters of this thesis for biomass determination. We observed that the relation between Photographic surface (PS) and Live weight (LW) was irrespective of the position of the animal in the photo or the number of pictures taken. Nonetheless, feeding conditions altered this relation, yielding higher PS in starved individuals due to the flattening effect observed in fasted earthworms.

The second technique that has been standardized is tagging earthworms with fluorescent elastomers. We obtained an asymptotic pattern while confronting survival to the size of the individuals, observing a survival percentage of 100 in sizes over 270 mg. Retaining time was surprisingly high since after 3 months 75 % of individuals remained labeled. Therefore, this method allows individual monitoring of earthworms within a population and, as has already been mentioned, has been used in the 3th and 4th chapters of this thesis.

There is a high influence of the presence of co-specific individuals on growth traits. In group-cultured organisms only 89.65 % of specimens showed a positive growth ($\Delta$ live weight > 0) and a change in growth patterns has been observed between both culture methods: isolated worms adjusted primarily to a linear pattern, whereas grouped organisms displayed primarily asymptotic growth curves. In this line, maximal live weights (MLW, in mg) and growth rates (r, mg day$^{-1}$) were higher in individualized organisms: 1453.08 *vs.* 598.52 mg for MLW and 16.53 *vs.* 7.39 mg day$^{-1}$ for growth rate. Part of the ingested energy is sent to reproduction (matting cost, formation of cocoons, etc.) and in this work the asymptotic point of the growth curve (maximal live weight) for group-cultured organisms was related to the detention of clitellum, observing a halt in somatic tissue production when arriving to the reproductive stage. So, the allocation of absorbed energy to reproduction and the lower food availability per individual associated with crowded environments explain the differences in growth rates and patterns between animals kept individualized or in group.

In our research no variation in the relationship between oxygen consumption ($\mu L$ $O_2$ $h^{-1}$) and dry weight (mg) has been observed for both culture conditions. Nevertheless, the elevation remained different being 2.63 and 3.19 in earthworms cultured in group or isolated respectively. This indicates that metabolic level was increased by a factor of 1.21 in isolated individuals. This is explained by the earlier development of reproductive structures and by the intraspecific competition for food and space that take place in group-cultured organisms.

Regarding the experiment with cut-grass composted for different periods, survival of hatchlings decreased linearly with composting period of grass and two groups became evident regarding maximal size attained: 120 mg in grass composted for 1 to 3 weeks (OM % over 80) vs. 60 mg in grass composted for more than 7 weeks (OM % below 77.7). From our results, standing crop of *E. andrei* in grass litter appears determined by the proportion of readily available carbohydrates extracted from plant cells by the assemblage between earthworms and soil microbiota. Impoverishment of quality associated to aging appears linked to the decline of

hydrated, readily extractable sugars within the fibre pool redounding in decreasing survival and growth.

In the experiment with soil belonging to the vegetation units, apart from substrates mention above, two control substrates were employed: Horse manure (HM) and Coconut Coir (CC). Initial characteristics of substrates displayed the highest organic matter percentage in CC, followed by HM and the lowest organic content was obtained in scrub (SC); however, CC displayed the lowest figures for soil respiration (virtually zero). A negative relation was established between substrate respiration ($\mu$L $O_2$ $h^{-1}$ $g^{-1}$) and fibre (% of organic matter), with the exception of HM. Survival percentage differed highly between substrates being 100 %, 90 %, 80 %, 67 % and 60 % in HM, *Eucalytus* plantation (EP), *Quercus robur* forest (FQR), SC and CC respectively. Growth patterns also differed largely among substrates and we obtained average maximal live weights (MLW) of 247, 117, 56, 19 and 10 mg in HM, EP, FQR, SC and CC respectively for hatchlings and 1473, 881, 479, 282 and 100 in HM, EP, FQR, SC and CC respectively for juveniles. Survival of newly hatched specimens and growth of hatchlings and juvenile individuals were positively related to substrate organic matter percentage (except CC) and to substrate respiration ($\mu$L $O_2$ $h^{-1}$ $g^{-1}$). A decrease of the non-fibre organic content (% of dry matter) of substrates and an increase of the proportion of their fiber content (% of organic matter) would account for the lower digestibility of the organic matter for both soil microorganism and earthworms, this relation being enhanced in lignin enriched substrates. Due to the mutualistic relation between earthworms and microbiota, in substrates with high microbial biomass, degradation of the most refractory pool of organic matter would be enhanced and also provides food in the form of microorganisms. So, a decrease of the proportion of easily metabolizable components in soil, with a concomitant reduction in soil microbial content, would reduce live expectancy and growth of these epigeic earthworms.

# Chapter 1

## 1. General introduction /

## Introducción General

# General introduction

## 0. Thesis structure and objectives

The present chapter is the general introduction in which a description of the specie, *Eisenia andrei*, is made from anatomical, physiological and ecological point of view. The last part of this section provides information about the industrial and conservational applications that the studies with this type of animals could offer.

The following 5 chapters are inter-connected either because the same culture conditions have been employed or the same animals have been used. Nevertheless, each chapter is independent, with specific objectives and the structure of the chapter is similar to that found a research paper with abstract, introduction, material and methods, results, discussion and conclusions.

In the chapter 7 of the present work, with the aim to join the information obtained, a general discussion is performed, followed by the chapter 8 in which the main conclusions of the study are presented. References used in this work are summarized in the last chapter.

**This work deals with three main objectives:**

1. Development of two different techniques in order to facilitate and allow for achieving the rest of the objectives of this thesis. In chapter 2 a photogrammetric method to obtain biomass measurements of tubular organisms was developed whereas in the first half of the chapter 3 a tagging method allowing individual monitoring of earthworms was standardized.

   The titles of the chapters comprising this block are: ***"Biomass assessment in annelids: A photogrammetric method suitable for hatchlings and adults developed in Eisenia andrei"*** for the chapter 2 and ***"Individual growth of Eisenia andrei in mesocosm at low density: Tagging with fluorescent elastomers"*** for the chapter 3.

2. Study of the effects of isolation and population density in growth traits and metabolism of *Eisenia andrei* specimens. This block is constituted of two chapters (3 and 4), where

a comparative study of growth dynamics and oxygen consumption was made in earthworms submitted to two culture conditions: grouped and individualized.

The first chapter of this block (Chapter 3) is called ***"Individual growth of Eisenia andrei in mesocosm at low density: Tagging with fluorescent elastomers"***, whereas the title of the second chapter (Chapter 4) is ***"Oxygen consumption of the earthworm Eisenia andrei: Effect of isolation"***.

3.  Study of the effects of the diet, in terms of organic matter quality and quantity, on life cycle traits of *E. andrei* specimens. This section is comprised of 2 chapters (5 and 6), in which the life cycle traits of *Eisenia andrei* specimens where studied in earthworms cultured in different natural substrates, using horse manure as a reference.

In chapter 5 substrates consisting in cut-grass pre-composted for different periods were employed. Substrates used in chapter 6 were soil collected from three vegetations units: *Eucalyptus globulus* plantation, deciduous forest dominated by *Quercus robur* and scrub dominated by *Crataegus monogina*.  The titles of the chapters of this block were: ***"Life cycle traits of Eisenia andrei dwelling in aging grass: An approach to food quality of litter for epigeic earthworms"*** for the chapter 5 and ***"Eclosion, survival and individual growth of Eisenia andrei in soil from three different vegetation units"*** for the chapter 6.

## Specific objectives

**Chapter 2**. *"Biomass assessment in annelids: A photogrammetric method suitable for hatchlings and adults developed in Eisenia andrei".* The objective of this chapter is to standardize conditions leading to obtain reliable quantification of individual biomass, length, surface, volume, and hydration level for long-term surveys of tubular soft-bodied organisms by means of photogrammetric techniques.

**Chapter 3**. *"Individual growth of Eisenia andrei in mesocosm at low density: Tagging with fluorescent elastomers".* This chapter has two objectives:

**Objective 1.** Standardize the use, in *Eisenia andrei*, of fluorescent elastomers as a tagging method, allowing individual monitoring of worms within a population.

**Objective 2.** Identify individual growth dynamics with special emphasis on shift points from juveniles to sub-adults and their connection with density evolution in *E. andrei specimens* reared in horse manure, performing a comparative study between earthworms cultured in group or individualized.

**Chapter 4.** *"Oxygen consumption of the earthworm Eisenia andrei: Effects of isolation".* The objective in this chapter is to ascertain whether the metabolism, measured in terms of oxygen consumption ($\mu L\ O_2\ h^{-1}\ animal^{-1}$), of individuals of the species *Eisenia andrei* is altered by the present of co-specific specimens.

**Chapter 5.** *"Life cycle traits of Eisenia andrei dwelling in aging grass: An approach to food quality of litter for epigeic earthworms".* The objective in this chapter is to test the suitability of mowed grass composted for different periods to support active populations of the epigeic earthworm *Eisenia andrei*, providing information of the nutritional requirements of this species since this approach results in substrates of different organic content (food quantity) and digestibility (food quality).

**Chapter 6.** *"Eclosion, survival and individual growth of Eisenia andrei in soil from three different vegetation units".* In this chapter our aim is to analyze suitability of soil from different vegetation types to support active populations of the earthworm *Eisenia andrei* using soil from *Eucalyptus globulus* plantations, forest dominated by *Quercus robur* and Scrub dominated by *Crataegus monogina* focusing in the nutritional value of their litter layer for epigeic earthworms.

# Introducción general

## 1. Estructura de la tesis y objetivos

Este primer capítulo se trata de la introducción general, en el cual se describe la especie de estudio *Eisenia andrei* (Bouché 1972), desde un punto de vista anatómico, fisiológico y ecológico. En la última sección del capítulo se detallan las aplicaciones tanto industriales como conservacionistas que ofrecen los estudios con este tipo de animales.

A la introducción general le siguen 5 capítulos que están conectados entre sí, bien porque se han mantenido las mismas condiciones de cultivo o bien porque incluso se utilizan los mismos animales. Son, sin embargo, capítulos independientes, cada uno con su estructura propia y dirigidos a cumplir unos objetivos específicos. De esta forma, cada capítulo constituye una unidad autoexplicativa y contiene los apartados de: resumen, introducción, material y métodos, resultados, discusión y conclusiones.

Se ha realizado una discusión general (Capítulo 7), con el objetivo de unificar la información obtenida de los distintos estudios expuestos a lo largo de la tesis, a la cual le siguen las conclusiones finales expuestas en el capítulo 8. En el último capítulo de la tesis se detalla la bibliografía utilizada para la elaboración del manuscrito.

**Este trabajo presenta tres objetivos principales**:

1. Desarrollo de dos técnicas para facilitar y permitir la consecución del resto de objetivos de la tesis. En el capítulo dos, se desarrolla un método de estimación de medidas de biomasa en organismos tubulares basado en técnicas fotográficas, mientras que en la primera mitad del tercer capítulo se realiza una estandarización del uso de elastómeros fluorescentes para el seguimiento individualizado de lombrices.

   Los títulos de los capítulos que conforman este bloque son: *"**Biomass assessment in annelids: A photogrammetric method suitable for hatchlings and adults developed in Eisenia andrei"**, para el capítulo 2, y *"**Individual growth of Eisenia andrei in mesocosm at low density: Tagging with fluorescent elastomers"**, para el capítulo 3.

**2.** Estudio del efecto del aislamiento y densidad poblacional sobre el crecimiento y el metabolismo de individuos de la especie *E. andrei*. Este bloque lo constituyen dos capítulos (3 y 4), en los cuales se realiza un estudio comparativo de las dinámicas de crecimiento y del consumo de oxígeno en individuos sometidos a dos condiciones de cultivo diferentes: en grupo o aislados.

El primer capítulo de este bloque (capítulo 3) se titula ***"Individual growth of Eisenia andrei in mesocosm at low density: Tagging with fluorescent elastomers"***, mientras que el título del segundo (capítulo 4) es ***"Oxygen consumption of the earthworm Eisenia andrei: Effect of isolation"***.

**3.** Estudio del efecto de la dieta, en términos de cantidad y calidad de materia orgánica, en el ciclo de vida de la especie *E. andrei*. Esta sección está formada por dos capítulos (5 y 6), en los que se estudian los parámetros del ciclo de vida de los individuos mantenidos en substratos naturales, usando como substrato de referencia la bosta de caballo.

En el quinto capítulo se utiliza hierba compostada por distintos periodos como substrato mientras que en el sexto se emplean muestras de suelo procedentes de tres unidades de vegetación (plantación de *Eucalyptus globulus*, bosque dominado por *Quercus robur* y matorral dominado por *Crataegus monogyna*). Los títulos de los capítulos que conforman este bloque son: ***"Life cycle traits of Eisenia andrei dwelling in aging grass: An approach to food quality of litter for epigeic earthworms"*** y ***"Eclosion, survival and individual growth of Eisenia andrei in soil from three different vegetation units"*** para el capítulo 5 y 6 respetivamente.

**Objetivos específicos:**

**Capítulo 2**: ***"Biomass assessment in annelids: A photogrammetric method suitable for hatchlings and adults developed in Eisenia andrei".*** El objetivo de este trabajo es estandarizar las condiciones que permitan una cuantificación fiable de medidas de biomasa, longitud, superficie, volumen y grado de hidratación en estudios a largo plazo de animales tubulares de cuerpo blando, utilizando para ello técnicas fotográficas.

**Capítulo 3**: *"Individual growth of Eisenia andrei in mesocosm at low density: Tagging with fluorescent elastomers".* Este capítulo tiene dos objetivos principales:

**Objetivo 1**. Estandarizar el uso de los elastómeros fluorescentes como método de marcaje en poblaciones de *E. andrei*, lo cual permite el seguimiento individualizado de lombrices dentro de una población.

**Objetivo 2**. Identificar los patrones de crecimiento individual de especímenes de *E. andrei* mantenidos en bosta de caballo, con especial atención en las etapas de paso de juveniles a adultos, y su conexión con la evolución de la densidad poblacional. En esta línea, se realiza un estudio comparativo entre individuos mantenidos en grupo o individualizados.

**Capítulo 4**:*"Oxygen consumption of the earthworm Eisenia andrei: Effects of isolation".* El objetivo de este capítulo es determinar el efecto del aislamiento sobre el metabolismo respiratorio, medido en términos de consumo de oxígeno ($\mu$L $O_2$ $h^{-1}$ $animal^{-1}$), de individuos de la especie *E. andrei*.

**Capítulo 5**: *"Life cycle traits of Eisenia andrei dwelling in aging grass: An approach to food quality of litter for epigeic earthworms".* El objetivo es testar la calidad de la hierba compostada por distintos periodos de tiempo como substratos para sostener poblaciones de la lombriz epigea *E. andrei*. Dado que en este estudio se utilizan substratos con distinto contenido de materia orgánica y distintos grados de digestibilidad de la misma, este trabajo proporciona información de los requerimientos nutricionales de esta especie.

**Capítulo 6**: *"Eclosion, survival and individual growth of Eisenia andrei in soil from three different vegetation units".* El objetivo de este capítulo es analizar la idoneidad de muestras de suelo procedentes de distintas unidades de vegetación (plantación de *Eucalyptus globulus*, bosque dominado por *Quercus robur* y matorral dominado por *Crataegus monogyna*) para sostener poblaciones activas de la lombriz epigea *E. andrei*, centrándonos en la calidad nutricional del substrato.

# 2. La especie

## 2.1. Taxonomía

La especie de lombriz sujeta al estudio presentado en esta tesis es *Eisenia andrei* (Bouché 1972), también conocida con el nombre de lombriz roja. Pertenece a la familia Lumbricidae, una de las familias más importantes de lombrices de tierra. La posición taxonómica de la especie sería la reflejada en el cuadro 1.

**Cuadro 1**. Posición taxonómica de la especie *Eisenia andrei*.

**Reino**: Animalia

**Subreino**: Eumetazoa

**Filo**: Annelida

**Clase**: Euclitellata

**Subclase**: Oligochaeta

**Orden**: Haplotaxida

**Familia**: Lumbricidae

**Género**: *Eisenia*

**Especie**: *Eisenia andrei*

A lo largo de la historia ha habido gran controversia acerca de la separación de la especie *Eisenia foetida* en dos especies diferentes, *Eisenia fetida* (Savigny, 1926) y *Eisenia andrei* (Bouché 1972) y que actualmente están catalogados en dos especies distintas (Pérez-Losada *et al.* 2005). *E. fetida* responde a un morfotipo rallado, sin pigmentación intersegmentaria, mientras que *E. andrei* presenta una coloración rojiza a lo largo de toda su parte dorsal (Domínguez *et al.* 2005). A parte de las diferencias de pigmentación, se han encontrado diferencias tanto bioquímicas (Roch *et al.* 1980) como genéticas (McElroy & Diehl, 2001), sugiriendo que *E. andrei* deriva de *E. fetida* por la pérdida de algunos alelos (Jaenike 1982; Henry 1999; Albani *et al.* 2003). Por otro lado, a pesar de que los requerimientos ecológicos y

el ciclo de vida sean similares en las dos especies, se ha encontrado que tanto la tasa de crecimiento como la producción de capullos son ligeramente más elevadas en *E. andrei* (Elvira *et al.* 1996a; Domínguez & Edwards 2011). Por último, en un estudio llevado a cabo por Domínguez *et al.* (2005), se demostró la existencia de aislamiento reproductivo post-zigótico entre ambas especies. Dado que la separación de estas dos especies es tardía y comparten ciclo de vida y requerimientos ecológicos, en muchas ocasiones a lo largo de esta tesis se utilizarán referencias de estudios con *E. fetida*, sobre todo al referirnos a aspectos biológicos, ecológicos y fisiológicos.

## 2.2. Anatomía

### 2.2.1 Anatomía externa

Los ejemplares de *E. andrei* presentan un cuerpo cilíndrico con simetría bilateral. Los individuos recién nacidos se caracterizan por una coloración rosa tenue, mientras que a medida que el ciclo vital se sucede la tonalidad se va oscureciendo hasta alcanzar una pigmentación rojiza uniforme a lo largo de toda la superficie dorsal del animal. Su intensa pigmentación sirve como camuflaje frente a la depredación y proporciona protección frente a los rayos ultravioleta.

Al igual que el resto de especies de lombrices, los ejemplares de *E. andrei* se encuentran externamente divididos por segmentos a lo largo del cuerpo, coincidiendo con los septos que dividen al animal internamente y que se describirán en el siguiente apartado. Los segmentos o metámeros son similares entre ellos por lo que se puede decir que presentan metamería homónoma con un número medio de 102 segmentos por individuo (Moreno & Borges 2004). La boca se encuentra localizada en el primer segmento o peristomio, al cual se le superpone una estructura denominada prostomio, que en el caso de nuestra especie presenta una disposición entre epilóbica y tanilóbica (Moreno & Borges 2004). El ano se sitúa en el pigidio, que no es un verdadero segmento, y está situado en la parte terminal del cuerpo (Shimizu & Nakamoto 2001).

## Quetas

Las quetas son estructuras que nacen en los folículos de la parte exterior del cuerpo y su capacidad para contraerse y retraerse, gracias a la acción de la musculatura anclada a la base de los folículos, les otorga gran importancia en la función locomotora (Edwards & Lofty 1977; Brusca & Brusca 2003). La disposición de las quetas y la distancia a la que están colocadas entre sí es constante para cada especie, de modo que cada especie presenta su propia fórmula quetal. *E. andrei* presenta una disposición lombriciana, con 8 quetas estrechamente pareadas por segmento, siendo 3.6:1.1:3.6:1:15 su fórmula (Moreno & Borges 2004).

## Genitales externos y aperturas de la pared corporal

A lo largo del cuerpo del animal se encuentran unas aberturas llamadas poros dorsales. Situados en las zonas intersegmentarias en posición medio-dorsal, son una vía de comunicación con la parte interna del cuerpo (celoma). Los anillos anteriores suelen estar desprovistos de ellos y la posición del primer poro dorsal, intersegmento 4-5 en el caso de *E. andrei*, es utilizada en sistemática como criterio de identificación de especies de la familia Lumbricidae (Sims & Gerard 1985).

Situados justo al término de los surcos intersegmentarios, con una orientación lateral en el cuerpo del animal, se encuentran los nefridioporos, que son las aberturas externas de los órganos excretores.

Debido al carácter hermafrodita de las lombrices, presentan aberturas masculinas y femeninas. Son parejas de poros situados en la cara ventro-lateral del cuerpo, y en el caso de *E. andrei* los poros masculinos se encuentran el segmento 15 y los femeninos en el 14. En los surcos intersegmentales de los segmentos 9-10 y 10-11 presenta dos pares de poros de la espermateca (Sims & Gerard 1985).

El clitelo se desarrolla al llegar a la edad adulta y cosiste en un abultamiento glandular asociado a la producción de capullos. Es una estructura anular que ocupa de 5 a 6 segmentos y se sitúa en los segmentos (26) 27-30 (31) (32) (Edwards & Lofty 1977). Presenta un color más pálido que el resto del cuerpo y es fácilmente diferenciable. En esta zona se desarrollan los tubérculos

pubertarios, engrosamientos glandulares con forma ovalada situados a ambos lados sobre el clitelo en posición ventral, cuya función, se cree, consiste en mantener a los animales juntos durante el proceso de cópula y ayudar en la transferencia de espermatozoides.

## Pared corporal

La pared corporal está compuesta por la cutícula, epidermis, capa de tejido conjuntivo, musculatura circular, musculatura longitudinal y peritoneo (Edwards & Lofty 1977).

La cutícula es muy estrecha y está compuesta por fibras de colágeno. De color transparente, se encuentra perforada por poros de pequeño tamaño. La cutícula se estrecha aún más cuando se superpone a los órganos sensoriales epiteliales y está perforada por poros de tamaño muy pequeño a través de los cuales se proyectan hilos finos desde las células sensoriales.

A continuación de la cutícula, se sitúa la epidermis, formada por una única capa de distintos tipos de células:

Células de soporte: De forma columnar, además de servir como soporte secretan material para la formación de la cutícula.

Células basales: Se encuentran en la base interna de la epidermis y son células de recambio.

Células glandulares: básicamente son células mucosas que se encargan de la segregación de moco hacia el exterior de la cutícula previniendo la desecación y facilitando la locomoción.

Células sensoriales: Son células que se agrupan para conformar los órganos sensoriales de la epidermis y responden a estímulos táctiles. Son más numerosas en la parte dorsal que en la ventral.

Células fotoreceptoras: Son capaces de distinguir diferentes intensidades de luz y son más numerosas en la zona anterior que posterior del cuerpo.

Debajo de la epidermis se encuentra una capa de tejido conjuntivo situándose a continuación la musculatura. Primeramente se encuentra la capa de músculo circular que se extiende alrededor de la circunferencia del cuerpo del animal excepto en los lugares intersegmentales, situándose a continuación la capa de músculo longitudinal compuesta por musculatura clasificada como

intermedia. Por último, en la base interna de la pared corporal se encuentra el peritoneo, también llamado epitelio celomático o somatopleura.

## 2.2.2. Anatomía interna

La cavidad interna está dividida por septos transversales formados por fibras musculares, coincidiendo cada septo con la metamería externa del animal. Cada uno de los segmentos, delimitados por los septos transversales, está separado en dos partes (derecha e izquierda) por los mesenterios dorsal y ventral.

### Celoma

Es una gran cavidad que se extiende a lo largo del cuerpo del animal y está relleno de líquido celomático. Rodeado por el peritoneo de la pared corporal exteriormente y por el peritoneo del canal alimentario interiormente, constituye una estructura de soporte del animal además de tener un papel importante como medio interno. Los septos transversales dividen al celoma en porciones comunicadas entre sí por poros que permiten la circulación del líquido celómático a lo largo del cuerpo. Estos poros están controlados por esfínteres musculares que mantienen los compartimentos celomáticos aislados, teniendo un papel fundamental en la locomoción (Brusca & Brusca 2003). El líquido celomático puede ser expulsado hacia el exterior como respuesta al estrés pudiendo prevenir la desecación, promover la respiración cutánea y proporcionar protección frente a los depredadores (Edwards & Lofty 1977).

Algunas células peritoneales de la pared intestinal se encuentran modificadas formando el tejido cloragógeno cuyas células están caracterizadas por la presencia de glóbulos amarillentos llamados cloragosomas (Edwards & Lofty 1977). Se cree que la función de este tejido es lo más parecido al hígado en vertebrados ya que actúa como lugar de metabolismo intermediario donde se produce la síntesis y almacenamiento del glucógeno y lípidos, así como la desaminación de proteínas (Brusca & Brusca 2003; Cholewa *et al.* 2006). Del mismo modo, también parece tener un papel importante en la excreción (Edwards & Lofty 1977; Brusca & Brusca 2003).

Los cuerpos celulares presentes en el celoma (celomocitos) se pueden clasificar en dos tipos principales (Vetvicka *et* Sima 2009; Mazur *et al.* 2011):

Amebocitos: Se cree que derivan del tejido mesenquimal y se subdividen en granulares e hialinos.

Eleocitos: Son cuerpos que derivan del tejido cloragógeno y se subdividen en cloragocitos ergastoplásmicos (precursores de los demás tipos), cloragocitos y eleocitos libres.

Los celomocitos participan activamente en la repuesta inmune del organismo (Adamowicz & Wojtaszek 2001; Cholewa *et al.* 2006; Vetvicka & Sima 2009). Presentan capacidad fagocítica y bactericida y en respuesta a una invasión externa participan en la formación de cuerpos multicelulares que encapsulan las sustancias ajenas al cuerpo del animal (Mazur *et al.* 2011).

Otra de las funciones de los celomocitos son, por un lado ayudar en el mantenimiento del pH y balance osmótico, y por otro participar en el transporte de substancias nutricionales (Adamowicz & Wojtaszek 2001; Vetvicka & Sima 2009).

Además de los cuerpos celulares, el celoma presenta inclusiones inorgánicas de carbonato cálcico cuyos niveles están regulados por las glándulas calcíferas que se describen en el siguiente apartado.

## Sistema digestivo

El aparato digestivo consiste en un tubo recto con partes que presentan distintos grados de especialización. En la parte más anterior se encuentra la boca que se abre en el primer segmento del cuerpo y da paso al digestivo anterior que comienza en una cavidad bucal, seguida de la faringe y el esófago. La parte posterior del esófago está diferenciada en un buche y a continuación una molleja en la cual se tritura el material ingerido. El resto del tubo digestivo está formado por el intestino, diferenciándose el digestivo medio en la parte anterior y el digestivo posterior en la parte más distal. El aparato digestivo termina en el ano que se sitúa en el pigidio en la parte posterior del cuerpo.

En las paredes del esófago se encuentran unas evaginaciones tapizadas por tejido glandular que son las glándulas calcíferas. Estas glándulas se abren en el esófago (Edwards & Lofty 1977) y extraen el calcio del material ingerido. Se cree que pueden ser las responsables del mantenimiento del pH de la sangre y celoma regulando sus niveles de iones calcio y carbonatos.

El exceso de calcio precipita en el interior de estas glándulas en forma de calcita, que, envueltas en mucus, son devueltas a la luz intestinal. El intestino no es capaz de reabsorber esta forma amorfa de calcio, de modo que es eliminado en las heces (Brusca & Brusca 2003).

El buche, que actúa como lugar de almacenamiento del alimento ingerido, da paso a la molleja, muy muscularizada y tapizada por una cutícula, que, ayudada por las partículas minerales ingeridas, es la encargada de triturar mecánicamente los alimentos. La fuerte contracción muscular de las paredes de la molleja hace que las cutículas de las superficies opuestas se junten y machaquen el alimento que pasará así al intestino.

El resto del aparato digestivo lo constituye el intestino, que es un tubo recto en casi toda su longitud que experimenta pequeñas constricciones al pasar por cada septo. Presenta dos capas de musculatura, circular en la parte más interna y longitudinal en la más superficial. El epitelio intestinal está compuesto básicamente por células glandulares secretoras y células ciliadas no glandulares. La superficie del intestino se ve incrementada por un pliegue de la pared, denominado tiflosol, que se proyecta desde la pared dorsal. La parte anterior del intestino es principalmente secretora y hay evidencias de secreción de proteasas (Brown *et al.* 2000), celulasas (Brown *et al.* 2000), quitinasas, amilasas y lipasas (Edwards & Fletcher 1988). No hay evidencias de la presencia de enzimas intrínsecas capaces de digerir la lignina o los compuestos fenólicos. Sin embargo, en *E. fetida* se constató actividad peroxidasa intrínseca que podría ayudar a destruir los enlaces aromáticos de la lignina (Hassett *et al.* 1988).

Los alimentos digeridos son absorbidos principalmente en la mitad posterior del intestino, denominada zona de absorción, donde la materia no digerida es envuelta en una membrana peritrópica mucopolisacárida y es excretada por el ano en forma de heces.

El peristaltismo producido por los músculos de la pared digestiva y los movimientos peristálticos asociados a la locomoción, desplazan el contenido del tubo digestivo a lo largo del cuerpo del animal pudiéndose completar la digestión de la materia ingerida.

Por último, es importante mencionar la función, antes descrita, del tejido cloragógeno como lugar en el que se sucede el metabolismo intermediario.

## Sistema circulatorio

El sistema vascular de los anélidos oligoquetos es un sistema de circulación cerrado. Consta de tres vasos sanguíneos principales que recorren el cuerpo en toda su longitud. El vaso dorsal, cuyas paredes están muy muscularizadas, está asociado al tubo digestivo en todo el cuerpo excepto en la zona más anterior que se encuentra separado por un mesenterio. En la parte ventral, suspendido de un mesenterio situado debajo del tubo digestivo se encuentra el vaso ventral. Por último, debajo de la cadena nerviosa ventral se sitúa el vaso subneural.

Los vasos comisurales, que forman anillos alrededor de los segmentos, conectan el vaso dorsal con el ventral y en sus respectivos recorridos van irrigando la pared corporal, el tubo digestivo y los nefridios. En la zona del esófago encontramos cinco pares de vasos periesofágicos, muy muscularizados, llamados corazones laterales por los que la sangre pasa del vaso dorsal al ventral. Los intercambios se realizan mediante plexos capilares que presentan ramas aferentes y eferentes de los tejidos.

La sangre circula en dirección posteroanterior en el vaso dorsal. La mayoría de la sangre pasa al vaso ventral por medio de los corazones laterales. En el vaso ventral la sangre circula en dos direcciones: en dirección a la cabeza y hacia el resto del cuerpo. La sangre de los órganos anteriores es recogida por ramas del vaso subneural y circula en dirección anteroposterior por él. Los vasos comisurales dorsoneurales permiten el paso de la sangre desde el vaso subneural hasta el dorsal. Debido a la muscularización de las paredes, el vaso dorsal y los vasos periesofágicos son los responsables de propulsar la sangre y hacer que la presión sanguínea se mantenga. Tanto el vaso dorsal como los corazones laterales están dotados de válvulas que aseguran el flujo sanguíneo en una única dirección.

La sangre presenta hemoglobina lo cual contribuye enormemente a aumentar la cantidad de oxígeno transportable por volumen de sangre. Esto tiene implicaciones adaptativas en que se discutirán más adelante.

**Sistema excretor**

Los principales órganos excretores son los nefridios, encontrándose en *E. andrei* un par de metanefridios en cada segmento excepto en los metámeros más anteriores y posteriores (Edwards & Lofty 1977).

Los nefridios presentan una forma tubular y constan de un nefrostoma anterior al septo que está en contacto con el celoma. Este nefrostoma presenta un tubo que atraviesa el septo y conecta con el resto del nefridio. La parte postseptal del metanefridio se encuentra dividida en 4 partes. Un túbulo estrecho seguido de un túbulo medio que actúa como riñón de acumulación. A continuación se dispone un túbulo ancho y le sigue un túbulo muscular que se abre al exterior por el nefridioporo, cuya apertura está controlada por un esfínter.

El fluido celomático que contiene las substancias de excreción pasa a través del nefrostoma y a lo largo del tubo nefridial por acción ciliar. El nefridio actúa como un filtro diferenciador ya que la proporción de urea y amonio con respecto a las proteínas es mayor en la orina producida que en el fluido celomático. La presión osmótica de la orina varía a su paso por el nefridio (Fig. 1), obteniéndose en la mayoría de los casos, una orina hipotónica con respecto al medio interno. Este dato, tiene implicaciones fisiológicas y ecológicas que se atenderán cuando hablemos de la osmoregulación. Esta diferenciación en la presión osmótica encontrada en el nefridio no está claro si está producida por la reabsorción de sales, por la secreción de agua o por ambas.
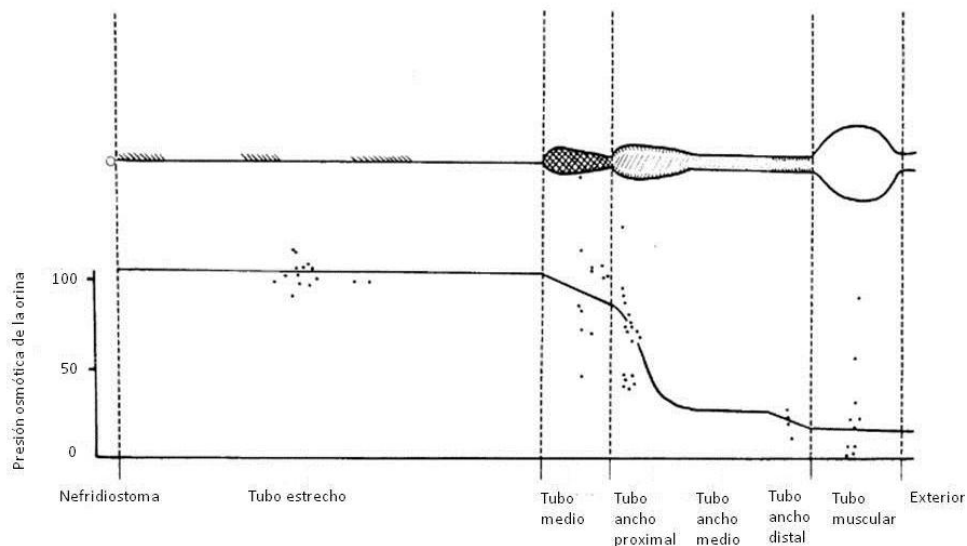


**Fig 1.** Cambios en la presión osmótica de la orina en *Lumbricus terrestris* a su paso por el nefridio (Original de Ramsay 1949).

El tejido cloragógeno, como ya se ha adelantado, parece tener un papel fundamental en la excreción (Laverack 1963). La mayoría de los productos de desecho del metabolismo llegan a la sangre y celoma de donde tienen que ser eliminados. Las células del tejido cloragógeno, se encuentran asociadas a los vasos sanguíneos y recolectan unos gránulos de color amarillento llamados cloragosomas. Se cree que las células cloragógenas, una vez liberadas en el celoma, forman vacuolas que se segregan en el fluido celomático. Otras células celomáticas las desintegran completamente y su contenido, principalmente urea y amonio, aunque también presentan grasas y glucógeno, es liberado en el celoma para su posterior excreción, bien directamente por los nefridios, o bien por medio de la encapsulación llevada a cabo por los amebocitos del fluido celomático (Edwards & Lofty 1977). Por otro lado, no se debe olvidar la importancia del tejido cloragógeno como lugar de metabolismo secundario y su papel en el mantenimiento de la homeostasis de la sangre y el celoma.

Por último, se debe mencionar que aproximadamente el 50 % de la excreción de compuestos nitrogenados se realiza a través de la producción de substancias mucosas que se segregan en la superficie corporal.

## Sistema respiratorio

*E. andrei* no presenta órganos respiratorios especializados. Estos animales realizan una respiración cutánea en la que el oxígeno y el carbono dióxido se difunden a través de la cutícula y epidermis hacia la sangre (Barnes *et al.* 1993). Para que este proceso fisiológico pueda tener lugar, la superficie corporal debe mantener una capa de agua donde el oxígeno pueda disolverse. La pared del cuerpo se mantiene húmeda por la segregación de mucus por parte de las glándulas epiteliales, por exudados del fluido celomático a través de los poros dorsales (en condiciones de desecación) y por las excretas metabólicas que salen al exterior por los nefridioporos (Edwards & lofty 1977). El oxígeno disuelto en la superficie permea a través de la cutícula y la epidermis y es recogido por pequeños vasos sanguíneos de la pared corporal.

La sangre presenta hemoglobina disuelta en el plasma lo cual contribuye enormemente a la cantidad de oxígeno transportable (Laverack 1963). Estos pigmentos respiratorios cogen el oxígeno y lo transportan a otros lugares del cuerpo. En un trabajo de finales de los años 50

realizado con *Lumbricus terrestris* se constató un porcentaje de saturación del 95 % a presiones parciales de oxígeno ($P_{O2}$) tan bajas como 22 mm Hg a 20 ºC (Haughton *et al.* 1958). Además, el fluido celomático presenta una $P_{O2}$ de 14 mm Hg, lo cual permite que el oxígeno pueda llegar a los lugares más profundos del cuerpo incluso cuando la $P_{O2}$ en el medio es muy baja.

## Sistema reproductor

Las lombrices, como ya se ha comentado, son hermafroditas por lo que presentan órganos sexuales masculinos y femeninos.

El aparato reproductor femenino presenta un par de ovarios que son, al igual que en vertebrados, los órganos productores de gametos femeninos (óvulos u oocitos). Estos son liberados al celoma y maduran en unas pequeñas bolsas de las paredes de los septos intersegmentarios llamados ovisacos (Brusca & Brusca 2003). Una vez maduros, son captados por los embudos femeninos y, por medio de los oviductos, salen al exterior a través de los poros femeninos situados en el segmento 14. Presenta además dos pares de espermatecas con aberturas (poros) a ambos niveles en los intersegmentos 9-10 y 10-11 (Sims & Gerard 1985; Moreno & Borges 2004). Éstas, son unas estructuras que actúan como receptáculo seminal después de producirse la cópula y servirán para almacenar el esperma hasta el momento de la formación del capullo.

El principal órgano masculino son los testículos. *E. andrei* presenta 2 pares de testículos productores de espermatozoides. Se describen 4 pares de vesículas seminales situadas en los segmentos 9, 10, 11 y 12, formadas por evaginación del peritoneo de los septos intersegmentarios, y son el espacio donde maduran los gametos masculinos (Sims & Gerard 1985; Moreno & Borges 2004). Los espermatozoides, una vez maduros, son captados por los embudos espermáticos, y son transportados por los espermiductos hasta los poros masculinos, situados en el segmento 15, en el momento de la cópula.

Hay evidencias de formación de espermatóforos (Moreno & Borges 2004), que son pequeñas cápsulas que se adhieren a la pared corporal y se llenan de espermatozoides. Se cree que tienen un papel importante en la transferencia del esperma impidiendo la digestión de los gametos en la espermateca y favoreciendo la fertilización del huevo durante la formación del capullo (Díaz-Cosín *et al.* 2011). Sin embargo, en un estudio llevado a cabo por Monroy *et al.* (2003) con su

especie hermana *E. fetida*, no se encontró ningún efecto de estas estructuras sobre su éxito reproductivo, de modo que la función de estas cápsulas no está del todo definida (Díaz-Cosín *et al.* 2011).

Al llegar la madurez sexual, se desarrolla una estructura glandular que forma un anillo alrededor del cuerpo del animal abarcando de 5 a 6 segmentos llamada clitelo, siendo la característica morfológica principal que sitúa a esta especie dentro de la clase Euclitellata. Es el encargado de la formación del capullo, una de las principales estructuras de la reproducción sexual, donde se produce la fecundación y desarrollo de los óvulos fecundados. Superpuestos al clitelo, a ambos lados del cuerpo, se desarrollan los tubérculos pubertarios que mantienen unidos a los ejemplares durante la cópula.

## 2.3. Fisiología

### 2.3.1. Metabolismo

La respiración es uno de los parámetros más importantes relacionados con los flujos de energía del organismo ya que medir la respiración implica medir el coste de la vida de un animal. Suele determinarse como cantidad de oxígeno consumida por unidad de tiempo ($\mu$L $O_2$ h$^{-1}$).

El consumo de oxígeno de un individuo depende principalmente del tamaño. El crecimiento alométrico de los distintos órganos a lo largo del crecimiento del animal (disminución de la relación superficie/volumen…) impiden que el metabolismo sea directamente proporcional al peso del individuo, y por tanto, la tasa respiratoria alcanza valores relativamente menores en animales de mayor tamaño.

La temperatura aumenta el metabolismo haciendo que el animal requiera una mayor cantidad de oxígeno para realizar sus funciones, de modo que, un aumento en la temperatura repercute en un incremento de la tasa de respiración. Sin embargo, Moment & Habermann (1979) encontraron un intervalo de termo-independencia para *E. fetida* entre 20 y 25 $^{\circ}$C, que como se verá más adelante, coincide con los óptimos de temperatura para la especie *E. andrei*.

Por otro lado, es importante mencionar también los efectos de la disponibilidad de oxígeno sobre la tasa de respiración. En aire, se precisa una disminución drástica de la presión parcial de

oxígeno para que tenga un efecto sobre la respiración. Esta situación de disponibilidad limitada es improbable que ocurra ya que se trata de una lombriz epigea, cuyo nicho se sitúa en los primeros centímetros del suelo (ver apartado de hábitat) donde la presión parcial de oxígeno es similar a la del aire libre. Sin embargo, algo que sí es posible que ocurra es un encharcamiento o inundación ocasional del hábitat. En este caso, la amplia superficie respiratoria sumada a la presencia de la hemoglobina en la sangre, que aumenta enormemente la cantidad de oxígeno transportable, permiten a este animal vivir sumergido en agua (relativamente oxigenada), siendo la falta de comida el factor limitante para la supervivencia y no así la escasez de oxígeno o problemas de osmorregulación (ver apartado de osmorregulación).

La humedad del suelo tiene también una fuerte influencia sobre la tasa de respiración. En un trabajo realizado con *E. fetida* (Diehl & Williams 1992), se demuestra una disminución de la respiración cutánea bajo condiciones de poca disponibilidad hídrica, explicado por lo expuesto anteriormente sobre la necesidad de mantener una película húmeda en la superficie del animal.

En relación a la disponibilidad de alimento, en ese mismo trabajo (Diehl & Williams 1992) se observa una influencia significativa de la cantidad de alimento sobre el consumo de oxígeno.

## 2.3.2. Relaciones hídricas: Osmorregulación

En la vida de los oligoquetos terrestres el agua es un factor limitante. La respiración, como hemos comentado, es cutánea, por lo que requieren una película húmeda en su pared corporal para que el intercambio gaseoso tenga lugar, luego, la falta de agua repercute negativamente sobre la respiración aeróbica (Presley *et al.* 1996). En condiciones de estrés hídrico, producido por falta de agua, el fluido celomático es expulsado al exterior para poder mantener húmeda la superficie corporal. Como consecuencia, el animal pierde agua y su turgencia disminuye. Se constata que pueden llegar a perder el 75 % del agua de su cuerpo sin que se produzca la muerte, sin embargo, como la actividad locomotora depende del aparato hidrostático, la actividad del animal disminuye drásticamente (Edwards & Lofty 1977; Stovold *et al.* 2003). A este respeto, Kretzschmar & Bruchou (1991) constataron una pérdida en peso del 60 % asociado a condiciones de baja disponibilidad hídrica que seguía siendo compatible con la actividad del animal. Dada la importancia de la conservación de agua, en muchos animales los productos de

excreción han cambiado favoreciendo la excreción de las substancias nitrogenadas en forma de moléculas menos tóxicas que el amonio (Barnes *et al.* 1993). Las lombrices de tierra producen principalmente amonio y urea como productos de excreción, pudiéndose favorecer la excreción de nitrógeno en forma de urea en condiciones de estrés hídrico causado por la desecación, produciendo una orina relativamente hipertónica con respecto a la orina amoniotélica (Brusca & Brusca 2003). De hecho, el aumento de la producción de urea parece estar correlacionada con la cantidad de agua presente en el medio, sugiriendo que los anélidos son capaces de cambiar sus patrones de excreción dirigiendo el ciclo metabólico a la producción de urea en lugar de amonio (Laverack 1963). Del mismo modo, en condiciones de estrés alimenticio, como puede ser el ayuno o la disponibilidad limitada de alimento, producen una orina ureotélica.

En el ambiente terrestre, las condiciones hídricas pueden ser inversas a las descritas hasta ahora, es decir, situaciones de exceso de agua produciendo un encharcamiento o inundación en el nicho del animal. En anélidos, se ha constatado una gran capacidad de osmorregulación bajo estas condiciones. De hecho, en un trabajo realizado sobre *Lumbricus terrestris* (Carley *et al.* 1983), la presión osmótica del fluido celomático se mantenía regulada aproximadamente en 150 mOsm en lombrices sometidas a distintos niveles de salinidades (de 3 a 150 mOsm); y, en un experimento no publicado realizado en nuestro laboratorio con *E. andrei* sometido a distintas concentraciones diluidas de salinidad (0, 50, 100, 150 y 200 mOsm/L), no se registraron variaciones significativas en el peso incluso en las concentraciones más diluidas. Por lo tanto, estos individuos son capaces de mantener la presión osmótica interna por encima de la del medio externo y producir una orina hipotónica con respecto a la de la sangre o celoma (Edwards & Lofty 1977). Este tipo de osmorregulación se consigue eliminando un gran volumen de orina hipotónica con respecto al medio interno, reabsorbiendo las sales y eliminando el exceso de agua por medio del aparato digestivo (Brusca & Brusca 2003). Bajo condiciones de encharcamiento, no sería de extrañar que la molécula de excreción de compuestos nitrogenados mayoritaria fuera el amonio ya que requiere más cantidad de agua para su eliminación y la cantidad de energía requerida para formar la molécula es menor que en el caso de la urea (Barnes *et al.* 1993). A pesar de que las lombrices osmorregulan muy bien en condiciones

diluidas de salinidad, no lo hacen así cuando están sometidas a altas concentraciones salinas luego, su forma de osmorregulación se podría asemejar a la de los animales de agua dulce.

No hay que olvidar que estos animales presentan adaptaciones de comportamiento tanto para evitar la desecación como las condiciones de exceso de agua (Brusca & Brusca 2003). De hecho, el nicho de estos individuos es una interfaz entre la vida terrestre y la vida dulceacuícola (Holmstrup & Westh 1995), por lo que se encuentran en hábitats relativamente húmedos escapando de la aridez y de condiciones de encharcamiento del suelo.

### 2.3.3. Locomoción

La locomoción en los oligoquetos es posible gracias a su esqueleto hidrostático. El aislamiento de cada uno de los segmentos por los tabiques intersegmentarios permite que cada compartimento funcione más o menos independientemente de los demás, de modo que la contracción de un segmento del cuerpo no implica el flujo de líquido celomático hacia otro (Stovold *et al.* 2003). Cada compartimento tiene un volumen de líquido constante, de modo que una disminución en grosor implica un aumento en la longitud y viceversa. Estos cambios de forma se desplazan hacia delante en el cuerpo como ondas peristálticas y son posibles gracias a la contracción alterna de la musculatura circular y longitudinal de cada uno de los segmentos. Las quetas sirven como elementos de anclaje en las zonas engrosadas del cuerpo y permiten al animal moverse hacia delante (Brusca & Brusca 2003).

### 2.3.4. Ciclo de vida, estrategias reproductivas y crecimiento

### Ciclo de vida

Es una especie que presenta un desarrollo directo, sin estadios larvarios, luego los individuos recién nacidos, son morfológicamente similares a los adultos. Los huevos son depositados en los capullos siendo el número de recién nacidos, por lo general, inferior al número de huevos depositado. Son lombrices de ciclo de vida corto, estrategia tipo r, en el que en aproximadamente 3 meses después de la eclosión se alcanza la madurez y se reproducen (Presley *et al.* 1996; Toccalino *et al.* 2004), sin embargo, en condiciones óptimas se puede llegar a completar el ciclo de vida en 50 días (Domínguez & Gómez-Brandón 2010). Este tipo

de estrategia deriva en una alta prolificidad y en la presencia de altas tasas de reproducción y crecimiento, siendo ambas tasas superiores que las de su especie hermana *E. fetida* (Elvira *et al.* 1996a; Domínguez *et al.* 2003). Tienen una esperanza de vida de unos 5 años (Domínguez & Gómez-Brandón 2010), aunque la vida media de una lombriz de tierra es de aproximadamente 600 días. Son iteróparas, con reproducción continua a lo largo del año, no obstante bajo condiciones adversas (humedad, temperatura y disponibilidad de alimento), pueden entrar en un estado de diapausa facultativa con el consiguiente parón en la producción de capullos (Edwards & Lofty 1977; Hartenstein *et al.* 1979; Domínguez & Gómez-Brandón 2010). Venter y Reinecke (1988) en un estudio con *E. fetida*, afirman que se mantienen reproductivamente activas hasta 500 días después de alcanzar la madurez sexual.

## Estrategias reproductivas

Esta especie, como ya se ha comentado, presenta **una reproducción sexual cruzada** en la que se requiere la transferencia de gametos de un individuo a otro mediante cópula. No obstante, en un experimento con *E. andrei* se encontró un caso de auto-inseminación (Domínguez *et al.* 2003). Uno de los órganos reproductores más importantes es el clitelo y sus estructuras adyacentes como los tubérculos pubertarios. El clitelo presenta tres tipos de células glandulares cada una de las cuales segrega un componente importante para la reproducción. Por un lado, segregan el moco que facilita la cópula; por otro lado, segregan las substancias que forman la cubierta externa del capullo; y finalmente, segregarán la albúmina del interior del capullo que sirve de alimento para los huevos fecundados durante la embriogénesis.

En el proceso de cópula, las lombrices se sitúan una enfrente de la otra en direcciones opuestas, poniendo en contacto sus caras ventrales. En este momento, se produce la liberación de los espermatozoides por el poro masculino que a través de los surcos seminales llegarán hasta los poros de las espermatecas de la pareja. Esta transferencia de esperma suele ser bidireccional lo cual optimiza la energía empleada durante el proceso de cópula, de hecho en un estudio realizado por Domínguez *et al.* (2003) se observa dicha transferencia bidireccional en el 88.2 % de los apareamientos. En un estudio llevado a cabo por Porto *et al.* (2012) constatan un aumento del éxito de eclosión de los capullos asociado a un mayor número de cópulas, sin observar

efectos negativos sobre el número de capullos depositados o sobre la biomasa de los recién nacidos.

Después de la cópula, si las condiciones son favorables, el clitelo segrega un anillo de mucosa de naturaleza proteica que formará la cubierta coriácea del capullo. La albúmina se segrega entre la superficie del clitelo y la manga mucosa la cual se va desplazando hacia la cara anterior y a su paso por los poros femeninos se liberarán los óvulos mientras que los espermatozoides son recogidos a su paso por los poros de las espermatecas. Los extremos abiertos de la manga mucosa se contraen y se cierran al salir por el extremo anterior del cuerpo para formar el capullo, en cuyo interior se produce la fecundación de los huevos. Los capullos son depositados cerca de la superficie del suelo, encontrándose a profundidades mayores bajo condiciones adversas. En un estudio realizado por Domínguez *et al.* (2005) se observó una media por animal de 2.8 capullos depositados por semana en individuos cultivados en estiércol bovino, mientras que los valores encontrados por Sheppard (1988) son ligeramente inferiores (1.34). El tiempo de incubación oscila entre 14 y 26 días (Toccalino *et al.* 2004; Domínguez & Gómez-Brandón 2010) y es dependiente de los factores ambientales. Así mismo, el número de recién nacidos por capullo es muy variable, pero se estima una media de 2 a 4 individuos (Domínguez et al 2005; Domínguez & Gómez-Brandón 2010).

En cuanto a la partenogénesis, a pesar de que hay trabajos en los que se han encontrado capullos en medios de crecimiento en lo que los animales se encontraban individualizados (Domínguez *et al.* 1997; Nakagawa *et al.* 2002), la partenogénesis en *E. fetida y E. andrei* no está demostrada. Los taxones con partenogénesis se caracterizan por ser poliploides (Beukeboom *et al.* 1998), y a pesar de que otras especies del género *Eisenia* lo son, *E. fetida y E. andrei* son diploides (Mundal 1952), por lo que es improbable que puedan ser especies partenogenéticas. La existencia de puesta en individuos que se encuentran individualizados podría deberse a la auto-inseminación (Domínguez *et al.* 2003).

**Crecimiento**

Los individuos recién nacidos son morfológicamente copias de los individuos adultos y sólo cuando se alcanza la madurez sexual se aprecian cambios morfológicos externos. En un trabajo

realizado sobre *E. fetida* (Moment 1953) se descubre que el número de segmentos de los individuos recién nacidos es el mismo que el de los individuos adultos, encontrándose solo en la fase prenatal un aumento en el número de segmentos. En base a esta evidencia, a pesar de que en otras especies de lombrices el crecimiento tiene lugar por adición de segmentos, en la especie *E. fetida* (hermana de la especie *E. andrei*) el crecimiento se produce por aumento del tamaño de los segmentos.

El crecimiento en esta especie es continuo y se ajusta a un modelo de crecimiento logístico que se caracteriza por la presencia de distintas etapas: fase lag, fase exponencial, fase de crecimiento rectilíneo, fase potencial y fase asintótica. La duración de cada fase dependerá de las condiciones ambientales y de las características genéticas de cada individuo.

# 3. El medio

## 3.1. Hábitat

La lombriz objeto de estudio se clasifica en la categoría ecológica de las lombrices epigeas. Viven en el horizonte orgánico, o cerca de la superficie del suelo, alimentándose de la materia orgánica en descomposición. Su pequeño tamaño, su alta tasa metabólica y su prolificidad le proporcionan gran plasticidad a la hora de adaptarse al medio. A continuación se muestra una síntesis de los factores abióticos y bióticos más importantes que afectan al ciclo de vida de esta especie.

### 3.1.1. Factores abióticos

El ciclo de vida de las lombrices de tierra está condicionado por los factores ambientales, siendo la temperatura, humedad y la fuente de alimentación las más estudiadas (Presley *et al.* 1996; Toccalino *et al.* 2004; Domínguez & Edwards 1997; Reinecke & Venter 1987). Son parámetros que afectan al metabolismo del animal, medido en términos de consumo de oxígeno, que en definitiva van a condicionar el crecimiento y reproducción de los individuos de la especie.

**Temperatura y humedad**

Como ya se ha adelantado en el apartado de fisiología, la disponibilidad hídrica resulta clave para los oligoquetos terrestres. Aunque esta especie se caracteriza por presentar un amplio rango de tolerancia de **temperatura y humedad,** se han definido unos óptimos de 20-25 ºC y 80-85 % HR para el cultivo de *Eisenia* (Toccalino *et al.* 2004; Domínguez & Gómez-Brandón 2010). Presley *et al.* (1996) constataron que la temperatura y la humedad presentaban una alta influencia sobre el crecimiento y la reproducción en *E. fetida* y se demostró mediante análisis estadísticos la interacción entre estas dos variables explicativas. Los óptimos de estas variables variaban según la ontogenia del animal, siendo los cambios de temperatura los que presentaban mayor influencia sobre el crecimiento y el "fitness" (Presley *et al.* 1996), ya que los valores de crecimiento y reproducción más altos se alcanzaban siempre a humedades del substrato altas.

Debido a su tolerancia a los cambios de temperatura y a la capacidad de aclimatación que presentan (Presey *et al.* 1996; Domínguez & Gómez-Brandón 2010) es una especie ubicua. Sin embargo, su capacidad de dispersión es bastante reducida ya que la actividad de la lombriz se ve disminuida en condiciones de desecación (ver metabolismo y osmorregulación).

**pH**

Las lombrices son altamente sensibles a la concentración de iones hidrógeno del substrato, luego no es de extrañar que las variaciones en el pH del medio limiten su distribución y abundancia. En cuanto al género *Eisenia*, se ha constatado que individuos de la especie *E. fetida* prefieren suelos con un pH situado entre 7 y 8 (Edwards & Lofty 1977), no tolerando pH-s por debajo de 5 (Edwards & Arancon 2004).

**Alimentación**

Las lombrices se alimentan de **materia orgánica** en descomposición determinándose su abundancia en función de la cantidad de materia orgánica disponible (Vliet *et al.* 2007). En concreto, las lombrices epigeas (Bouché 1977) se alimentan de la materia derivada del aporte de la vegetación ingiriéndose conjuntamente tanto partículas minerales, que facilitan la molienda del material vegetal facilitando la asimilación de nutrientes (Curry & Schmidt 2007), como

meso y micro biota del suelo (Curry & Schmidt 2007). Se encuentran mayores densidades en hábitats de bosques de hoja caduca o en praderas en las cuales hay un aporte de material herbáceo derivado de la siega. No obstante, su abundancia en bosques de hoja caduca puede estar condicionada a la discontinuidad en el aporte de materia por parte de la vegetación, limitando así la disponibilidad de alimento.

A pesar de que la presencia de oligoquetos detritívoros terrestres venga determinada por la disponibilidad de materia orgánica, no es tanto la cantidad de alimento como la **calidad** del mismo la que determina la abundancia de estos macroinvertebrados. Tasas de crecimiento y abundancias poblacionales altas se correlacionan positivamente con la disponibilidad de carbohidratos fácilmente asimilables (Curry 1998; Tiunov & Scheu 2004) y negativamente con el contenido de lignina del alimento (Hendriksen 1990). En este contexto, las tasas de crecimiento más altas las reportan trabajos realizados con estiércol animal (Kaushik & Garg 2004; Siddique *et al.* 2005) las cuales presentan un alto contenido de materia orgánica, en el caso del estiércol de caballo alrededor del 85 %, correspondiendo una gran parte de ella a la fracción de carbohidratos solubles. También, se ha encontrado correlación positiva entre el contenido de nitrógeno y proteína del suelo y la abundancia de lombrices, estando el nitrógeno considerado como un factor limitante para el crecimiento de las lombrices de tierra (Curry 1998).

En cuanto a las preferencias nutricionales, la palatabilidad es variable en los distintos tipos de hojas, correlacionándose negativamente con el contenido de polifenoles (Satchell & Lowe 1967). Los fenoles en las plantas se encuentran glucosidados, lo cual reduce enormemente su toxicidad. Al producirse la necrosis del tejido vegetal, la fracción glucosídica se hidroliza liberándose así estos compuestos aromáticos tóxicos para los organismos del suelo (Pridham 1965) que podrían producir un rechazo a un alimento con un alto contenido de polifenoles. En un estudio de la influencia de la dieta sobre el crecimiento y la reproducción de *E. andrei* (Domínguez *et al.* 1997) las tallas máximas (MLW) alcanzadas por los animales en cultivos individualizados se distribuían de la siguiente manera: MLW en paja > MLW en acículas de pino > MLW en corteza de pino = MLW en hojas de roble > MLW en helecho. Los autores

atribuían estas diferencias a la cantidad de polifenoles de los substratos y a la digestibilidad de la materia orgánica.

## 3.1.2. Factores bióticos

En este apartado se hablará de dos de los factores bióticos más importantes que van a determinar el hábitat y el ciclo de vida de esta especie: Competencia intraespecífica y mutualismo con los microorganismos del suelo.

### Competencia

Hablaremos de competencia en términos de competencia intraespecífica. En este sentido, la **densidad poblacional** es otro factor que va a condicionar los parámetros del ciclo de vida de la lombriz. Numerosos estudios han demostrado una disminución en el peso individual a mayor densidad poblacional (Domínguez & Edwards 1997; Kammenga *et al.* 2003; Unuofin & Mnkeni 2014). Los efectos adversos sobre el crecimiento en densidades altas son debidos a la competencia intraespecífica por el substrato y al proceso de la reproducción.

### Mutualismo

Existe una relación mutualista entre los microorganismos del suelo y las lombrices (Trigo *et al.*1999). Esta relación podría resumirse en que mientras que las lombrices proporcionan un medio idóneo que favorece la actividad microbiana, los microorganismos ayudan en la degradación de la materia orgánica, proporcionando metabolitos fácilmente asimilables para las lombrices, aumentando así su capacidad digestiva a una tasa conforme a sus demandas metabólicas (Lavelle *et al.* 1997). Brown *et al.* (2000), en un extraordinario análisis de las interacciones entre los distintos componentes del suelo, relatan que esta relación mutualista está basada en la "paradoja de la Bella Durmiente":

> "The basis of this paradox is that soil microbial communities (the 'Sleeping Beauties') have the ability to digest almost any organic substrate yet are dormant most of the time, because they need assimilable carbon (food resources) but have a limited ability to move throughout the soil in order to reach these resources. Earthworms (the 'Prince Charming') secrete mucus ('the Kiss' = resources), move within the soil and provide the suitable temperature, moisture and organic resources within their guts for microbes to be activated. This activation by an extra contribution of assimilable C is what Jenkinson (1966) called a 'priming effect'."

> (Extraído de Brown *et al.* 2000)

La relación mutualista no tiene únicamente lugar en el interior del las lombrices. Los anélidos funcionan como transformadores del mantillo vegetal: reducen el tamaño de partícula a su paso por la molleja, añaden azúcares y moco y proporcionan sustancias derivadas de la excreción. Todo ello hace que las heces de las lombrices sean muy ricas en carbohidratos solubles y sustancias nitrogenadas, de modo que proporcionan una fuente externa de recursos para los organismos del suelo (Domínguez *et al.* 2009). La reducción del tamaño de partícula es de especial interés cuando el material vegetal es rudo y correoso, y los microorganismos no pueden atacarlo directamente. Por otra parte, en ocasiones la materia orgánica requiere un procesamiento previo llevado a cabo por la microbiota del suelo (p. ej. sustancias con alto contenido de fenoles) antes de que pueda ser asimilada por las lombrices (Curry 1988).

Además de todo lo expuesto, la microbiota del suelo constituye una fuente de alimento vivo para las lombrices, ya que se han encontrado evidencias de procesamiento de estos microorganismos en el tubo digestivo de las lombrices (Curry & Schmidt 2007).

Debido a la relación mutualista, las lombrices tienen un alto impacto en la estructura de las comunidades microbianas (Postma-Blaauw *et al.* 2006; Gómez-Brandón 2011). Todo ello, repercute en los ciclos de la materia orgánica y en la estructura física y química del suelo que pasaremos a explicar en el siguiente apartado.

## 3.2. Relaciones con el medio

Las lombrices de tierra, junto con los microorganismos edáficos, son considerados ingenieros ecológicos ya que modifican las propiedades físicas y químicas del suelo (Ojha & Devkota 2014). El grupo ecológico al que pertenece esta especie, debido a su naturaleza epigea, afecta a la capa más superficial del suelo, en la que la materia orgánica se va acumulando por aporte de la vegetación, luego la actividad de estos organismos va a afectar principalmente a los ciclos de materia orgánica y a la biodisponibilidad de nutrientes. Por otra parte, es importante mencionar su implicación en la génesis del suelo.

### 3.2.1. Efecto de las lombrices sobre los ciclos de materia orgánica

Las lombrices de tierra, en especial el grupo ecológico de las lombrices epigeas, consumen gran parte de la materia orgánica que se deposita en el suelo anualmente (Curry & Schmidt 2007), luego es indiscutible la importancia que tienen sobre los ciclos de materia orgánica (Lemtiri *et al.* 2014). La primera fase en la que actúan es en la fragmentación de la materia. A pesar de que los microorganismos del suelo pueden procesar directamente el tejido vegetal más lábil, los restos vegetales más enriquecidos en lignina y celulosa requieren un procesamiento previo antes de que puedan ser atacados por los microorganismos. En este contexto, las lombrices epigeas fragmentan la materia orgánica y se produce el primer ataque enzimático. Como se ha mencionado previamente, durante el paso por el tubo digestivo, la materia orgánica se va modificando: se produce una adición de azúcares, se modifica la composición y la actividad microbiana y se homogeniza y modifica la materia por acciones enzimáticas, tanto intrínsecas como por actividad microbiana endosimbionte (Domínguez *et al.* 2009).

Como resultado de la actividad de las lombrices se produce una reducción de la hojarasca y de la materia orgánica acumulada en el suelo, se incrementa la actividad de los microorganismos, aumenta la disponibilidad de nutrientes y se produce una modificación de la comunidad edáfica microbiana (Brown *et al.* 2000). Las lombrices de tierra también están implicadas en el proceso final de degradación de la materia orgánica, produciendo una aceleración del proceso de humificación gracias a la acción de su flora endosimbionte (Edwards & Lofty 1977). Todo ello, presenta gran implicación en la fertilidad del suelo.

### 3.2.2. Efecto de las lombrices sobre la fertilidad del suelo

La actividad de las lombrices aumenta la cantidad de nitrógeno mineralizado en el suelo (Cortez *et al.* 2000), haciéndolo biodisponible para las plantas. Para que las plantas puedan asimilar el nitrógeno, es necesario que el ratio C/N se sitúe en valores por debajo o iguales a 20/1 (Edwards & Lofty 1977). Los anélidos son capaces de disminuir ese ratio mediante los procesos metabólicos de consumo de carbono (respiración) en el cuerpo del animal (Edwards & Lofty 1977). Además, las excretas metabólicas (urea y amonio) y los productos de defecación están

muy enriquecidos en nitrógeno mineral (Dash & Patra 1977, 1979) y el mucus producido por la actividad excavadora de las lombrices presenta un bajo ratio C/N por su alto contenido en sustancias glicoproteícas y aminoácidos de bajo peso molecular (Martin *et al.* 1987). Por otra parte, en suelos con presencia de lombrices se ha encontrado una mayor capacidad de intercambio catiónico: las heces producidas por las lombrices se encuentran muy enriquecidas en iones Ca, Mg, P y K intercambiable lo cual enriquece enormemente la fertilidad del suelo (Lemtiri *et al.* 2014).

### 3.2.3. Pedogénesis y estructura del suelo

Las lombrices, como habitantes edáficos tienen un papel importante en la formación de nuevas capas de suelo (Ojha & Devkota 2014). En el contexto de las lombrices epigeas, que habitan los primeros centímetros del suelo, los procesos de degradación y mineralización de la hojarasca ayudan a la formación de la estructura del suelo: los productos de egestión de las lombrices, "cast", y su actividad excavadora superficial, contribuyen a la formación y estabilización de agregados en el suelo. Estos agregados pueden pasar a capas más internas del suelo mediante la acción de otros grupos ecológicos (endogeas y anécicas) proporcionando materia orgánica parcialmente degradada a las capas del suelo menos enriquecidas.

# 4. Aplicaciones: Industria y conservación

## 4.1. Industria del vermicompostaje

*E. andrei*, junto con su especie hermana *E. fetida*, han sido dos de las especies más utilizadas en la industria del vermicompostaje. A este respecto, se han realizado múltiples estudios con distintos tipos de substratos como estiércol de caballo (Sangwan *et al.* 2008), de vaca (Kaushik & Garg 2004; Siddique *et al.* 2005; Yadav & Garg 2011), de oveja (Siddique *et al.* 2005), de cerdo (Reeh 1992; Domínguez *et al.* 1997), de ganado en general (Bansal & Kapoor 2000; Gunadi *et al.* 2002), de aves de corral (Yadav & Garg 2011), heces humanas (Yadav *et al.* 2011), lodos residuales (Hartenstein & Hartenstein 1981) y mezclas de los mismos con diversos materiales como papel, cartón, hierba, serrín y residuos de comida (Domínguez *et al.* 2000), residuos de cocina (Tripathi & Bhardwaj 2004), residuos de la industria alimenticia (Yadav &

Garg 2011), residuos vegetales (Frederikson *et al.* 1997), compuestos lignocelulósicos (Vincelas-Akpa & Loquet 1997), restos de las cosechas (Bansal & Kapoor 2000) y residuos de la industria del algodón (Albanell *et al.* 1988), entre otros.

Esta industria, además de proporcionar un excelente abono natural para las plantas, puede llegar a tener una gran importancia en el reciclaje de los residuos sólidos derivados de la actividad humana. Una última aplicación podría ser la utilización de la carne de lombriz para elaboración de piensos de animales.

## 4.1.1. Abonos naturales: vermicompost

Después de la revolución verde, la comunidad científica ha abandonado su interés por los sistemas de agricultura que se basan en la utilización de fertilizantes artificiales y pesticidas, para centrarse en sistemas en los que se usan materiales orgánicos compostados como medio para incrementar la fertilidad del suelo (Jack & Thies 2006). Este actual interés por la aplicación de esta técnica de tratamiento de los abonos y residuos vegetales, deriva de la concienciación pública acerca de la pérdida de nutrientes y la eutrofización de los ecosistemas acuáticos por la sobre-aplicación en los suelos de abonos de origen animal sin previo tratamiento. A pesar de que el compostaje implica una pérdida global de nutrientes (Sommer 2001), numerosos estudios han demostrado efectos beneficiosos de este tipo de tratamientos para las plantas y el ecosistema en comparación con sistemas en los que se aplican el material orgánico sin compostar o fertilizantes sintéticos (Buckerfield *et al.* 1999; Arancon *et al.* 2003; Loecke *et al.* 2004; Lazcano *et al.* 2009).

Existen dos tipos principales de tratamientos: Compostaje, que requiere una fase de calentamiento; y, vermicompostaje. El **compostaje** es un proceso biooxidativo controlado que, a partir de un substrato orgánico heterogéneo en fase sólida y pasando por una fase termofílica y una liberación temporal de fitotoxinas, conduce a la producción de dióxido de carbono, agua, minerales y materia orgánica estabilizada (Zucconi & De Bertoldi 1987). Los encargados de llevar a cabo este proceso son los microorganismos. Por otro lado, el **vermicompostaje** ha sido definido como el proceso de biooxidación y estabilización de la materia orgánica gracias a la acción conjunta de lombrices y microorganismos (Aira *et al.* 2002). A diferencia del compostaje

común, en el vermicompostaje no tiene lugar una fase de calentamiento y permite mantener una amplia diversidad de organismos a lo largo de todo el proceso. Existe una categoría intermedia que consiste en combinar ambas técnicas, en la que inicialmente tendría lugar una fase de pre-compostado parcial a altas temperaturas para terminar con una fase final de vermicompostaje (Frederikson *et al.* 1997).

Entre los beneficios del vermicompost, en comparación con el compostaje común y obviamente con la aplicación del material orgánico sin compostar, se citan (Gajalakshmi & Abbasi 2004):

1. Aumento de la capacidad de retención de agua del suelo

2. Incremento de nutrientes en formas asimilables por las plantas

3. Aumento de la densidad de microorganismos

4. Reducción del ratio C/N

5. Presencia en las excretas de las lombrices de hormonas y encimas que estimulan el crecimiento de las plantas y disuaden a los patógenos

La demanda de compost en agricultura y jardinería se encuentra en auge y permite contemplar la creación de plantas de producción de compost. Las importaciones de turbas para la incorporación a enmiendas y sustratos alcanzan anualmente una cifra en torno a 165.000 Tm. que, en el futuro podrán ser sustituidas por compost de calidad. Hay que señalar que por ausencia de controles de calidad en los suministros de materias primas, de normativa para los procesos de compostaje y de instrucciones de uso e informaciones sobre el compost, las salidas comerciales de estas producciones no cuentan con la necesaria imagen de prestigio entre los consumidores y tampoco hay diferenciación clara de productos que signifique transparencia de cara a la demanda. Entre las medidas que se priorizan para optimizar el sector se encuentra la definición de procedimientos y analítica para el control sistemático y seguimiento del proceso de compostaje y la promoción de plantas de compostaje locales, en las que participen organizaciones de agricultores (p. ej. cooperativas) y otros interesados en el sector de abonos, con objeto de atender mercados de ámbito local y comarcal. En la actualidad, no existen apenas plantas de compostaje de dimensión pequeña o media, gestionadas por agricultores y promotores locales, que aprovechen residuos agro-ganaderos y agro-industriales de la propia

zona, y tengan una comercialización con bajos costes, atendiendo la demanda agrícola de su entorno.

## 4.1.2. Reciclaje de los residuos

Dada la actual gestión de residuos, y pese a la cada vez mayor separación y reciclaje de los mismos, los residuos sólidos, levantan polémica tanto por su tratamiento mediante incineradoras como por su almacenamiento en vertederos. Ya que el porcentaje de materia orgánica presente en los mismos alcanza el 50-60 %, se puede plantear el vermicompostaje como forma de transformación de un residuo en dos productos: vermicompost y carne de lombriz. De hecho, en numerosas comunidades españolas las instituciones locales (ayuntamientos, mancomunidades) están fomentando el compostaje individual como un método integrado en las políticas de desarrollo sostenible. Si bien los beneficios del vermicompost parecen evidentes, y, como se ha comentado anteriormente, aumenta la literatura sobre compostaje con *E. fetida y E. andrei* sobre gran variedad de substratos, muy habitualmente en países emergentes, p. ej. Sudamérica, en los que el compostaje de residuos orgánicos de basuras y procedentes en muchos casos del sector primario como plantaciones de caña de azúcar, efluentes ganaderos etc. (Castillo *et al.* 2000; Hernández *et al.* 2006; Gutiérrez-Vázquez *et al.* 2007) aparece como una alternativa económica a la gestión de residuos, no abundan los trabajos que intenten sistematizar la metodología y características del vermicompost y su influencia sobre los gusanos (Leroy *et al.* 2007), lo cual resta posibilidades de comercialización estable como abono. Desde este punto de vista, esta tesis proporciona información sobre los procesos fisiológicos del agente biotecnológico animal (*E. andrei* como ingeniero ecológico) enfrentado a distintos substratos. Si bien este trabajo se centra en substratos de origen natural (bosta de caballo, hierba y suelos), proporciona información que podría ser utilizada en el procesamiento de residuos de la agricultura, así como en tratamientos de abonos.

## 4.1.3. Carne de lombriz para elaboración de piensos

Como ya se adelantaba en el apartado anterior, uno de los subproductos del vermicompostaje es la carne de lombriz. Dada la alta prolificidad y tasas de crecimiento tanto de *E. andrei* como *E.*

*fetida*, en condiciones óptimas lombrices de 200 mg pueden llegar a duplicar su biomasa en menos de dos semanas. Por otra parte, aproximadamente el 70 % de la biomasa seca corresponde a sustancias proteicas, a menudo limitantes en los piensos por su alto coste. Además, la carne de lombriz es muy rica en aminoácidos esenciales tales como lisina y metionina, siendo sus valores mayores que en la carne o el pescado (Edwards 1985). Por todo ello, se postula su utilización como fuente de proteína para la elaboración de piensos comerciales. No obstante, para que esta práctica sea rentable, es necesario sistematizar la producción manteniendo los cultivos de lombrices bajo un riguroso control de las condiciones ambientales y, los residuos (fuente de alimentación) deben ser añadidos periódicamente para permitir a las lombrices explotar al máximo la materia orgánica. Esta práctica podría resultar de especial interés en la industria de la acuicultura, ya que en muchas ocasiones en las piscifactorías se utilizan harinas de pescado como fuente de alimentación, que suponen un alto coste y, como se ha mencionado anteriormente, presentan una menor cantidad de aminoácidos esenciales que la harina de lombriz.

## 4.2. Conservación

En el apartado dedicado a las relaciones de estos organismos con el medio, ha quedado constancia de su alta implicación en el mantenimiento de las propiedades del medio edáfico. Desde este punto de vista, las lombrices son organismos clave en la conservación de la calidad del suelo y podrían utilizarse como herramientas de manejo del medio.

En concreto, en ecosistemas agrarios, el establecimiento de poblaciones activas de lombrices, ayudaría al mantenimiento de la fertilidad y calidad del suelo. De hecho, Lavelle *et al.* (1989) lo confirman en un artículo reflexivo sobre el manejo de las lombrices en dichos ecosistemas y Jouquet *et al.* (2014) realizan una revisión acerca del manejo de las lombrices de tierra para la restauración de la funcionalidad de los ecosistemas dañados. En relación a las lombrices epigeas, como ya se ha comentado en apartados anteriores, tienen un papel muy importante como fertilizantes naturales del suelo, proporcionando nutrientes fácilmente asimilables por las plantas. De este modo, en ecosistemas muy explotados, en los que la lixiviación de nutrientes
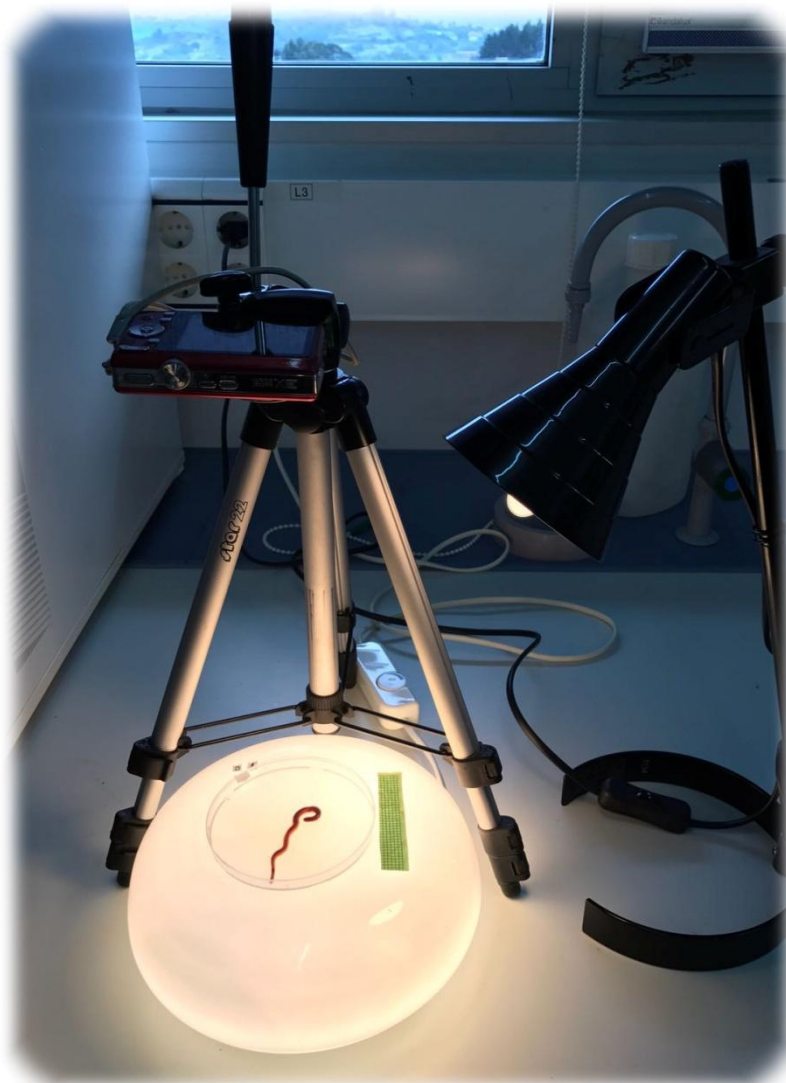
hacia las aguas subterráneas y la acumulación de metales pesados constituyen un problema global, la introducción de lombrices acompañada de la adición de materiales orgánicos, como pueden ser abonos de origen animal, podría revertir la situación crítica a la que se ha llegado tras años de explotación agraria.

Por otra parte, su capacidad para eliminar del medio substancias tóxicas, como herbicidas (Cao *et al.* 2015), pesticidas (Kelsey *et al.* 2008), metales pesados (Li *et al.* 2010), hidrocarburos aromáticos (Natal-Da-Luz *et al.* 2012) etc., les otorga una función biorremediadora. La introducción de lombrices en el medio facilita la creación de nuevas capas de suelo y el establecimiento de la vegetación. Así mismo, promueve la productividad primaria y la movilización de los  metales pesados. Sin embargo, estos efectos beneficiosos son muy poco predecibles, ya que son muy variables en función de las características del suelo y de la especie de lombriz en cuestión. Una de las mayores desventajas de la utilización de estos individuos para la restauración de ecosistemas dañados es que los metales que acumulan pueden pasar a otros niveles de la cadena trófica (Jouquet *et al.* 2014). Además, resulta complicada la extracción de estos organismos bioacumuladores del suelo con el fin de eliminar las substancias nocivas, bien por la dificultad para capturarlos o bien por el impacto que dicha extracción podría tener sobre el ecosistema edáfico. Por otra parte, estos organismos podrían utilizarse también como bioindicadores de calidad de suelo (Reinecke & Reinecke 1998). De hecho, existe un protocolo establecido por la OECD (1984) para la realización de experimentos toxicológicos con lombrices de tierra. En esta línea, son numerosos los trabajos realizados con *E. andrei* y *E. fetida* en suelos contaminados en los que se analiza el efecto de un determinado contaminante sobre los parámetros fisiológicos de estos organismos (Landrum *et al.* 2006; Xiao *et al.* 2006; Wang *et al.* 2012; Piola *et al.* 2013, entre otros). De este modo, realizando este tipo de experimentos en suelos en los que se pueda sospechar de la presencia de algún tipo de contaminante, se podría determinar su posible efecto sobre los organismos del suelo, y sobre la cadena trófica en general, mediante la extrapolación del efecto que causa en las lombrices, pudiendo determinar, parcialmente, la calidad de un determinado medio edáfico.

# Chapter 2

## 2. Biomass assessment in annelids:

## A photogrammetric method suitable for

## hatchlings and adults developed in *Eisenia andrei.*

# Biomass assessment in annelids: A photogrammetric method suitable for hatchlings and adults developed in *Eisenia andrei.*

## Abstract

A simple photogrammetric, non-destructive method, to measure individual biomass in tubular soft-bodied organisms has been developed in *Eisenia andrei*. Photographic procedures can be easily performed with low cost digital cameras and number of pictures to be processed can be reduced to two per animal (VC $\leq$ 3.5 %) even at sizes ~10 mg live weight. Image analysis has been undertaken using CobCal 2.0© software. No bias is induced by body position and accuracy in terms of the regression coefficient of the equation ($y=a*x^b$) relating portrayed area (mm$^2$) to live weight (mg) is 98 %. Two different procedures have been designed for laboratory and field uses and since no differences between methods appear at sizes over eight mg live weight a common function relating image area to live weight results (slope = 0.681; intercept = 3.27). Below 8 mg, weight exponent remains unchanged but the value for the elevation rises to 4.21 indicating an increase of surface exposure to camera lens in newly-hatched worms: visible area regarding geometric area (considering a cylinder shape) enlarges from 34 % to 43.52 %. As a conclusion this non-invasive procedure has proved suitable in worms ranging from sizes of 0.2 to 3 000 mg live weight to determine biometric parameters such as length, volume, surface or body weight, that are key factors to interpret physiological responses underlying growth patterns.

# 1. Introduction

The utilization of non-disturbing sampling techniques which would provide a permanent historic record of photo-samples has been long encouraged in the context of surveys of conservation status particularly as regards populations of marine benthic organisms (Littler & Littler 1985; Ponder *et al.* 2002). More recently, developments in software and imaging technology have supplied easy managing and inexpensive tools that provide accurate and repeatable measures that have extended the scope of photogrammetric methods to quantitative field studies of biomass evolution and growth rates in aquatic invertebrates (Bernardini *et al.* 2000; Vanaverbeke *et al.* 2003; Page *et al.* 2005; Abdo *et al.* 2006). Advantages of these methods in the context of growth studies include the possibility of accessing to early stages of the life cycle minimizing damage associated to frequent handling, allowing simultaneous recording of different biometric parameters and providing the means for long-term individual registers of biomass in order to study population dynamics. Concerning terrestrial invertebrates, Perea *et al.* (2008) have applied it to evaluate growth in small juveniles of *Cantareus aspersus* snails. In this work undertaken with *Eisenia andrei* (Bouché 1972), our objective has been to standardize conditions leading to obtain reliable quantification of individual biomass, length, surface, volume, and hydration level for long-term surveys of tubular soft-bodied organisms by means of photogrammetric techniques. For this purpose, we have established the minimum number of images to be taken to obtain area measurements, evaluated accuracy and precision in laboratory conditions and explored the effectiveness under field conditions.

# 2. Material and methods

## 2.1. Biological material and maintenance condition

Specimens of *Eisenia andrei* within a size range from 0.2 to 3 132 mg of live weight (n = 474) reared in captivity were individually weighed (live and dry weight), measured (volume, length and radius) and repeatedly portrayed. The annelids were maintained in darkness in a growth chamber under controlled conditions of temperature (20 ± 2 ºC) and humidity (80 ± 3% RH),

and fed with horse manure (86 % organic matter, pH = 7.413 ± 0.023) collected from horses maintained in an Atlantic meadow under semi extensive conditions.

## 2.2. Length, volume and weight measurements

A group of 60 worms ranging from 2.3 to 681 mg of live weight were hand sorted from culture vessels and individually measured for length (L = mm) and radius (*r* = mm) with a 0.05 mm precision manual calliper. Geometric Lateral Surface (GS) and Geometric Volume (GV) were calculated according to the formula $2\pi rL$ and $\pi r^2 L$ respectively. Live and dry weights (LW and DW) were obtained using a $10^{-5}$ digital analytical balance. Prior to live weight determinations, animals were carefully cleaned according to the following procedure: short immersion in a small Petri dish containing de-ionized water and drying with a moist cloth verifying no injuries were inferred and removing any external traces of water. A subsample of 57 individuals was used to calculate volume by immersion according to Archimedes principle.

## 2.3. Photogrammetric method

Photographs were taken with a Kodak© Easyshare M763 digital camera (Focal length equivalent from 34 to 102 mm; Optical sensor size 5.744; Maximum aperture F2.8-F5.1). In order to favour simplicity in data acquisition camera parameters were fixed preferentially in the automatic mode: sensor resolution 7MP (3072 x 2304); Light sensitivity: ISO auto (64-320); Exposure Metering: Multi-segment; Shutter speed: automatic (from 4 to 1/1400 sec); Optical zoom 3X; Macro Focus range from 10 to 70 cm; Focus adjustment: autofocus multi-area; Focal distance: 25 cm (tripod).

Animals were placed in Petri dishes with a graduated reference (a grid: 4 mm² per square). Dishes of different diameters adjusted to worm size were required (Fig. 1).

Two photograph taking procedures were used. In the, from now on called "Field Method" (FM), the grid was situated under the Petri dish, while it was placed aside in the alternative "Laboratory Method" (LM). Caution to keep the ventral surface of every individual in contact with the bottom of the Petri dish was kept in both procedures.
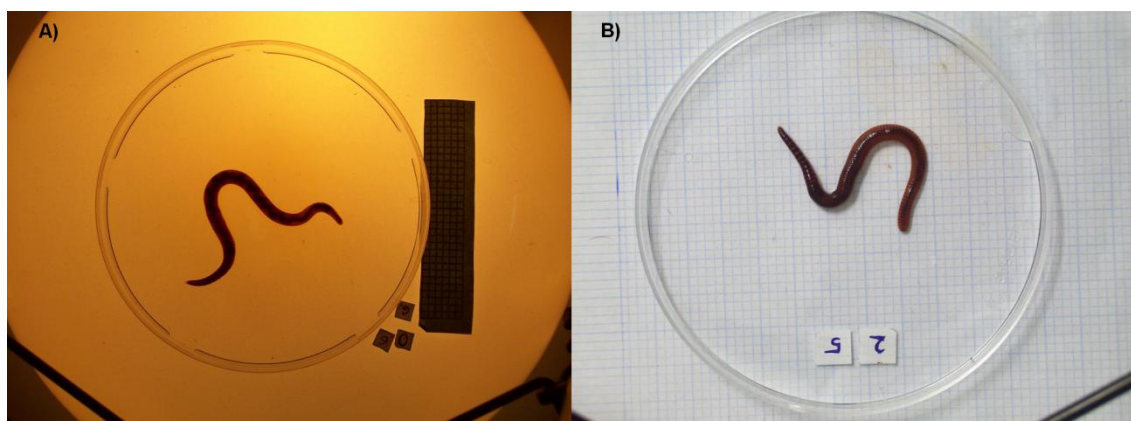
**Fig. 1**. Digital photographs of *Eisenia andrei*. A: Laboratory Method (LM). B: Field Method (FM).

In the LM worms (n = 294) were carefully cleaned from debris prior to taking pictures and illumination was situated underneath the animals (Fig. 1 A) allowing direct determination of surface displayed in the image. In contrast, in the FM, a total number of 123 individuals were readily portrayed, either with light situated over the animals or without any artificial illumination (Fig. 1 B) and photographs required subsequent image treatment with Adobe® Photoshop® to define clear borders.

Since the final objective was transforming surface data into weight figures across a wide size gradient while retaining maximum accuracy at the lower segment of worm population (newly hatched $\leq 0.8$mg live weight), three sources of variability were tested. Initially, effect of position and body shape and minimum number of images required to attain least possible inter-pictures variation was evaluated using the FM. Later, differences in feeding and digestive condition have been analyzed by means of the LM.

### 2.3.1. Influence of body shape and position on surface determination

Observation and careful handling of worms led us to standardize 3 positions in which pictures could be taken: fully extended, coiled and intermediate (Fig. 2). Since expected error would presumably increase at lower sizes, 72 individuals ranging from 10.1 to 334.7 mg of LW were selected to obtain details of precision preferentially in smaller animals.
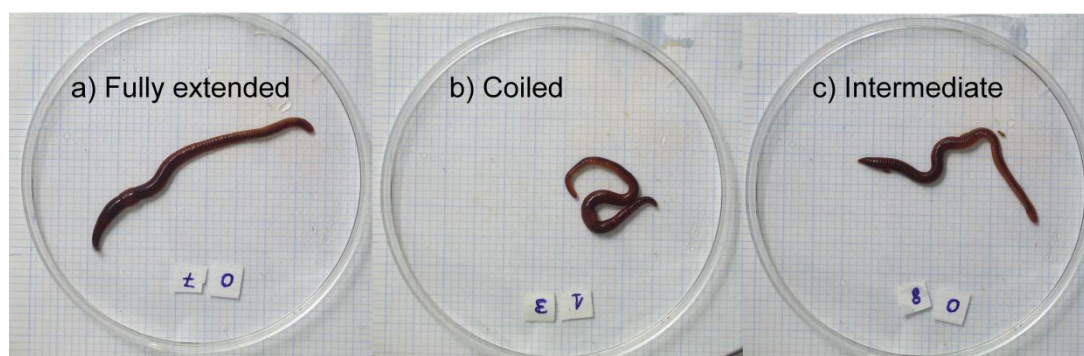
**Fig. 2**. *Eisenia andrei* images taken in the three standardized positions.

Two different procedures were assayed. A preliminary experiment undertaken with a group of 20 individuals involved photographing single specimens having been forced to adopt the three positions alternatively, and more than one picture was taken in every position in most cases. Later, a broader experiment was performed in which specimens within a group of 72 individuals were repeatedly portrayed (5 pictures per worm to a total of 561 pictures) of which 371 adopted the intermediate position, 138 were coiled and 52 fully extended.

### 2.3.2. Number of images taken

A set of 80 individuals (10.1 to 446.7 mg of live weight) was distributed in 7 groups (n = 10-14) according to the number of pictures to be taken: 2,3,4,5,6,7 and >7 (8,9,10). Size differences within groups were favoured as well as no differences as regards mean LW. Pictures of worms spontaneously appearing as fully extended or in the intermediate position were employed. FM conditions pertained to this trial.

### 2.3.3. Influence of feeding conditions on surface determination and weight

Routine rearing of worms precludes knowing feeding activity of a given organism. In order to test the influence of feeding conditions on both live weight and image based size calculations, 38 individuals were fasted along 48h and live weight registered and pictures taken at daily intervals (0, 24 and 48 hours) the latter according to the LM. Starvation took place in individual chambers with a moist cloth that was kept at saturation and faeces were removed periodically.

### 2.3.4. Photographic Surface (PS) estimations

Digital images were transferred to the computer in JPG format and individually processed using CobCal® 2.0 software. The programme, easily handled, is based in pixelization and

colorimetric analysis of images where the selected shape to be measured is encoded as a positive colour and identified against a background (negative colour). Precision is set up by default at the highest level and results are given as a fraction of total delimited area (in our case a circle of adequate diameter).

## 2.4. Biomass conversion and statistical analysis

Standard procedures for DW determinations (dehydration after oven drying 24 hours at 80 ºC) were applied to animals under two feeding regimes: currently fed (n = 50) and fasted (n = 49) along 48 hours (digestive voided). A wide size range of animals with similar mean LW was employed and photographic surface (PS), LW and DW were simultaneously recorded.

PS measurements ($mm^2$) were related to LW (mg) by means of the equation $Y = a*X^{\,b}$. Linear regression procedures (after logarithmic$_{10}$ transformation of data) followed by ANCOVA and TUKEY analysis, have been used to ascertain significance of differences between both regression coefficients and intercepts.

# 3. Results

## 3.1. Biomass determinations

Scaling of dry to live weight (in mg) was undertaken separately in two groups of worms, fed and fasted along 48 hours. While in fed worms both live and dry weights included gut contents, in fasted animals two relationships were established: one excluding gut contents in which DW related to final LW (after 48 h starvation) and one in which entire (initial) LW related to DW. Linear regression equations obtained after log-log transformations are shown in Table 1.

LW regression coefficients closely approached unity in both feeding conditions. Nevertheless, high $R^2$ values (over 99 %) and sample size resulted in significance differences between slopes:

      (F1)  Fed: $DW = 0.1*LW^{\,1.025}$

      (F2)  Fasted $_{(both\ voided)}$: $DW = 0.16*LW^{\,0.986}$

**Table 1**. Coefficients of Log-Log regression equations of Dry Weight (DW = mg) vs. Live Weight (LW = mg) of *Eisenia andrei*. Differences in individual equations for gravimetric measurements in different feeding conditions are analyzed through ANCOVA. SD = standard deviation; DF = degrees of freedom; $CI_{95\%}$ = Confidence intervals.

| Feeding conditions | N | Mean Live Weight | SD | Size range (mg) | $R^2$ | Intercept ($CI_{95\%}$) | Slope ($CI_{95\%}$) |
|---|---|---|---|---|---|---|---|
| Fed | 50 | 186.62 | 138.03 | 10.10 - 446.7 | 0.996 | (-0.996) ± 0.043 | 1.025 ± 0.019 |
| Fasted | 49 | 197.57 | 139.58 | 1.70 - 648.6 | 0.992 | (-0.793) ± 0.055 | 0.986 ± 0.025 |

| ANCOVA | DF | F-Value | P-Value |
|---|---|---|---|
| **Slope** | 1, 94 | 6.168 | 0.0148 sig. |

**Table 2**. Coefficients of Log-Log regression equations of Geometric and Immersion Volumes ($mm^3$) vs. Live Weight (mg) in *Eisenia andrei*. Differences between individual equations are analyzed through ANCOVA. DF = degrees of freedom; $CI_{95\%}$ = Confidence intervals.

| Method | N | Size range (mg) | $R^2$ | Intercept ($CI_{95\%}$) | Slope ($CI_{95\%}$) |
|---|---|---|---|---|---|
| **Immersion Volume** | 57 | 18.4 - 471.3 | 0.960 | 0.014 ± 0.111 | 0.995 ± 0.057 |
| **Geometric Volume** | 30 | 202.3 - 414.2 | 0.550 | 0.052 ± 0.850 | 0.983 ± 0.342 |

| ANCOVA | DF | F-Value | P-Value | Common Values |
|---|---|---|---|---|
| **Slope** | 1, 83 | 0.003 | 0.9549 No. Sig | 1.001 ± 0.040 |
| **Intercept** | 1, 84 | 0.168 | 0.6829 No. Sig | 0.004 ± 0.086 |

Assuming an isometric relationship between dry and live weight (see $CI_{95\%}$ for the slopes), flesh DW (mg) with digestive tube full represented 10 % of entire LW (mg) (F1) and the proportion of body water reduced from 90 % in fed animals (F1) to 84.3 % in fasted worms (F2). At this respect, in the sub sample set to eliminate intestinal content, LW (mg) change along starvation related to initial weight (mg) according to the linear function: *Live Weight* $_{(48h\ starved)}$ = 0.8668*Initial Live Weight* ($R^2$ = 0.979), indicating loss of 13.32 % of initial weight irrespective of size.

In order to transform LW data of currently cultured individuals into DW of the carcass, the following relationship relating voided DW (mg) to fed LW (mg) was calculated:   Fasted $_{(entire\ LW)}$: $DW = 0.13*LW^{0.994}$ ($R^2$ = 0.989; n = 50). In fact, ANCOVA comparisons between this equation and (F2) resulted in no differences between slopes (*p-value = 0.6962*) which described and isometric relationship, remaining the intercepts significantly different (*p-value < 0.001*). As a conclusion DW of flesh represented 13.5 % of entire LW of an active and presumably feeding worm.

Mean diameter was 2.98 mm ($\pm$ 0.80) and 76.67 % of specimen ranged from 2 to 3.5mm while mean length stood for 38.51 mm ($\pm$ 22.78) for a total distribution between 4.5 and 80mm. Length (mm) increased with size (mg) according to the allometric equation: *Length=* 0.8921*Live Weight* $^{0.6683}$ ($R^2$ = 0.9028; n = 72).

Diameter changes along growth showed an isometric pattern presenting a weight exponent of 0.3352. The combination of patterns exhibited by length and diameter indicated worms become slimmer while length /volume rise.

Since volume and weight are alternative measures to biomass computing, and considering that a cylinder is a good approach to worm shape, we initially tested two procedures to determine volume across a wide range of sizes: geometric calculations based on length parameters determined with a manual calliper and measures of water displaced by immersion. Results were analyzed by linear regression treatments, previous log-log transformation, and presented in Table 2. No statistical differences were evident as regards to both slope and intercept relating volume to LW and a common regression equation irrespective of method was calculated (Table 2; Fig. 3).
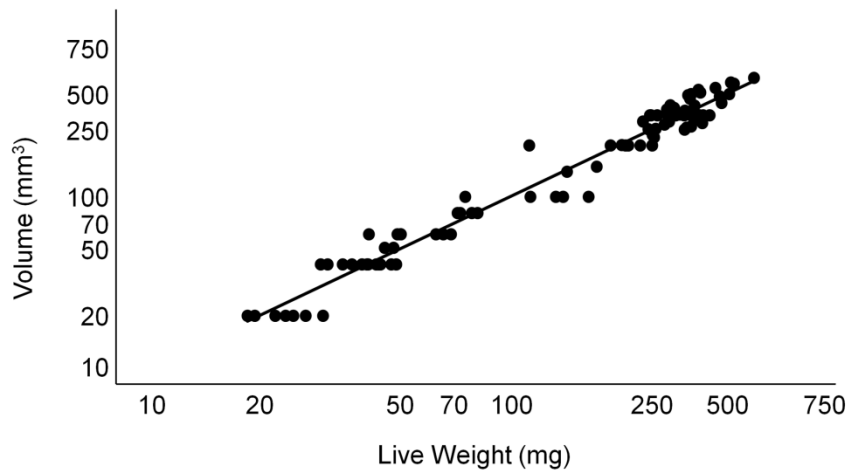
**Fig. 3**. Volume (mm$^3$) vs. Live Weight (LW = mg) in *Eisenia andrei*:
*Volume* = 1.009*$LW^{1.0011}$ (R$^2$ = 0.9671; n = 87).

Confidence intervals indicated $y = x$ (i.e. volume = LW) since slope and intercept were not significantly different from unity and zero respectively. As a consequence, density calculations reproduced water figures: LW: 1 mg; Volume: 1,009 ml; *Density* = $1/1,009$ = 0.991 ≈ 1 mg/ml. This result agrees with determinations of water content of worms above explained.

Surface to Volume ratio (S/V = mm$^2$ cm$^{-3}$) declined along with LW (mg) increase according to the equation: *S/V* = 9.0977*$Live\ weight^{-0.3239}$ (R$^2$ = 0.9573), lowering by a factor of 3 between 10 and 200 mg.

## 3.2. Photographic Surface (PS)

### 3.2.1. Number of pictures required

Analysis of accuracy in PS determination as related to the number of individual pictures taken (from 2 to 10) is indissolubly linked to simultaneous examination of inherent variation in body positioning of worms. Since as explained later, shape adopted while portraying exerted no significant effect, we tested accuracy according to the following procedure: groups of 7 to 14 worms were classified as regards the number of pictures taken to every individuals within the group (from 2 to 10 per worm) and a variation coefficient was calculated for each individual. Later, a mean variation coefficient was computed for each of the seven groups established (Fig. 4). Results of ANCOVA analysis of the means of variation coefficient, including LW as

covariate and an interaction term for the product of LW and number of pictures taken, are shown in Table 3. No significant differences were evident among the 7 groups and *p-values* largely exceed 0.05 for every factor: a common variation coefficient of 6 % was found irrespective of the number of pictures taken (Fig. 4).

**Table 3**. Results of ANCOVA analysis of the means of variation coefficient, including live weight (mg) as covariate and an interaction term for the product of live weight and number of pictures taken (between 2 and more than 7 pictures)

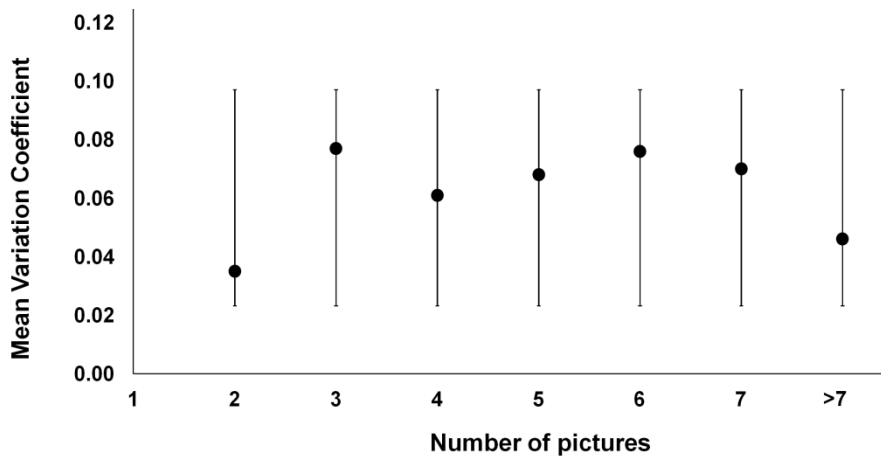| ANCOVA | F-Value $_{6, 64}$ | P- Value |
|---|---|---|
| **Number of pictures** | 0.370 | 0.8951 No. Sig |
| **Live Weight** | 1.752 | 0.1903 No. Sig |
| **Number of pictures*Live Weight** | 0.823 | 0.5565 No. Sig |



**Fig. 4**. Mean Variation Coefficients (MVC) vs. number of pictures taken per individual.

### 3.2.2. Photogrammetric *vs.* Geometric procedures

In order to calculate the proportion of total surface portrayed in a picture, photogrammetric measurements of surface were compared with GS of the cylinder ($2\pi rL$) obtained from calliper measurements (Fig. 5).
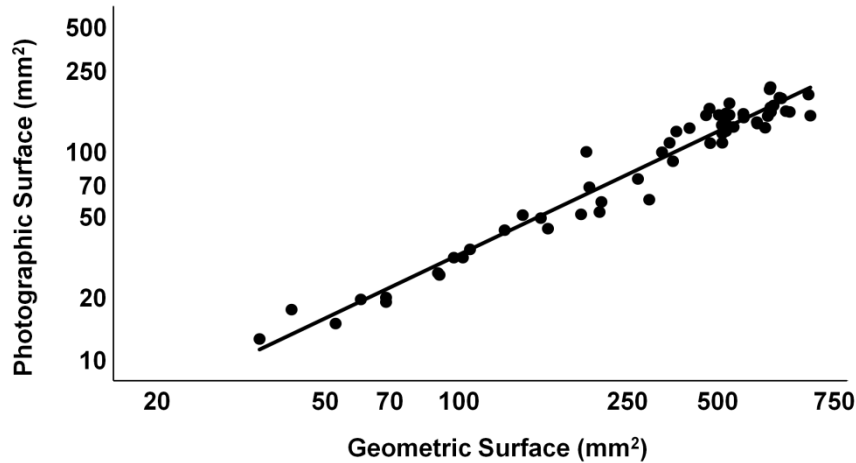
**Fig. 5**. Photographic Surface (PS = mm$^2$) vs. Geometric Surface (GS = mm$^2$) in *Eisenia andrei*: *PS* = 0.34*$GS$ $^{0.987}$ (R$^2$ = 0.958; n = 60). CI$_{95\%}$ is ± 0.055 for the slope and ± 0.1062 for the elevation.

A good correlation coefficient explaining 96 % of variability resulted for the isometric relationship between both sets of data and intercept and slope estimations were highly significant ($P < 0.001$). As a conclusion, the image displayed 34 % of GS (mm$^2$): *GS = PS /* 0.3404.

Photogrammetric measurements of surface can also be used to calculate volume as an alternative to the geometric or immersion methods: *Volume = GS*r / 2*, so, *Volume = PS*r /* 0.6808. Volumes so obtained, compare well with geometrical estimations (ANOVA: *p-value =* *0.4277*, $F_{1, 118}$ = 0.633).

## 3.3. Field method (FM)

Distribution of size frequencies (LW in mg) of the laboratory-cultured batch of *Eisenia andrei* used in this experiment is shown in Fig. 6. Since body size is directly related to age, we selected individuals from different sizes (from 8 mg to 464 mg) including a variety of growing stages. Two cohorts became evident: newly hatched and non-clitellated juveniles ranging from 8 to 110 mg (Mean = 45.941; SD = 30.324) and larger juveniles as well as clitellated and non-clitellated adults with LW from 140 mg to 464 mg (Mean = 293.537; SD = 77.06). Larger adults were excluded since expected error is likely to decrease at larger sizes.
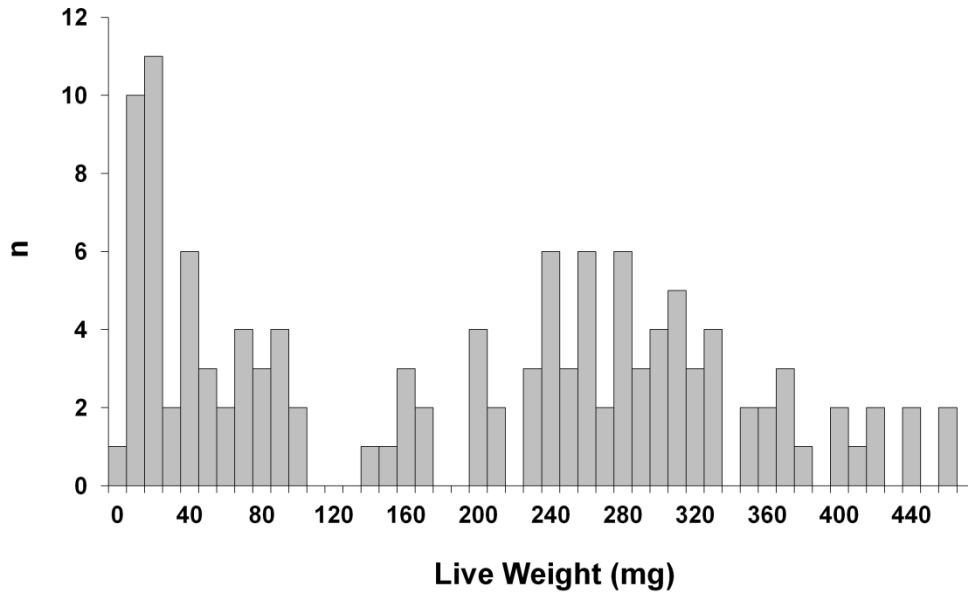
**Fig. 6**.Histogram for the frequency distribution of Live Weight (LW = mg) of specimens of *Eisenia andrei* used in the Field Method (FM).

### 3.3.1. Analysis of the effects of position and body shape

The possible influence of corporal position in the photogrammetric estimations of body surface and its effect on the relation with body weight was undertaken in two separate experiments summarized in Table 4. Results from the design in which a given worm (n = 20) was forced to adopt alternatively fully extended, coiled or intermediate position showed no differences in both parameters of the lineal regression equation relating estimations of PS to LW. Consequently, a common function with regression coefficient of $0.676 \pm 0.014$ and intercept of $0.472 \pm 0.029$ explaining 98 % of experimental variation was calculated.

In the next step, where images of 72 specimens within the same range of sizes were recurrently taken, identical results were obtained (Table 4). As a consequence, surface determinations based on digital images had a common relationship with live body weight irrespective of position, body shape or number of pictures taken per individual worm within a size range of 10 to 460 mg of LW. So, all data appearing in fig. 6 (n = 123) was pooled in a single equation shown in Fig. 7. The equation explained 98 % of variation and coefficients were highly significant ($p < 0.001$).

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**Table 4**. Coefficients of Log-Log regression equations of Photogrammetric Surface (PS = mm$^2$) vs. Live Weight (LW = mg) of *Eisenia andrei*. Differences in individual equations for photogrammetric measurements of body surface in different positions are analyzed through ANCOVA. DF = degrees of freedom; CI$_{95\%}$ = Confidence intervals.

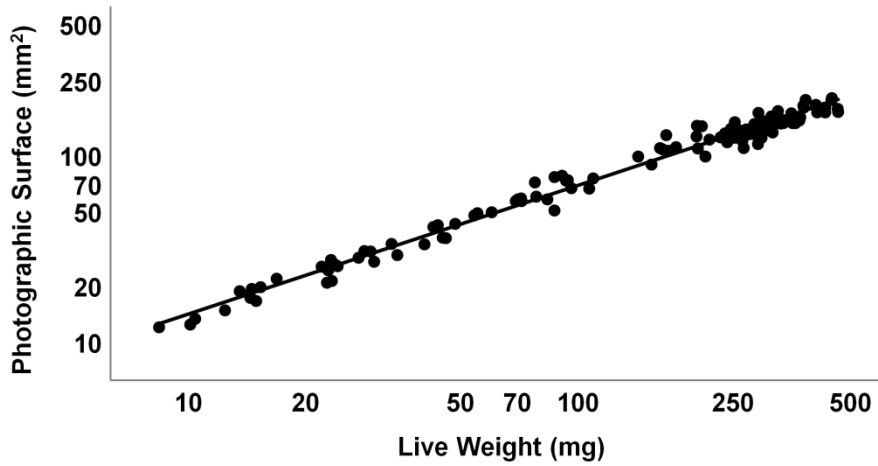| Method | Position | n | Size range (mg) | R$^2$ | Intercept (CI$_{95\%}$) | Slope (CI$_{95\%}$) |
|---|---|---|---|---|---|---|
| **Single worm exhibiting every position** | Fully extended | 27 | 10.1 - 334.7 | 0.978 | 0.463 ± 0.092 | 0.682 ± 0.041 |
| | Coiled | 41 | 10.1 - 334.7 | 0.989 | 0.459 ± 0.048 | 0.638 ± 0.023 |
| | Intermediate | 78 | 10.1 - 334.7 | 0.984 | 0.481 ± 0.040 | 0.671 ± 0.020 |
| **Spontaneous positions** | Fully extended | 52 | 10.1 - 405.8 | 0.980 | 0.501 ± 0.059 | 0.672 ± 0.027 |
| | Coiled | 138 | 10.1 - 445.0 | 0.984 | 0.512 ± 0.030 | 0.666 ± 0.014 |
| | Intermediate | 371 | 10.1 - 461.9 | 0.980 | 0.485 ± 0.022 | 0.678 ± 0.010 |
| | **ANCOVA** | **DF** | **F-Value** | **P-value** | **Common Values** | |
| **Single worm exhibiting every position** | Slope | 2, 140 | 0.314 | 0.7312 No. Sig | 0.676 ± 0.014 | |
| | Intercept | 2, 142 | 0.081 | 0.9224 No. Sig | 0.472 ± 0.029 | |
| **Spontaneous positions** | Slope | 2, 555 | 0.760 | 0.4684 No. Sig | 0.674 ± 0.007 | |
| | Intercept | 2, 557 | 0.322 | 0.7250 No. Sig | 0.494 ± 0.017 | |

**Fig. 7**. Photographic Surface (PS = mm$^2$) *vs.* Live Weight (LW = mg) in *Eisenia andrei* obtained using the Field Method: $PS = 2.924*LW^{0.689}$ ($R^2$ = 0.9849; n = 123).CI$_{95\%}$ is ± 0.015 for the slope and ± 0.22 for the elevation.

## 3.4. Laboratory Method (LM)

Frequency distribution of LW (mg) of individuals used in the LM appears illustrated in Fig. 8. A number of 294 animals covering the whole range of sizes found in our breeding scheme were analyzed in two separate groups. Newly hatched worms (n = 107) within less than one day after hatching (LM$_{NH}$< 8 mg) with a mean LW value of 2.2 ± 1.472 mg (Fig. 8A) on one hand, and the rest of the population (LM$_P$, n = 187) in which two cohorts were evident: from 9 mg to 175 mg (Mean = 79.157; SD = 44.93) and from 175 mg to 570 mg (Mean = 316.663; SD = 88.584). A low proportion of the population (5.88 %, n = 11) exceeding 600 mg is not shown in Fig. 8B.
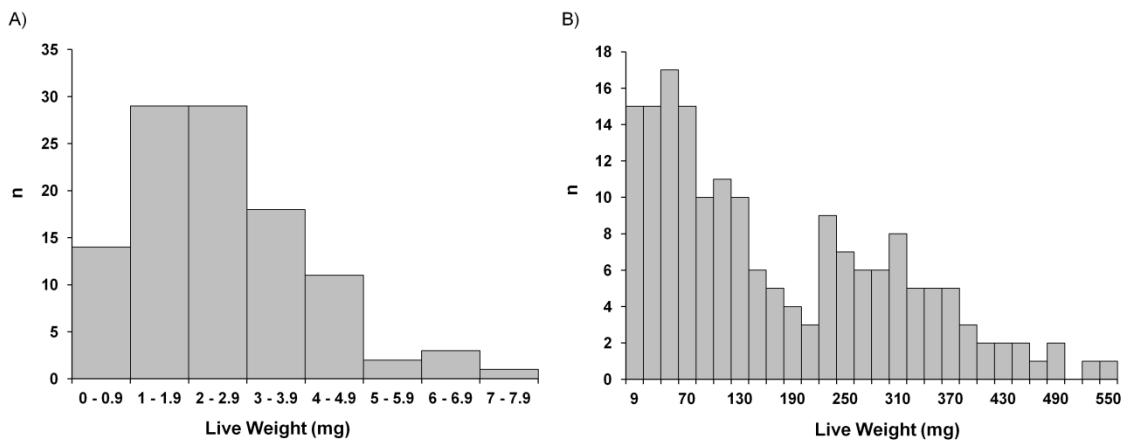


**Fig. 8**. Histograms for the frequency distributions of Live Weight (LW = mg) of specimens of *Eisenia andrei* used in the Laboratory Method. A): Newly Hatched specimens < 8 mg ( Mean = 2.2 ± 1.472). B): Population with sizes ranging from 9 mg to 3132 mg. Individuals larger than 570 mg (n = 11; 5.88%) do not appear in the figure.

## 3.4.1. Influence of feeding conditions

Evaluation of the effects that different amounts of food being processed inside the digestive tube could have in the actual proportion of portrayed surface was undertaken standardizing 3 feeding conditions: Fed, 24 and 48 hours fasting respectively.

As shown in Table 5, a common relationship between image surface estimations ($PS = mm^2$) and LW (mg) was obtained irrespective of the length of starvation explaining 98 % of experimental variation: $PS = 4.623*LW^{0.647}$.

Nevertheless, fasted individuals showed significant differences with the fed group regarding elevation 0.638 *vs.* 0.582 with a common slope of 0.66:

$$(F3) \quad \text{Fasted: } PS = 4.34*LW^{0.66}$$

$$(F4) \quad \text{Fed: } PS = 3.819*LW^{0.66}$$

Unfed animals visible surface increases by a factor of 1.136.

**Table 5**. Coefficients of Log-Log regression equations of Photogrammetric Surface ($PS = mm^2$) vs. Live Weight ($LW = mg$) of *Eisenia andrei* for different feeding conditions. Differences in individual equation for photogrammetric measurements of body surface between two different feeding conditions (Common Fasted & Fed) analyzed through ANCOVA. DF = degrees of freedom; $CI_{95\%}$ = Confidence intervals.

| Feeding conditions | n | Size range (mg) | $R^2$ | Intercept ($CI_{95\%}$) | Slope ($CI_{95\%}$) |
|---|---|---|---|---|---|
| **Fasted 24 h** | 38 | 19 - 681.8 | 0.981 | $0.657 \pm 0.065$ | $0.644 \pm 0.030$ |
| **Fasted 48 h** | 38 | 19 - 681.8 | 0.978 | $0.669 \pm 0.072$ | $0.653 \pm 0.033$ |
| **Fed** | 187 | 10.1 - 3132.0 | 0.974 | $0.574 \pm 0.035$ | $0.663 \pm 0.015$ |
| | | | | | |
| **Fasted 24h Vs. 48h** | **ANCOVA** | **DF** | **F-Value** | **P-Value** | **Common Values** |
| | **Slope** | 1, 72 | 0.186 | 0.6676 No. Sig | $0.647 \pm 0.023$ |
| | | | | | |
| | **TUCKEY** | **DF** | **q** | **Significance** | |
| | **Intercept** | 72 | 0.76615 | No .Sig | $0.665 \pm 0.052$ |
| | | | | | |
| **Fed Vs. Fasted** | **ANCOVA** | **DF** | **F-Value** | **P-Value** | **Common values** |
| | **Slope** | 1, 259 | 0.961 | 0.3279 No. Sig | 0.660 |
| | **Intercept** | 1, 260 | 68.036 | <0.0001 Sig | |
| | | | | **Fasted** | 0.638 |
| | | | | **Fed** | 0.582 |

## 3.5. Field vs. Laboratory method

Linear regression analysis of the relationship between PS (mm$^2$) and LW (mg) in the different groups of individuals used along the experiments are summarized in Table 6.

All equations were highly significant and regarding the regression coefficient, slight differences appeared when extreme disparity in size range applies: $LM_{NH}$ vs. $LM_P$ or FM. Examination of confidence intervals revealed that a common significant function could be established for $LM_P$ and FM ($PS = 3.27*LW^{0.681}$, $R^2 = 0.97$) indicating that resolution was similar for both methods. Contrasting this common equation for larger animals with $LM_{NH}$ showed that regression coefficient would be comparable (mean slope of 0.681) whereas intercepts remained significantly different, increasing the body surface pictured by a factor of 1.29 in smaller animals ($PS = 4.21*LW^{0.681}$, $R^2 = 0.865$). This finding appears related to results obtained for fed and fasted worms and the same considerations would be appropriate.

**Table 6**.Coefficients of Log-Log regression equations of Photogrammetric Surface (PS = mm$^2$) vs. Live Weight (LW = mg) of *Eisenia andrei* for different methods. Differences in individual equation for photogrammetric measurements of body surface between two groups of sizes (Common FM+LM$_P$ & LM$_{NH}$) analyzed through ANCOVA. LM$_{NH}$= Laboratory Method Newly Hatched; LM$_P$= Laboratory Method Population over 8 mg Live Weight; FM= Field method; DF= degrees of freedom; CI$_{95\%}$= Confidence intervals.

| Method | n | Size range (mg) | R$^2$ | Intercept (CI$_{95\%}$) | Slope | CI$_{95\%}$ (Slope) |
|---|---|---|---|---|---|---|
| **LM$_{NH}$** | 107 | 0.2 - 7.0 | 0.870 | 0.614 ± 0.024 | 0.715 | 0.661 - 0.769 |
| **LM$_P$** | 187 | 10.1 - 3132.0 | 0.974 | 0.574 ± 0.035 | 0.663 | 0.648 - 0.679 |
| **FM** | 123 | 8.4 - 463.5 | 0.985 | 0.466 ± 0.033 | 0.689 | 0.674 - 0.704 |
| **Common (FM+LM$_P$)** | 310 | 8.4 - 3132.0 | 0.972 | 0.524 ± 0.028 | 0.677 | 0.664 - 0.690 |

| Common Vs LM$_{NH}$ | ANCOVA | DF | F-Value | P-Value | Common values | |
|---|---|---|---|---|---|---|
| | **Slope** | 1, 413 | 2.973 | 0.0850 No. Sig | 0.681 | |
| | **Intercept** | 1, 414 | 57.366 | <0.0001    Sig | | |
| | | | | **LM$_{NH}$** | 0.625 | |
| | | | | **Common (FM+LM$_P$)** | 0.515 | |

# 4. Discussion

Our breeding routine of *Eisenia andrei* fed on horse manure corresponds to optimal conditions (Domínguez & Edwards 2011) and characteristics of our ongoing culture are similar to those reported in the literature regarding minimum and maximum sizes attained for laboratory cultures grown in cow manure: between 6.5 to 1 000 mg live weight (Domínguez *et al.* 1997; Domínguez *et al.* 2000 for *E. andrei* and Gunadi *et al.* 2002 for *E. foetida*). Data from newly hatched organisms is not so frequent ranging from 6.5 to 11 mg live weight for *E. andrei* and *E. foetida* and our lower limit is lowest in the literature: 0.2 mg live weight with a size distribution reaching 7 mg for worms born within 24 h surveying. This aspect is of considerable interest since the objective of the work was focused on providing a simple and reliable tool to allow long-term individual growth studies from earliest stages of life cycle.

## 4.1. Live weight measurements: body water, gut content & biomass

Lowe & Butt (2005) and more recently Fründ *et al.* (2010) have reviewed in detail the relationships between moisture content of soil and physiological status of earthworms, strongly connected to hydration level of tissues. In this work, soil moisture under culture conditions was kept constant (80 % RH) precluding changes in water potential that could alter tissue hydration hindering biomass inference from live weight determinations: Kretzschmar & Bruchou (1991) report significant live weight losses up to 60 % of initial weight associated to decreasing humidity that were still compatible with activity. More recently Stovold *et al.* (2003) demonstrate this water loss resides basically in the middle and end segments of *Aporrectodea caliginosa,* allowing maintenance of radial pressure on the anterior end and concomitantly burrowing activity.

Our data for *E. andrei* obtained over a wide size range (2.3 to 681.8 mg live weight) indicates water content relates isometrically to body weight reaching 90 % in fed worms and decreasing to 84 % after a period of 48 h starvation. These figures, obtained through equations explaining over 99 % of variability, relate well to general results for lumbricids (Laverack 1963; Florkin 2012) and are in coincidence with early data of 83.3 % for *E. foetida* reported by O´Brien

(1957a; 1957b). Regarding water losses associated to short-term starvation Martin (1986) reported similar results for three species of pasture lumbricidae. Furthermore, our results show that gut content removal after 48 h fasting implies 13.32 % weight loss, in agreement with data for *Lumbricus terrestris* obtained along an identical starvation period by Eisenhauer *et al.* (2009) appearing in *Eisenia andrei* as size-independent. Intestine voidance took place primarily along the initial 24h accounting for 90.41 % of total faeces production in accordance with Martin (1986) and egestion dynamics reported by Dalby *et al.* (1996) for various species of *Aporrectodea* with size ranges included in our study.

## 4.2. Morphometrical determinations of biomass

### 4.2.1. Length, Diameter and Volume

Length scales allometrically to live weight and a weight exponent of 0.6683 indicates growth involves volume gain (i.e. live weight) that exceeds, in relative terms, length augmentation. Consequently Surface to Volume ratio becomes increasingly reduced according to a weight exponent of -0.3239. Mean body diameter scales to live weight isometrically: weight exponent is 0.3352 according to theoretical predictions for linear dimensions (b = 0.333). Simultaneous consideration of weight exponents exhibited by length and diameter derives in relatively thinner specimen in the course of development. Kurth & Kier (2014) have recently reported analogous results for *Lumbricus terrestris* in the comparison of juveniles (1-3 g live weight) with fully grown adults (3-10 g) with both weight exponents departing from isometry which, in terms of a ratio Length/Diameter represents a 128 % to 163 % increase. In *E. andrei* a sharp difference appears between juveniles (mean live weight 43.8 mg) and sub-adults and mature specimens (mean live weight 308.6 mg): Length/Diameter evolves from 3.67 to 21.45 respectively and reaching a body length of 34 mm implies reducing diameter to ~1/3.

Two alternative approaches to volume determination, geometric calculations from calliper measurements and water displacement by immersion have produced identical results regarding relation with live weight: isometric relation with elevation value of 0. Hence, volumetric estimations can be directly derived from live weight measurements.

## 4.2.2. Surface evaluation through image analysis

Frequent handling of organisms may lead to damage and variable water loss rendering direct measurements of live wet weight unreliable as a parameter to estimate biomass and follow growth along time. In fact, procedures involved in pictured taking proved less time consuming (a factor of 3 per worm) than safe manipulation of worms undertaken to obtain accurate gravimetric determinations of live weight or linear parameters while establishing the reference scaling of dry biomass to live weight. Nevertheless extreme care while dealing with animals resulted in exponential functions with $R^2$ >99 % for both fed and fasted (48 h) organisms.

Since determining minimum number of pictures to provide repeatable and reliable estimations of size as well as deciding on the body position more suitable to those purposes was essential, initial design took into consideration body shape and a good number of pictures were taken per individual. In fact, the shape adopted by the animals on the Petri dish (always with the ventral side down) had no effect on accuracy in terms of linear regression equations relating area ($mm^2$) to live weight (mg) and a common function explaining 98 % of variation has been calculated. Regression equations relating area determined through image analysis to dry weight have been used in pond macroinvertebrates ranging from 0.01 to 77 mg dry weight (Bernardini *et al.* 2000) which includes lower range shown by newly hatched *E. andrei* (0.02 mg dry weight) represented by worm-shaped *Dugesia*. In their work, area appears as the best predictor of biomass against other parameters (perimeter, axis), showing higher values for the correlation coefficient for every species, especially for smaller organisms. Regarding the required number of pictures to be processed, no differences in precision were found in a range of 2 to 10 pictures inspected. Variation coefficient remaining 6 % irrespective of size: worms are small (mean diameter 2.98 mm ± 0.7979) and as related to fixed focal distance (25cm) minimum and maximum diameters represent 0.2 % and 2 % respectively. Under these conditions, photogrammetric surface represents 34.04 % of geometric surface ($R^2$= 96 %). Comparable studies have been performed in sponges, where the number of pictures inspected involve 2 (Page *et al.* 2005), 3 (Koopmans & Wijffels 2008) or 5 (Abdo *et al.* 2006) considering the bewildering display of morphologies and the underwater picture constraints. Perea *et al.* (2008)

working with juvenile land snails (*Cantareus aspersus*), of similar size range of *E. andrei* used in our experiment, examine 3 to 5 images. Two replicates appear thus appropriate for *E. andrei* considering their consistent cylindrical shape, in coincidence with Vanaverbeke *et al.* (2003) that analyzed nematode biomass in sand bank marine communities in terms of 2-3 replicates.

## 4.2.3. Surface determinations and feeding conditions

Starvation (24 to 48 hours) implies a reduction of 13.32 % in live weight irrespective of body size which does not influence weight exponent and fed and fasted worms present a common slope of 0.66. The elevation, however, increases from 3.82 in fed animals to 4.34 in starved specimen. Since water acts as a hydrostatic skeleton in worms, water loss derived from starvation implies loss of turgidity and flattening and, consequently, larger proportion of surface exposure to camera lens (a factor of 1.136).

## 4.2.4. Surface determination and size: Laboratory *vs.* Field method

No differences between laboratory or field method appear when size exceeds 8 mg live weight and a common linear function relating area to live weight with a weight exponent of 0.681 and an elevation of 3.27 results. Below 8 mg weight exponent remains unchanged but the value for the elevation increases to 4.21 (factor increase 1.287). At this respect, surface to volume ratio increases in a continuum towards lower sizes i.e. hydrostatic skeleton ($mm^3$) per unit area ($mm^2$) decreases in smaller worms: for *E. andrei* of 10 mg live weight $1/$ (Surface/Volume) is 0.21 and reaches 0.70 for 400 mg organisms. So, some size depending flattening occurs becoming significant below 8 mg live weight. Although biomass predictions are similar with the two methods (over 8 mg live weight), laboratory method allows direct estimations of surface being less time consuming than the field method where image analysis requires Photoshop processing.

# 5. Conclusions

The main point has been demonstrating that images taken without special requirements can be a good tool to assess biomass of soft-bodied tubular organisms within a wide size range (starting on eight mg live weight for the field method) and this regardless of shape (body position) and number of pictures taken. Water loss derived from starvation implies loss of turgidity and flattening and, consequently, larger proportion of surface exposure to camera lens (a factor of 1.136). These characteristics imply the procedure can be applied to field surveys of growth and biomass estimations from earlier life stages since pictures of worms would be reliably related to their body mass as long as scaling of weight to portrayed surface is established for the particular occasion (around 20 to 30 individuals for a regression analysis). These practises minimize capture and sacrifice of animals associated to laboratory determinations, providing a permanent record that can be reviewed for posterior analysis.

# Chapter 3

## 3. Individual growth of *Eisenia andrei* in mesocosm at low density:

## Tagging with fluorescent elastomers

# Individual growth of *Eisenia andrei* in mesocosm at low density: Tagging with fluorescent elastomers

## Abstract

In this work we have evaluated individual growth of *Eisenia andrei* reared in horse manure in two conditions: in mesocosm at similar initial densities, and in isolated organisms. Our aim has been to identify individual growth dynamics, with special emphasis on shift points from juveniles to sub-adults, and its connection with density evolution, performing a comparative study between earthworms cultured in group or individualized. We have used fluorescent elastomers for individual identification in group-cultured organisms. Since tagging soft body organisms presents limitations, our second objective has been to standardise the use of fluorescent elastomers as a tagging method in *E. andrei*. For this purpose, 87 juvenile and adult individuals were marked, and 40 newly hatched specimens were used as a control. Elastomers remain in the earthworm's body for 3 months in 75 % of the cases. Mortality associated with the tagging method depends on body size: from 110 to 270 mg survival increases from 45 to 80 %, reaching 100 % in specimens larger than 270 mg. A sample of 51 individuals of *E. andrei* ranging from 34.66 to 1092.27 mg was used to ascertain density effects. From the tagged pool, 33 individuals were selected and groups of 11 specimens were placed in 1100 cm$^3$ containers with an average initial density of 56.84 mg live weight/ g dry horse manure. The remaining 18 juveniles (34.43 to 187 mg) were individually cultured in 450 cm$^3$ boxes. Growth was monitored for 100 days and biomass determinations were undertaken every 3-4 days. Results show that grouped specimens adjusted primarily to an asymptotic model (~50 %) whereas in isolated organisms a rectilinear pattern is defined in ~ 45 % of cases. Maximum weight achieved during the experiment correlates with growth model, being on average 552 mg and 1453 mg for grouped and individually cultured earthworms respectively. Maximum live weight recorded in individuals reared in group is associated with detection of clitellum (AP = 63.759* e $^{(0.005LWC)}$; R$^2$=0.897). Initial density increases over the experimental time according to an asymptotic function (*Density* = (111.27-55.102)*(1-e $^{(-0.032Time)}$) + 55.102; R$^2$=0.845) indicating that 111.27 mg live weight / g dry horse manure would be the maximum density the population can support. When higher values are attained, death or weight loss of some of the individuals occur, resetting density below hitherto referred threshold.

# 1. Introduction

*Eisenia andrei* is an epigeic annelid, ubiquitous with a worldwide distribution, with tolerance to temperature and moisture variability, and whose vital cycle exhibits high growth and reproduction rates. These characteristics explain the use of these organisms in vermicomposting in the context of waste management and also in ecotoxicological, ecological, physiological and genetic studies (Domínguez *et al.* 2005; Piola *et al.* 2013). Production of cast enriched with nutrients easily assimilable by plants contributes to the maintenance of soil fertility (Brown *et al.* 2000), and their interactions with top soil components and mutualistic relationship with soil microorganisms prevent soil degradation (Trigo *et al.* 1999; Edwards 2004). For these reasons, *E. andrei* is a very important species not only for waste management carried out in the vermicomposting industry, but also by their role in natural fertilization and protection of soil.

In a physiological context, many authors have reported that individual live weight is affected by population density, proving that worms reared in high density environments present lower growth rates, and individual live weight decreases (Domínguez & Edwards 1997; Kammenga *et al.* 2003; Unuofin & Mnkeni 2014).These negative effects of crowding conditions are due to intraspecific competition for food and space, and reproductive cost, that implies a translocation of energy to the formation of reproductive tissue and cocoons, in detriment of somatic growth. Previous studies on the growth of *E. andrei* have been performed in a population average basis. Due to the high interindividual differences in growth rates and patterns among worms, and their implications in waste management and in conservation of soil quality, a tool that would allow individual identification and monitoring is necessary.

Ringing in birds, and radio-tracking in birds and mammals, have been the most commonly utilised individual identification techniques, and tagging with fluorescent elastomers has been widely used in marine species, not only in fishes but also in soft-bodied invertebrates ( Zeeh & Wood 2009; Brewer & Norcross 2012). In the last ten years, studies in annelids have been undertaken. Gonzalez *et al.* (2006) published a study with *Pontoscolex corethrurus,* starting from an initial live weight of 200 mg. Lately, studies with species belonging to the family Lumbricidae, including *E. andrei*, (Butt & Lowe 2007; Butt *et al.* 2009) have been performed

with success within these species. Based on the fragility of *E. andrei* specimens, especially in the earliest stages of life, we understand it is necessary to analyse the possible effects of the tagging tech upon earthworms. We have put special emphasis on the effects of body size at tagging. Besides, in field monitoring experiments a short- to long-term tracking is required, so study of the remaining time of the tag becomes necessary.

Due to the high influence of population density on growth and reproduction in annelids, and the importance of interindividual differences, especially in the vermicomposting industry, our aim has been to identify individual growth dynamics with special emphasis on shift points from juveniles to sub-adults and their connection with density evolution, performing a comparative study between earthworms cultured in group or individualized. For this purpose, we have standardized the use, in *E. andrei*, of fluorescent elastomers as a tagging method, allowing individual monitoring of worms within a population.

# 2. Material and methods

A sample of 157 individuals of *Eisenia andrei* reared in horse manure (86 % organic matter, pH = 7.413 ± 0.023) in constant darkness at laboratory conditions (20 ºC; 80 % RH) was used in this experiment. Since the final objective of this work was to analyse the effect of population density upon individual growth, specimens of *E. andrei* required tagging in order to remain identified along the experimental period. Consequently, the experiment was performed in two steps. Initially, 127 *Eisenia andrei* specimens were used to analyse and standardise tagging conditions (87 tagged individuals and 40 control worms). Later, 51 specimens were employed in the population density experiment.

## 2.1. Tagging method

A total of 87 specimens were marked: 30 adult and sub-adult individuals, ranging from 90.94 to 1702.658 mg live weight, and 57 juvenile worms within a size range of 27 to 248.411 mg. Forty newly hatched individuals without tag, ranging from 0.315 to 6.234 mg live weight, were employed as a control group, individually maintained at culture at the same laboratory conditions.

In order to test the possibility of damage of the elastomers on the viability of earthworms, marked individuals were individually maintained and monitored for 10 days, with exhaustive inspection during that period. Deaths occurring between days 0 and 8 days were associated with the tagging method. In the control group, survival was monitored over 160 days. Two possible effects of tagging were analysed:

1. **Size of the animal**, where individuals were split into 9 and 10 groups of sizes within the control and experimental group respectively. In control group, weight measurements for 160 days were recorded in order to ascertain the influence of animal size in survival percentage. In the experimental group, a relation between animal size (weight at elastomer inoculation = initial live weight) and survival percentage was established.

2. **Number of elastomer inoculations**, 1 or 2, given to each animal. For this purpose we have compared survival, in terms of days, of marked earthworms, after tagging with one or two punctures, using initial live weight as a covariate.

## 2.1.1. Elastomer inoculation procedure

Fluorescent elastomers easily identifiable with ultraviolet light, consisting of a colored silicone mixed with a curing agent (1:10), were employed for marking earthworms. The kit, supplied by Northwestern Marine Technology Company, contained 7 different colours, which allowed having 21 colour combinations.

Prior to tagging, suitability of the different colour possibilities had to be tested. From the seven-colour kit, only red, green, orange, and blue were valid for the method, as red and green do not sufficiently differ from pink and yellow, respectively, and white was discarded because of the difficulties to differentiate the elastomer from some hypopigmented parts of earthworm body.

On the whole, we achieved a total of 16 different tags, which became our maximal n value for worms per mesocosm that this colour assortment permits. An insulin needle, with a diameter of 300 nm, was used to inoculate elastomers in animals larger than 100 mg live weight. For smaller earthworms we used an in vitro fertilisation needle with a cut in the tip (180nm) due to the density of the elastomer fluid.

Before elastomer injection, earthworms were anaesthetised (Butt & Lowe 2007) by immersion in a Holtfreter solution (Dales 1978) with ethanol at 5 % for 30 minutes (Cooper 1968; Marks & Cooper 1977). The injection was postclitellar (Butt & Lowe 2007) and intracoelomic (Butt *et al.* 2009), and one (n=33) or two (n=54) punctures were given to each animal in different positions of the body. For more accuracy, we utilised a stereoscopic microscope.

Photographs of every worm were taken after elastomer inoculation with a Kodak© Easyshare M763 digital camera (Focal length equivalent from 34 to 102mm; Optical sensor size 5.744; Maximum aperture F2.8-F5.1). In order to favour simplicity in data acquisition, camera parameters were fixed preferentially in the automatic mode: sensor resolution 7MP (3072 x 2304); Light sensitivity: ISO auto (64-320); Exposure Metering: Multi-segment; Shutter speed: automatic (from 4 to 1/1400 sec); Optical zoom 3X; Macro Focus range from 10 to 70cm; Focus adjustment: autofocus multi-area; Focal distance: 25cm (tripod).

## 2.2. Effects of population density

Two culture conditions were established to evaluate the effect of population density: grouped and isolated. For this purpose, a total sample of 51 individuals of *E. andrei* with live weight ranging from 34.43 to 1092.27 mg was used. A group of 33 tagged earthworms was randomly chosen from survivors to elastomer inoculation, ranging from 34.66 to 1092.27 mg live weight. Each group of 11 specimens was placed in a 1100 cm$^3$ container and fed 45 g of horse manure on a dry weight basis; three replicates were used, starting from an average initial density of 56.84 mg live weight per gram of dry horse manure. The remaining 18 juveniles (34.43 to 187 mg) were individually cultured in 450 cm$^3$ boxes and fed with 25 g of dry dung. Maintenance parameters were similar to culture conditions and in order to avoid a reduction in feeding availability, additional substrate was added every 20-25 days. Growth was monitored for 100 days and biomass was ascertained from pictures taken every 3-4 days (see Chapter 2).

## 2.3. Statistical analysis

Basic statistics (Frequency distribution, one-way ANOVA procedures and non-parametric Mann-Whitney U test) were employed to describe and compare samples of animals and culture

methods (STATVIEW 5.0.). ANCOVA analysis was used to ascertain the effect of number of punctures on survival with live weight as covariate (STATVIEW 5.0.).

Linear and non-linear regression procedures were performed with nlme R package (Pinheiro *et al.* 2015) and SYSTAT version 13, from Systat Software, Inc., San Jose California USA, www.sigmaplot.com.

# 3. Results

In this experiment, a total sample of 157 *E. andrei* specimens was used. Two different experiments took place. In the first part, an attempt to standardise the use of fluorescent elastomers was made; and for this purpose, 87 juvenile and adults individuals were marked and 40 newly hatched individuals were used as a control. Later, in order to detect the effects of population density on growth, from the tagged pool 33 earthworms were reared in group, and 18 individuals were individually cultured.

## 3.1. Tagging method

### 3.1.1. Size structure of samples

40 individuals from the earliest stages of life, within a size range from 0.315 to 6.23 mg live weight (Mean = 3.008; SD = 1.294), were chosen as a control (Fig. 1 A). Fig. 1 B represents the tagged population (n=87) with an initial size range from 27.606 to 1702.658 mg live weight (Mean = 224.6819; SD = 310.069).
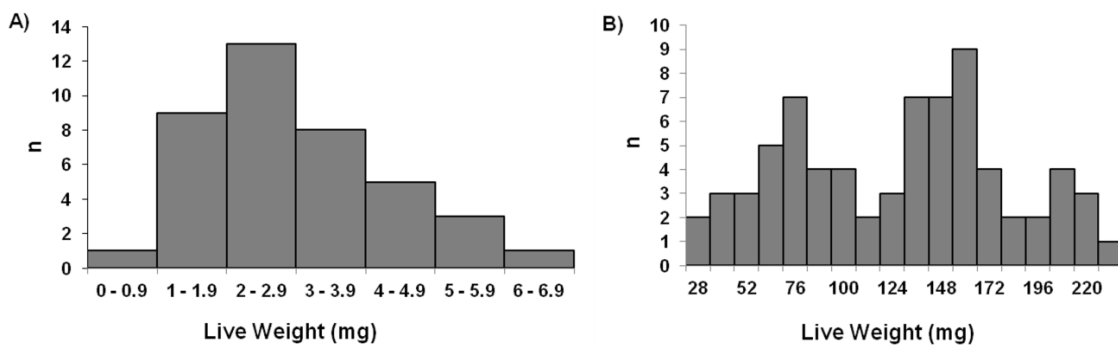


**Fig.1.** Histograms for frequency distribution of Live Weight (mg) in specimens of *E. andrei* used in the experiment. A): control group (Mean= 3.008 ; SD = 1.294). B): Tagged population group (Mean= 224.682; SD = 310.069). Individuals larger than 570 mg (n=15; 17.24%) do not appear in the figure.

### 3.1.2. Position and remaining time of the elastomer

Since growth experiments take a short to large time span ($\geq 30$ days), ascertaining tagging endurance became a key goal. At this respect, we focused on the position of the elastomer in the earthworm's body, and the time the tag remained in the organism.

Regarding position, in 100 % of tagged worms the elastomer changed its location during the experiment, always from front to back. So, the position of the label in the earthworm's body is not enough to distinguish one individual from others.

Focusing on remaining time of the elastomer, we can see in Fig. 2 that in one month of experiment, only 5 % of worms lost their mark. This percentage increased with time, but, at the end of the experimental time, 75 % of them were easily recognized. Nevertheless, the integrity of some tags changed and a few tags appeared to break up, persisting as a number of sub-units. The following sigmoid model shows (Fig. 2) a stabilisation of the number of animals without label in three months.



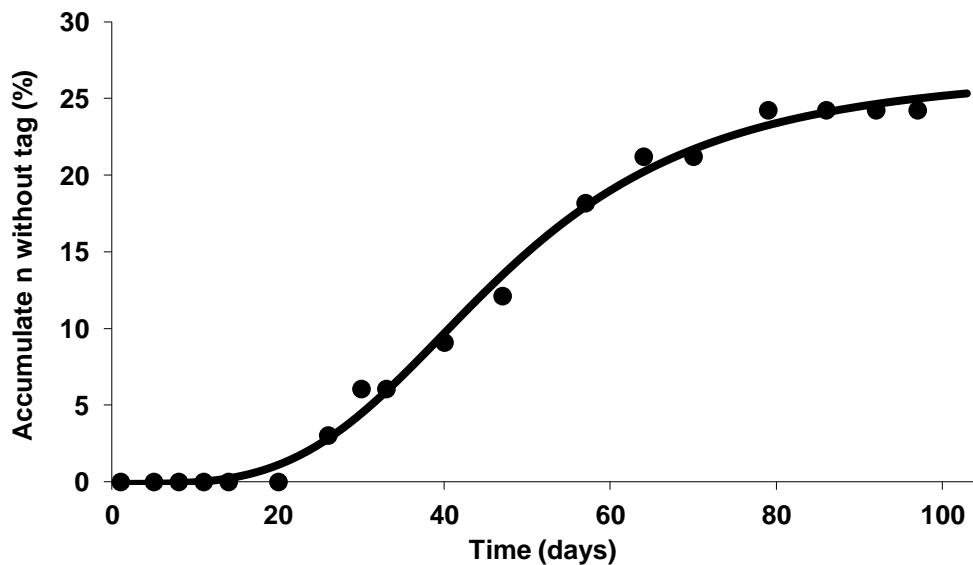**Fig. 2**. Sigmoid model for the accumulate % of individuals without a tag along time in marked worms of the species *Eisenia andrei*.
Individuals without tag $=-0,12789+(26,94989/(1+(Time/46,791)^{(-3,5747)}))$ ($R^2= 0,99746$ ; n=33).

### 3.1.3. Effect of the size on survival

Total survival of labelled animals was 77.01 % (n=67) within the first 8 days after tagging. A mortality of 6.89 % was detected after this period (n=6).

In the control group we found a survival percentage over 97.5 in individuals larger than 6 mg. Since the minimum initial size of marked earthworms was 27 mg, the mortality found in our experimental group was associated with the tagging method.

Our results showed no mortality in the first 8 days after tagging in individuals over 270 mg live weight, and survival approached 80 % in worms ranging from 110 to 270 mg live weight. In the smallest ones, survival percentage increases with size, being 44.45 % in individuals between 27 and 60 mg, 60 % from 60 to 80 mg, and 70 % from 80 to 110 mg.

Asymptotic curve (Fig. 3) predicts survival in relation to live weight (mg). Kruskal-Wallis non parametric test displayed statistical differences (*p-value* < 0.001) regarding initial live weight for the different survival percentages found in the study in marked population



**Fig. 3.** Asymptotic modelization of survival % *vs.* Initial live weight (mg) of worms of the species *Eisenia andrei* in the experimental group. Survival % = $100*(1-e^{(-0.012LW)})$; $R^2$=0.928.

## 3.1.4. Effect of number of punctures on survival

For this study we have evaluated difference regarding days of survival of earthworms after tagging between two groups: worms marked once, or twice.

Since size of the animal is relevant for survival in this method, as demonstrated above, T-test analysis showed no statistical differences in initial live weight between individuals marked once or twice (t-value=-1.813; *p-value* = 0.0734). Once difference regarding initial size was discarded between the two groups, an ANCOVA analysis for survival time of tagged

individuals was performed, with initial live weight (mg) as a covariant and number of punctures as a factor, and is shown in Table 1.

**Table 1.** Results of ANCOVA analysis of the survival time after tagging, including live weight (mg) as covariate, and an interaction term for the product of live weight and number of punctures given (one or two).

| ANCOVA | F-value $_{1,75}$ | P-value |
|---|---|---|
| **Number of punctures** | 0.003 | 0.9553 |
| **Initial Live Weight** | 4.181 | $< 0.01$ |
| **Number of punctures*Initial Live Weight** | 1.283 | 0.2609 |

No significant differences were evident (*p-value* $= 0.9553$), regarding the number of punctures given, upon the survival. However, as explained above, initial live weight was determinant (*p-value* $< 0.01$) for the viability of tagging in worms.

## 3.2. Study of the effects of population density

### 3.2.1. Density effects on growth

Our final objective in this study was to test differences in growth as a consequence of population density. Consequently, clitellated individuals (n=4) within an initial size range from 794.965 to 1092.27 mg were excluded from the analysis of growth, since no somatic biomass evolution was reported. Individuals used to test the effects of population density are represented in Fig. 4.
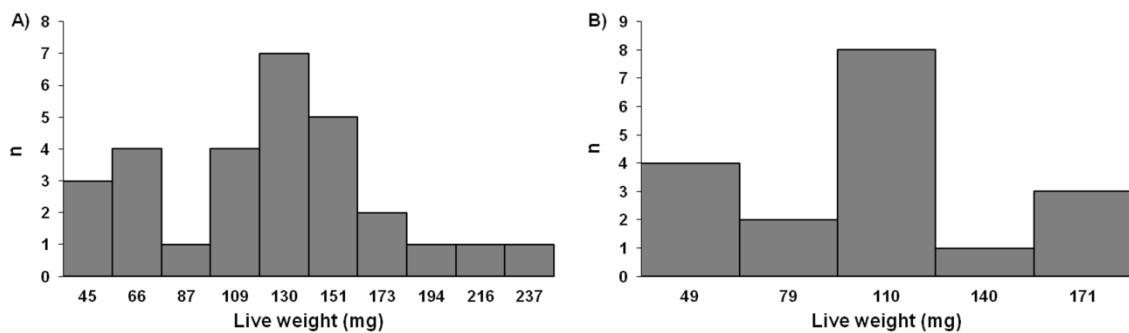


**Fig.4.** Histograms for frequency distribution of Live Weight (mg) in juvenile specimens of *E. andrei* used in the study of the effects of population density. A: Group-cultured individuals (Mean= 124.06; SD = 51.90). B: Isolated cultured individuals (Mean = 105.337; SD = 44.772).

Tagged juvenile individuals within an initial size range from 34.66 to 248.41 mg are shown on Fig. 4 A. Fig. 4 B represents isolated individuals ranging from 34.43 to 187 mg live weight.

Frequency histograms revealed similar distribution of initial live weight in both culture methods, and one-way ANOVA analysis revealed no statistical differences regarding mean initial live weight for individuals grown in group or individualized (*p-value*= 0.324).

From the 29 selected labelled juveniles, 89.65 % (n=26) had a positive growth, and 10.34 % (n=3) decreased along experimental time. Every worm grown isolated (n=18) increased in biomass during the experiment.

Focusing on worms with a positive growth, results obtained in both grouped and individualized settings revealed that approximately 25 % and 18 % of the individuals presented an exponential and power growth pattern respectively. Mesocosm specimens adjusted primarily to an asymptotic model (54 %), whereas in isolated organisms a rectilinear pattern is defined in 50 % of cases (Fig. 5).



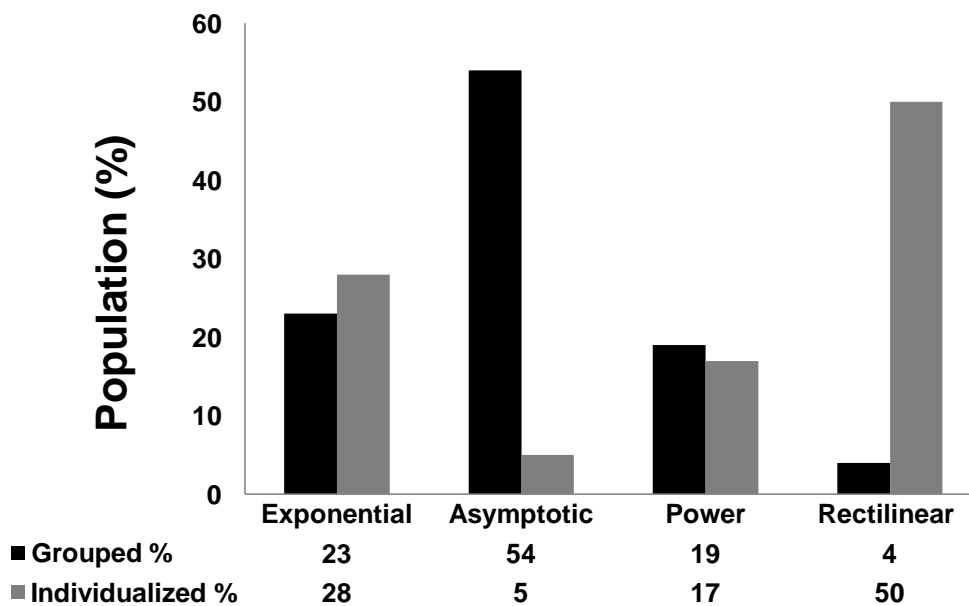| | Exponential | Asymptotic | Power | Rectilinear |
|---|---|---|---|---|
| ■ Grouped % | 23 | 54 | 19 | 4 |
| ■ Individualized % | 28 | 5 | 17 | 50 |

**Fig. 5**. Frequency histogram for the percentages of different growing patters found in individuals of *Eisenia andrei* cultured in group and individualized.

For the next step, we established the equations of the models for each specimen, joining those with similar parameters in a single equation. Table 2 summarizes the equations and growth

parameters found in our experiment, in both grouped and isolated cultures. In the same way, growth curves are represented in Fig. 6.



**Fig. 6.** Growth patterns for specimens of *Eisenia andrei* growing in group and individualized. A= Exponential (Y=a*(e$^{Xb}$); B= Rectilinear (Y=a+b*X); C= Power (Y=a*X$^b$); D= Asymptotic (Y =(a-c)*(1- e$^{Xb}$)+c).

In isolated earthworms, we found statistical differences (*p-value* = 0.034) regarding initial live weight between individuals with an exponential growth pattern (Mean= 65.522; SD=34.597) and the rest of the models, in which mean initial live weights of 128.789 mg (SD= 32.968) and 101.011 mg (SD=61.892) were found in rectilinear and power patterns, respectively. This suggests that those individuals with smaller weights displayed an exponential growth. Although we found no statistical differences in worms cultured in mesocosm, the smallest earthworms were also associated with exponential growth patterns with a mean live weight of 91.928 mg (SD=45.43), in contrast with those showing an asymptotic model (Mean=140.696; SD=48.83).

**Table 2**. Growth equations and parameters of *Eisenia andrei* individuals reared in group and individualized.

| Type of culture | Type of growth | Equation | $R^2$ | n | ISR (mg) | MIS (mg) | MMS (mg) | TMS (days) |
|---|---|---|---|---|---|---|---|---|
| Grouped | Exponential a | $LW = 142.32*e^{0.018t}$ | 0.89 | 4 | 42.94-152.25 | 108.30 | 780.41 | 82 |
| Grouped | Exponential b | $LW = 44.09*e^{0.023t}$ | 0.93 | 1 | - | 77.01 | 389.77 | 98 |
| Grouped | Exponential c | $LW = 55.29*e^{0.034t}$ | 0.99 | 1 | - | 41.34 | 599.70 | 70 |
| Grouped | Rectilinear a | $LW = 35.63 + 5.49*t$ | 0.91 | 1 | - | 34.66 | 454.35 | 98 |
| Grouped | Power a | $LW = 30.88*t^{0.665}$ | 0.85 | 4 | 59.3-115.18 | 88.76 | 628.27 | 81 |
| Grouped | Power b | $LW = 113.11*t^{0.432}$ | 0.85 | 1 | - | 179.45 | 919.70 | 92 |
| Grouped | Asymptotic a | $LW = (386.37-159.49)*(1-e^{(-0.082t)}) + 159.49$ | 0.70 | 4 | 132.24-218.17 | 162.73 | 431.31 | 60 |
| Grouped | Asymptotic b | $LW = (571.04-70.81)*(1-e^{(-0.017t)}) + 70.81$ | 0.89 | 5 | 75.76-158.03 | 123.42 | 514.50 | 82 |
| Grouped | Asymptotic c | $LW = (687.04-69.93)*(1-e^{(-0.02t)}) + 69.93$ | 0.89 | 3 | 69.39-146.96 | 107.27 | 612.32 | 80 |
| Grouped | Asymptotic d | $LW = (704.06-170.59)*(1-e^{(-0.034t)}) + 170.59$ | 0.88 | 1 | - | 248.41 | 632.98 | 69 |
| Grouped | Asymptotic e | $LW = (328.32-105.94)*(1-e^{(-0.032t)}) + 105.94$ | 0.85 | 1 | - | 131.47 | 352.88 | 97 |
| Individualized | Exponential a | $LW = 190.991*e^{0.021t}$ | 0.92 | 4 | 40.22-108.55 | 73.29 | 1453.71 | 100 |
| Individualized | Exponential b | $LW = 104.215*e^{0.026t}$ | 0.95 | 1 | - | 34.43 | 1084.78 | 100 |
| Individualized | Rectilinear a | $LW = 216.97 + 16.56*t$ | 0.95 | 5 | 111.34-187.00 | 141.34 | 1874.50 | 100 |
| Individualized | Rectilinear b | $LW = 164.69 + 12.38*t$ | 0.95 | 2 | 94.41-98.72 | 96.56 | 1385.67 | 96 |
| Individualized | Rectilinear c | $LW = 95.21 + 16.38*t$ | 0.98 | 1 | - | 141.72 | 1697.40 | 96 |
| Individualized | Rectilinear d | $LW = 163.54 + 8.756*t$ | 0.96 | 1 | - | 117.51 | 991.15 | 96 |
| Individualized | Power a | $LW = 47.75*t^{0.705}$ | 0.96 | 2 | 47.49-86.75 | 67.12 | 1283.25 | 98 |
| Individualized | Power b | $LW = 128.26*t^{0.447}$ | 0.93 | 1 | - | 168.78 | 1091.44 | 98 |
| Individualized | Asymptotic a | $LW = (640.39-105.8)*(1-e^{(-0.028t)}) + 105.8$ | 0.87 | 1 | - | 106.32 | 718.78 | 88 |

**ISR= Initial Size Range; MIS= Mean Initial Size; MMS= Mean Maximal Size Achieved; TMS= Time Maximal Size**

In group-cultured specimens with an asymptotic model, maximum live weight (asymptotic point) is associated with detection of clitellum, according to the model: *Asymptotic point =* 110.8* e $^{(0.00366 \text{ x } Live \text{ } weight \text{ } at \text{ } clitellum \text{ } detection)}$; $R^2=0.9$ (Fig. 7) .The exponential model showed that little increments in worm size at the moment of clitellum appearance resulted in an exponentially higher maximal live weight. Remarkably, the asymptotic models "a" and "e" (Fig. 6 D), corresponded to early breeding worms, with a prompt gonadal development in detriment of somatic growth.
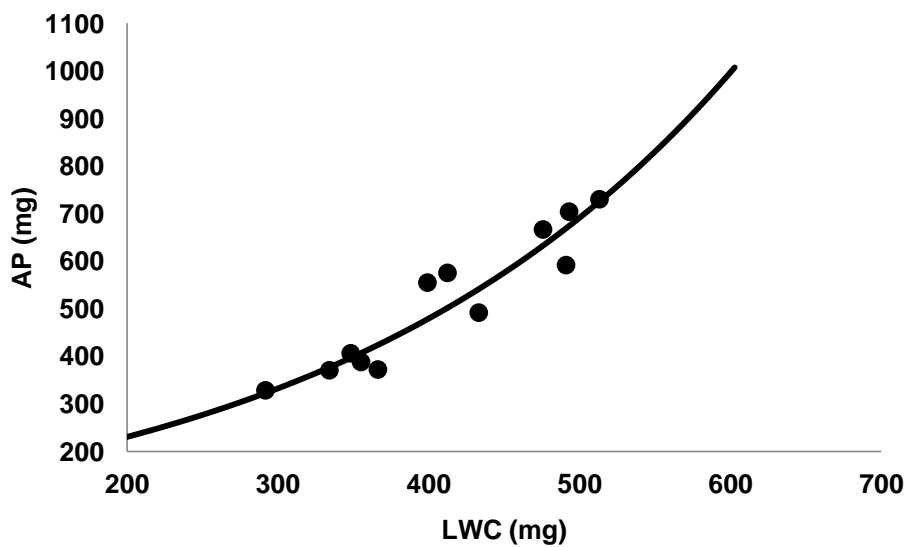


**Fig. 7.** Relation between asymptotic point (AP in mg) and live weight at clitellum detection (LWC in mg) in group-cultured *Eisenia andrei* specimens. $AP = 110.8*e^{(0.0036LWC)}$; $R^2=0.9$.

Maximal live weights achieved in individuals grown isolated (Mean= 1453.08; SD= 362.351) largely exceeded those found in worms cultured in mesocosm that exhibited a positive growth (Mean= 598.52; SD=151.938). These results were statistically tested by a Mann-Whitney non-parametric analysis (*p-value* < 0.0001). In the same line, growth rates of the two types of culture were compared by analysing the first month of growth, in which a linear biomass increase was found. Mann-Whitney U test revealed statistical differences (*p-value* < 0.001) between the two types of cultures, the mean growth rate of the isolated organisms being higher than the grouped ones by a factor of 2.23 (16.53 vs. 7.39 mg day$^{-1}$).

### 3.2.2. Density effects on carrying capacity

Three replicates with 11 tagged individuals per container were employed in the experiment, differences in initial biomass between the three containers not being significant (*p-value* = 0.9535). In mesocosm, density increased along the experimental time according to an asymptotic function: *Density* = $(111.27\text{-}55.102)*(1\text{-e}^{(-0.032Time)})$ + 55.102; $R^2$=0.845, indicating that 111.27 mg earthworm live weight / g dry horse manure would be the maximum density the population can support. When higher values are attained, death or weight loss of some of the individuals occurs, resetting density below the aforementioned threshold.



**Fig. 8.** *Eisenia andrei* density (mg Live Weight g$^{-1}$ dry horse manure) evolution along time (days) in mesocosm. *Density* = $(111.27\text{-}55.102)*(1\text{-e}^{(-0.032Time)})$ + 55.102; $R^2$=0.845.

## 4. Discussion

Results support our aim, since we have demonstrated the effects of population density on growth of *E. andrei* specimens. Also we have standardised tagging with fluorescent elastomers as a method to identify individuals within a population, providing a tool for medium to large time span experiments.

## 4.1. Tagging method

### 4.1.1. Colour, position and remaining time

This inexpensive and easy to apply method, allows short- to long-term experiments, since marks have remained in 75 percent of earthworms for a 3-month period, allowing field monitoring for a relatively long period. Increasing the number of colours, and their combinations provide a large range of labels available for identifying each worm within a population. In our experiment, 25 percent of individuals lost the mark along the experiment, and in some cases, the tag was reduced in size compared to the initial one. This could be due to the insertion of the elastomer in the coelomic cavity, where coelomocytes, which are involved in immune defence reactions (Valembois *et al.* 1992, 1994), recognise the elastomer as a foreign material and tend to eliminate it. Both cloragocytes and amebocytes, which are in fact two types of coelomocytes, encapsulate foreign substances (Adamowicz & Wojtaszek 2001) which waft towards the posterior part of the earthworm's body through the coelom. This encapsulation of the material can be complete when the amount of elastomer introduced is small, which results in the total loss of the tag, or partial, being the label size too large to be completely encapsulated and, consequently, external visibility of the mark is reduced but not lost (Butt *et al.* 2009). Sequestration by coelomocytes altered the position of the elastomer and helps us explain the migration of tags towards the rear (Gonzalez *et al.* 2006; Butt & Lowe 2007; Butt *et al.* 2009). Another explanation for the disappearance of the tags is by the accidental insertion of the label into the gut, since we found elastomer mixed with faeces.

### 4.1.2. Lethal effects of tagging

Since total survival of tagged individuals largely differed from the control group, being 77.01 % and 97.5 % respectively, we suggest retaining the individuals for a period to ensure their viability (Gonzalez *et al.* 2006; Butt *et al.* 2009) before starting the experiment.

In our study, survival depends on the size of the animal at the moment of tagging, since the fragility in the earliest stages of the vital cycle is larger than in the sub-adult and adult phases. The experiment showed a survival of 100 % for individuals larger than 270 mg live weight.

Data reported by Butt & Lowe (2007) in a study with *Lumbricus terrestris*, where a survival of 96 % along the experiment was shown, match ours, since the initial live weight of *Lumbricus* juveniles is on average 500 mg (Daniel 1991). Field studies carried out on *Pontoxcolex corethrurus* (Gonzalez *et al.* 2006) with an initial live weight of 200 mg, which represents the boundary line for survival success in our experiment, reported a mortality of 20 % of tagged individuals, which adjusts well with our results. Regarding newly hatched and juvenile worms, there is no evidence of previous studies in earthworms with such a small size range. Since the viability of small specimens (27 to 60 mg live weight) is limited (44.45 %), special care is required when introducing the elastomer and an adequate needle size is necessary. We have proposed a model which predicts survival related to size in order to know the initial number of individuals required for each experiment depending on the stage of the vital cycle studied.

No influence of the number of inoculations given to each earthworm has been found, so this suggests that the damage is not caused by the number of punctures, being probably the quantity of elastomer introduced, or the time spent introducing it, the factors affecting worm viability. Nevertheless, locations of the elastomer involving vital organs, i.e. nephridial tissue, can also damage the animal (Butt *et al.* 2009).

### 4.1.3. Sub-lethal effects of tagging

The study of growth in marked individuals demonstrates no effect of tagging on the viability of earthworms 8 days after exposure to the elastomer, since maximal live weights achieved are similar to those reported in previous studies in earthworms cultured in horse manure (Neuhauser *et al.* 1980; Sangwan *et al.* 2008; Garg *et al.* 2005; Domínguez & Edwards 2011).

## 4.2. Study of the effects of population density

### 4.2.1. Density effects on growth

In order to test the influence of the population density on the development of *E. andrei* specimens, two culture methods were employed: group-cultured specimens (n = 33), with an average initial density of 56.84 mg g $^{-1}$, and individualized. Since the main objective was the study of differences in growth as a consequence of population density, clitellated individuals

within an initial size range from 794.965 to 1092.27 mg, in which no somatic weight evolution was observed along the experiment, were excluded from the analysis.

In sum, growth analysis of 29 group-cultured individuals (from 34.66 to 248.41 mg) and 18 individualized juveniles (from 34.43 to 187 mg) was performed. Size distribution of individuals of both methods was homologous, and no differences between methods regarding initial live weight were observed.

Maximal live weights achieved in the experiment largely differ between both culture methods, being, on average, three times larger in isolated worms than in grouped ones. Moreover, growth rates (mg day $^{-1}$) observed in the first month of the experiment varied from 7.39 mg day $^{-1}$ for grouped organisms to 16.53 mg day $^{-1}$ for isolated individuals. Results suggest that some density-dependent mechanisms are operating in group-cultured organisms. Many studies have found a decrease in individual growth rate and individual biomass as population density increases (Neuhauser *et al.* 1980; Domínguez & Edwards 1997; Ndegwa *et al.* 2000; Kammenga *et al.* 2003; Siddique *et al.* 2005). Furthermore, Klok (2007) established a negative linear relation between adult weight and density (*Adult weight* = 1412 − 56.5*Density*) for individuals of *Lumbricus rubellus*. Baveco & DeRoos (1996) proposed density-dependent mechanisms to consist of competition for food and space, and reproductive cost. In our experiment food was added every 20 to 25 days so, although food limitation could be precluding growth in group-cultured organisms, crowding negative effect on growth might be principally due to the energy cost of reproduction.

Earthworms are hermaphrodite, and, although Domínguez *et al.* (2003) found one case of self-insemination in a study with *Eisenia fetida* and *E. andrei*, and parthenogenetic production of cocoons has been also reported for many species of earthworms (not proven in *E. andrei*), they most commonly reproduce by cross-fertilisation (Edwards & Lofty 1977). In our experiment no evidence of self-insemination was found, since no cocoons were recorded in cultures of individualized earthworms. Development of gonadal tissue and formation of cocoons require a great quantity of energy from the animal, so, as it commonly occurs in all species of animals, energy from ingesta is sent to provide resources for the reproductive process in detriment of

somatic growth (Hartenstein *et al.* 1979; Sibly & Calow 1986). Crowded environments promote reproduction, since mating probability is higher and the excretion of pheromones to the media might accelerate the development of the clitella. In fact, data from population density studies carried out with *E. andrei* (Domínguez & Edwards 1997) and *E. fetida* (Unuofin & Mnkeni 2014) reveal that individuals grown in high density cultures developed the clitellum at smaller sizes than those grown isolated. This explains growth divergence between the two culture methods and the existence of early breeding specimens in group-cultured animals.

Growth models have been established focusing on the logistic growth curve which is in fact divided in 5 consecutive stages: lag, exponential, rectilinear, power and asymptotic. Animal growth has been modeled according to the point of the curve where the animal was located along experimental time. Growth patterns are extremely linked to reproductive processes and, concomitantly, depend on population density. In our experiment, asymptotic growth models are represented by a continuous biomass increment followed by a halt in somatic growth, achieving the maximal live weight before concluding the trial. This pattern was observed in approximately 50 % of individuals grown in mesocosm, being the asymptotic point (maximal biomass) associated with detection of clitellum (Neuhauser *et al.* 1980; Domínguez & Edwards 1997; Kammenga *et al.* 2003; Siddique *et al.* 2005; Unuofin & Mnkeni 2014), when the translocation of energy from somatic growth to reproduction occurs (Sibly & Calow 1986). In contrast, data from worms cultured isolated reveal continuous biomass increment in ~95 % of individuals. This difference in the distribution of growth patterns is illustrated in Fig. 9.

No differences were found between both culture methods in the proportion of individuals with power or exponential patterns, being represented by ~20 percent and ~25 percent of individuals respectively.
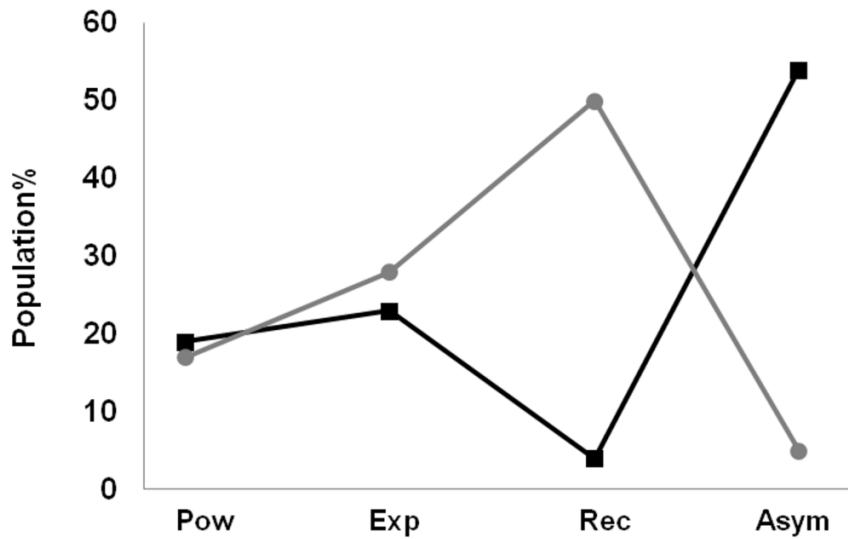
**Fig. 9.** Percentage of the population that adheres to each of the growth patterns in the species *Eisenia andrei*. Black squares: group-cultured; Grey circles: Individually cultured.

## 4.2.2. Density effects on carrying capacity

To test the influence of population density on stocking rate, three replicates with 11 tagged individuals per container were employed, differences between boxes regarding initial live weight not being significant (Mean initial density: 56.84 mg live weight / g dry horse manure). Our results demonstrate the existence of a maximal population density the culture can support, and match previous studies performed in *E. fetida* (Neuhauser *et al.* 1980; Ndegwa *et al.* 2000; Unouofin & Mnkeni 2014) which reported a loss in total biomass when achieving the maximal stocking rate. Moreover, Neuhauser *et al.* (1980) found a maximal density of 14.13 mg live weight / g wet substrate, which matches our results (13.113 mg live weight / g wet substrate). In sum, this decline in total biomass of the culture can be explained by the competition for food and space, toxicity of metabolites excreted by individuals, and the reproductive costs.

# 5. Conclusions

Relationship between clitellum acquisition and growth, and data from Domínguez & Edwards (1997) and Unuofin & Mnkeni (2014) explained above, let us understand differences in growth parameters found between individually and group-cultured worms. High differences in biomass increment were obtained in the different culture methods, observing average maximal live weights of 598.52 mg and 1453.08 mg in grouped and individualized cultured worms respectively. Due to the high influence of density on growth (Domínguez & Edwards 1997; Klok 2007; Unuofin & Mnkeni 2014), it is an important parameter to consider in the vermicomposting industry. In this context, tagging allows us to select lineages of animals with different growth patterns (early breeding, giants…), and, depending on the needs, selected individuals can be removed from the population and inoculated in the culture. Since no sub-lethal effects of the tagging method were observed, studies of individual growth and reproduction in animals from a population can be undertaken, and the method allows work with every stage of the vital cycle of *E. andrei*, from newly hatched to adults. This method is also useful in dynamic population studies or field monitoring of individuals, in behaviour experiments, and ecotoxicity estimation trials.

# Chapter 4

## 4. Oxygen consumption of the earthworm

## *Eisenia andrei*: Effect of isolation

# Oxygen consumption of the earthworm *Eisenia andrei*: Effect of isolation

# Abstract

Metabolic rate is a measure of the costs of living, and both endogenous and exogenous factors are liable to influence overall energy expenditure. In this work, we have focused on body size as the key endogenous factor whereas population density, understood as both added effects of competition for nutritional resources and reproductive behaviour, has been chosen as a mixture of exogenous and endogenous pressures. Within this frame, the purpose of this study has been analyzing whether respiratory metabolism (oxygen consumption, in $\mu L\ O_2\ h^{-1}$ animal$^{-1}$) in *E. andrei* would appear dependent on culture condition (grouped vs. individualized) introducing body weight as a covariate. In our experimental design monthly determinations of oxygen consumption have been performed in 51 individuals (18 individualized and 33 group-cultured). Group-cultured individuals (distributed in 3 groups) were labeled with fluorescent elastomers and kept in 1100 cm$^3$ capacity containers (11 individuals per container. Initial density: 56.84 mg live weight / g dry substrate), while individualized worms were maintained in 450 cm$^3$ boxes, horse manure being the substrate in both cases. A common weight exponent of 0.66 was obtained for both culture conditions and the influence of isolation resulted in increased metabolic rates (x 1.21). This is associated to the earlier development of reproductive structures in group-cultured organisms submitted as well to intraspecific competition for nutrients. An analysis of the influence of reproductive state on whole animal´s metabolic costs reveals a sharp difference between body mass exponents for mature and non-mature worms in both culture conditions. In isolated organisms an isometric relationship between oxygen consumption and weight was found in non-mature organisms whereas in mature individuals metabolism scales allometrically to weight (b=0.476). The opposite result for the group-cultured worms was obtained, being the slopes 0.592 and 0.96 for non-mature and mature organisms respectively.

# 1. Introduction

Earthworms, as heterotrophic organisms, obtain from the assimilated organic matter the energy to supply their vital functions. This energy is transferred to high energy content molecules (ATP) for the posterior use in endergonic reactions. Metabolic rate accounts for demands of matter and energy transformation rates within an individual (Meehan 2006) and measuring the metabolic rate implies measuring the costs of living (Hulbert & Else 2000).

Metabolic rate of a given organism is conditioned by several factors and among them, body mass is considered to be of major importance (Meehan 2006). It is reasonable to expect a higher respiration, in absolute terms, in big animals than in small individuals, but, since oxygen uptake is a surface dependent function and body surface increases in two dimensions while body mass increases in three dimensions, a negative allometry (b < 1) related to size increase could be expected (Rubner 1883). Many theories have been postulated regarding the fact that metabolism does not usually scale proportionally to body mass (values of the exponent: $^2/_3$ or $^3/_4$ power of weight), data coming principally from interspecific comparisons of endothermic animals (von Bertalanffy 1957; Glazier 2005; Moran & Wells 2007). At this respect, the present study provides data from routine metabolism of ectothermic individuals belonging to the same species.

Population density is a factor that affects growth and reproductive behavior in earthworms. Many studies have demonstrated an earlier development of gonadal tissue and decrement of individual body mass associated with crowded environments (Domínguez & Edwards 1997; Kammenga *et al.* 2003; Unuofin & Mnkeni 2014). However, few studies have been made to ascertain whether metabolism is affected by the presence of co-specific individuals. Uvarov & Scheu (2004) in an study of the effects of density on respiratory activity in *Lumbricus rubellus* and *Dendrobaena octaedra* found that respiration rates in *L. rubellus* did not differed between individuals kept in group or individualized, whereas respiration in isolated *D.octaedra* specimens was higher than respiration of individuals kept in group.

In the present study the objective was to ascertain whether the metabolism, measured in terms of oxygen consumption ($\mu L\ O_2\ h^{-1}$ animal$^{-1}$), of individuals of the species *E. andrei* was altered by the presence of co-specific specimens. For this purpose, two different culture conditions were established (Grouped and Isolated), and individuals were kept in horse manure for a 3 months period, taking monthly oxygen consumption measurements.

# 2. Material and methods

The influence of isolation on aerobic metabolism has been tested in this study. For this purpose, 33 earthworms labeled with fluorescent elastomers were placed in three containers with similar initial densities, whereas 18 other worms were individually cultured. Individual metabolic responses were determined by means of aerobic respiration, measured in terms of the oxygen consumption rate ($VO_2$ in $\mu L\ O_2\ h^{-1}$ animal $^{-1}$) according to the Warburg manometric method.

## 2.1. Experimental procedure

A total sample of 51 earthworms, from an ongoing culture maintained in darkness under control conditions of temperature (21 $^o$C ± 1) and humidity (85 % RH) fed with horse manure was used in the experiment. A group of 33 worms ranging from 34.66 to 1092.27 mg was chosen and labeled with fluorescent elastomers, following the procedure detailed in Chapter 3. Tagged earthworms were carefully split into three 1100 cm$^3$ capacity containers filled with 45 g of horse manure on a dry weight basis (11 earthworms per box), and caution to maintain similar initial population densities among containers was keep (Mean initial density= 56.84 mg worm live weight/ g dry substrate). The remaining 18 worms within an initial size range from 34.43 to 187 mg were individually cultured in 450 cm$^3$ capacity containers filled with 25 g of dry horse manure.

Animals were cultured for 3 months and maintaining parameters were similar to the ongoing culture. Additional food was added every 20-25 days in order to avoid a reduction in foodstuff availability. Individual biomass was ascertained from pictures taken every 3-4 days, according to a photogrammetric method (see chapter 2) in which a scale was set between biomass (live weight in mg) and image surface (body surface in mm$^2$). Dry weight (DW in mg) was estimated

from live weight (LW in mg) by means of the equation: *LogDW = 1.025\*LogLW - 0.99* (see chapter 2). Oxygen consumption ($VO_2$ in $\mu L$ $O_2$ $h^{-1}$ animal $^{-1}$) was monthly and individually determined (~ 4 determinations per individual), the first measurement being taken after 5-day permanence in the experimental culture conditions.
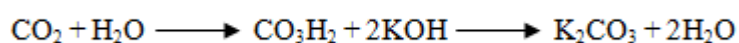
## 2.2. Respirometric determinations: The Warburg method

Oxygen consumption was individually measured using the Warburg manometric method which is based on the ideal gas law, and entails registering the changes in pressure of the gas in an enclosed measuring system (respirometric chamber).

$$P \cdot V = n \cdot R \cdot T$$

**P**: Pressure of the gas; **V**: Volume of the gas; **n**: Amount of substance of the gas; **T**: Temperature; **R**: Universal gas constant (0.082).

By measuring pressure changes in the chamber, it is possible to quantify variations on gas quantity (n) since volume (V) and temperature (T) are kept constant. The respiration process implies $O_2$ uptake and $CO_2$ efflux, so, in order to measure pressure variation due to $O_2$ consumption, $CO_2$ must be removed from the respirometric chamber. For this purpose a chemical trap, KOH dissolution, is used:

$$CO_2 + H_2O \longrightarrow CO_3H_2 + 2KOH \longrightarrow K_2CO_3 + 2H_2O$$

Manometers are composed of two branches: one connected to the respirometric chamber and the other open to the atmosphere. The first branch is equipped with a key to isolate the chamber from the atmosphere. Manometric liquid used to measure pressure variation was a Brodie solution obtained by adding 46 g of NaCl, 10 g of sodium iodine and 0.4 g of Evans Blue to 1 L of distilled water.

*E. andrei* specimens were carefully cleaned and weighed before being individually placed in respirometric chambers (12 $cm^3$), which, once connected to the manometer, were submerged during the experiment in a thermostatic bath at 20 $^o$C. A chamber without earthworms (thermobarometer) was used as a control. Respirometric chambers were maintained in constant darkness, and a cloth soaked in deionized water was placed inside in order to keep moisture

conditions, and also serving as worm-borrowing medium. While the respirometric chamber reaches the experimental temperature, gas inside the chamber will vary its volume, so a period of temperature adaptation is necessary for the inside of the chamber to attain experimental conditions. After that period, the two branches of the manometer are level at 150 mm of Brodie liquid, and the respirometric chamber is isolated from the atmosphere by closing the key.

Respiration of animals produces a decrement of the pressure inside the chamber. Consequently, the Brodie liquid of the branch connected to the respirometric chamber rises. To take the measurements, the branch of the manometer connected to the respirometric chamber was leveled to the point it was set at the beginning of the experiment (150 mm of Brodie) in order to keep a constant volume. Oxygen consumption was estimated from the difference in height between the liquids of the two branches, corrected by the control (thermobarometer). These differences represent pressure variation between the respirometric chamber and the atmosphere. Measurements were taken every 20 to 30 minutes, depending on the size of the animals, for a period from 1 to 2 hours.

Manometric measurements were transformed into oxygen consumption rates ($VO_2$ in $\mu LO_2\ h^{-1}$) by the following equation:

$$VO_2 = h * K * 1000$$

where *h* is the manometric height (mm/hour) and *K* the chamber constant:

$$K = V_g * 273 / T * P_0$$

$V_g$: volume of the gas phase in mL; $T$: experimental temperature in Kelvin degrees; $P_0$: atmospheric pressure measured in mm of Brodie liquid (10000 mm Brodie is equivalent to 760 mmHg).

## 2.3. Statistical analysis

As a consequence of the noticeable effect of animal size on the metabolic rate ($VO_2$ in $\mu L\ O_2\ h^{-1}$), data have been allometrically analysed according to the equation: $VO_2 = a * DW^b$, where $DW$ is the dry weight of the animal in mg, "b" the exponent of the weight and "a" the specific

coefficient. As the relation is a power law, it is possible to obtain a linear regression by a $Log_{10}$-$Log_{10}$ transformation of variables: Log $VO_2$ = Log$a$ + b *Log$DW$.

Linear regressions were obtained with a repeated-measures linear mixed-effect model test, using lmer4 (Bates *et al.* 2015) and MuMIn (Barton 2016) R packages. Later, regression simple analyses were performed (STATVIEW 5.0.).

Since the oxygen consumption rate of each earthworm was measured more than once along the experimental period, differences between the slopes of linear regressions have been analysed with a repeated measurements ANCOVA test (IBM Corp. Released 2013. IBM SPSS Statistics for Windows, Version 22.0. Armonk, NY: IBM Corp.). Later, differences between the parameters of the linear regression equations were analysed through a standard ANCOVA procedure (STATVIEW 5.0.). T-test was used to evaluate the isometry of slopes and the differences between the slopes obtained with the theoretical slope of 0.66. Tukey-Kramer test was used to ascertain differences between intercepts.

Basic statistics (Frequency distribution and ANOVA test) were employed to describe and compare samples of animals (STATVIEW 5.0.)**.**

# 3. Results

Metabolic responses ($VO_2$ in µL $O_2$ $h^{-1}$animal$^{-1}$) of *E. andrei* individuals from two different rearing conditions, individualized *vs*. group-cultured, have been analysed. Measurements for each individual earthworm have been performed at 1 month intervals (4 measurements).

## 3.1. Size structure of samples

Size distributions of individuals (live weight in mg) at the beginning of the experiment (day 1) are represented in Fig. 1. Tagged earthworms cultured in group (34.66 to 248.41 mg) appear in Fig. 1A where (for practical representation purposes) individuals larger than 700 mg (n = 4; Mean = 826.92 mg) are not shown. In Fig. 1B isolated specimens raging from 34.43 to 187 mg are represented. Earthworms were cultured for 5 days before starting metabolic measurements.
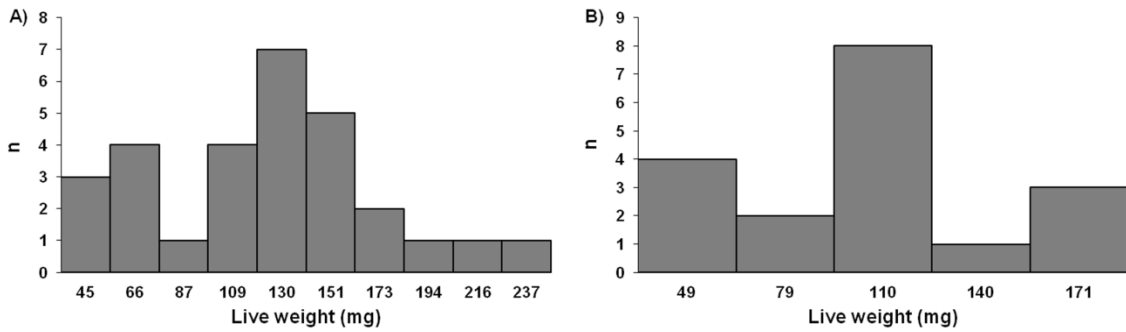
**Fig.1.** Histograms for frequency distribution of Live Weight (mg) of juvenile specimens of *E. andrei* used in the study of influence of isolation in metabolic responses. A): Group-cultured individuals (Mean= 124.06; SD = 51.90). B): Isolated cultured individuals (Mean = 105.337; SD = 44.772).

Isolated worms (n = 18) exhibited high growth rates and clitellum acquisition took place at a mean live weight of 530.28 mg (S.E = 21.92) so that virtually all the population (89 %) had matured after the initial 30 days of experiment (Table 1). In contrast, group-cultured organisms matured continuously along maintenance period and mean size at clitellum acquisition was significantly lower (p-value < 0.01): 423.68 mg live weight (S.E = 21.76).

**Table 1**. Dynamics of clitellum acquisition along the experiment in *E. andrei*: accumulated percentage.

| Culture conditions | Day 1 | Day 30 | Day 60 | Day 90 |
|---|---|---|---|---|
| Isolated | 0 | 88.8 | 100 | 100 |
| Grouped | 12.12 | 26.66 | 60 | 100 |

## 3.2. Analysis of metabolic responses

Since growth rate in isolated earthworms exceeded animals reared in group, size range along time differs between conditions and considering the whole time interval we had worms from 94.41 to 1867.43 mg in individually cultured worms and from 47.62 to 948.74 mg in those reared in group.

Regression equations (Log-Log transformation) between oxygen consumption (VO$_2$ in µL O$_2$ h$^{-1}$) and animal dry weight (DW in mg) were obtained after two different analyses (repeated-measures linear mixed-effect model test *vs*. Standard linear regression test) and identical results

for regression coefficients were obtained in both analyses. Parameters of linear regression equations covering actual size range for both culture conditions are summarized in Table 2.

On a first approach, a comparison of the slopes obtained for the two regression equations was performed using repeated measurements ANCOVA analysis. Results displayed homogeneity of the slopes since *p-value* for the interaction term for the product of the covariate (Log DW) and the factor (culture condition) largely exceed 0.05. Later, regression equations were tested using standard ANCOVA procedures (Table. 2) and a common slope of 0.624, not different from the theoretical 0.66, was calculated. This indicates that metabolic allometry was not affected by differences in size ranges attained in this experiment. Intercepts for both groups differed significantly appearing higher (0.503 vs. 0.419) for worms reared individually.

As a result we derive the following allometric equations, which indicate that isolation increases metabolism by a factor of 1.21:

Group-cultured individuals: $R^2 = 0.643 \quad VO_2 = 2.63 * DW^{0.66}$

Individually cultured individuals: $R^2 = 0.806 \quad VO_2 = 3.19 * DW^{0.66}$

### 3.2.1. Influence of reproductive condition

An analysis of worms reared in isolation according to developmental stage –Juvenile non-clitellated *vs.* Mature clitellated worms- resulted in an isometric relationship between $VO_2$ and DW for immature non-clitellated worms ranging from 94.41 to 370.05 mg live weight (t = -1.206, *p-value* = 0.247), indicating metabolism increases with body weight by a fixed factor (intercept) :$VO_2 = DW$, $R^2=0.873$. In contrast, mature worms (from 474.31 to 1867.4 mg live weight) exhibited negative allometry and adjust to the equation: $VO_2 = 7.8 * DW^{0.476}$, $R^2=0.45$ (Fig. 2).

**Table 2**. Coefficients of Log-Log regression equations of $VO_2$ ($\mu L\ O_2\ h^{-1}$) *vs.* Dry weight (DW in mg) of *Eisenia andrei* specimens individualized and group-cultured. Differences in Individual equation for oxygen consumption measurements between two culture conditions analysed through ANCOVA. Size range in mg of live weight. $CI_{95\%}$ = Confidence intervals. Intercept $_r$ = Recalculated value of the elevation for the common exponent of 0.66.

| Culture method | n | Size range (mg) | $R^2$ | Intercept (CI $_{95\%}$) | Slope (CI $_{95\%}$) | Intercept $_r$ |
|---|---|---|---|---|---|---|
| Individualized | 68 | 94.41 - 1867.43 | 0.81 | $0.483 \pm 0.15$ | $0.671 \pm 0.08$ | 0.503 |
| Grouped | 123 | 47.62 - 948.77 | 0.65 | $0.511 \pm 0.12$ | $0.599 \pm 0.08$ | 0.419 |
| ANCOVA | DF | F-value | P-value | Common values | | |
| Slope | 1,187 | 1.318 | 0.2525 | 0.624*[1] | | |
| Intercept | 1,188 | 15.991 | $< 0.0001$ | | | |

*[1] Slope (b) = 0.66 (t = -1.2141, *p-value* = 0.2262)

**Table 3**. Coefficients of Log-Log regression equations of $VO_2$ ($\mu L\ O_2\ h^{-1}$) *vs.* Dry weight (DW in mg) of the different size ranges found in non-mature *Eisenia andrei* specimens cultured in group. Differences in Individual equation for oxygen consumption measurements between size ranges analysed through ANCOVA Size range in mg of live weight. $CI_{95\%}$ = Confidence intervals. Intercept $_r$ = Recalculated value of the elevation for the exponent of 1.

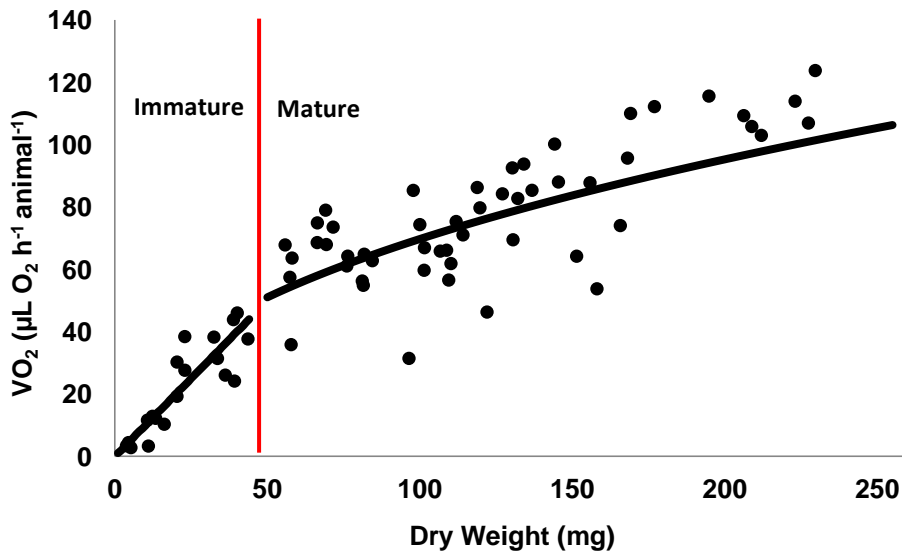| Size range (mg) | n | R2 | Intercept (CI $_{95\%}$) | Slope (CI $_{95\%}$) | Intercept $_r$ |
|---|---|---|---|---|---|
| 47 - 90 | 13 | 0.47 | $0.152 \pm 0.62$ | $1.065 \pm 0.75$ | 0.204 |
| 100 - 350 | 52 | 0.52 | $0.144 \pm 0.32$ | $0.899 \pm 0.24$ | 0.012 |
| 351 - 593 | 10 | 0.60 | $-1.030 \pm 1.79$ | $1.445 \pm 1.04$ | -0.262 |
| ANCOVA | DF | F-value | P-value | Common values | |
| Slope | 2,69 | 0.434 | 0.6468 | 0.933*[1] | |
| Intercept | 2,71 | 7.449 | $< 0.01$ | | |

*[1] Slope (b) = 1 (t = -0.6129, *p-value* = 0.5419).

**Fig. 2**. VO$_2$ (µL O$_2$ h$^{-1}$ animal $^{-1}$) *vs.* Dry weight (mg) of *Eisenia andrei* specimens reared isolated in horse manure. Left immature individuals: $VO_2 = DW$, R$^2$=0.873. Right mature individuals: $VO_2 = 7.8DW^{0.476}$, R$^2$=0.45.

Although size ranges in both groups (immature and mature worms) present a common factor of variation (~4) a reduction of mass specific metabolic costs associated to size increase was restricted to mature specimen where the slope (0.476) differed significantly from the common slope (b = 0.66) obtained for the total population (t = -2.4228, *p-value* < 0.05).

Concerning group-cultured organisms, mature worms exhibited an isometric relation with body weight (slope = 0.9623, not significantly different from unity: t = -0.211, *p-value* = 0.834) whereas the non-mature population (47 to 593 mg live weight) exhibited negative allometry (slope = 0.592, not different from 0.66: t = -1.096, *p-value* = 0.2767). A detailed analysis of oxygen consumption according to differences in size range in juveniles was undertaken and results appear summarized in Table 3. ANCOVA analysis revealed a common slope of 0.933, not different from unity, indicating metabolism increases with body weight by a fixed factor within size ranges. Nevertheless, intercepts remained different, metabolic level decreasing with size rise.

# 4. Discussion

Oxidation reactions in heterotrophic organisms (i.e. aerobic respiration) account for the energy loss as heat, representing the fraction of exchanged energy spent in maintaining animal life. Among the several methods available to evaluate metabolic costs in animals, advantages of using determinations of oxygen consumption largely exceed the benefits of the other techniques and vast literature for different phyla has been reported since the beginning of the 20th century. Modification of anabolic or catabolic demands associated to variations in environmental conditions (stress factors, food limitation, temperature etc.) and/or changes during ontogeny such as development of gonadal tissue or somatic growth would require adjusting of physiological responses in a compensatory direction altering metabolic rates of oxygen consumption. As population density alters life cycle traits of this epigeic earthworm (Chapter 3), the main objective in this work was to understand the effect of isolation on metabolic responses, in terms of oxygen consumption ($\mu L$ $O_2$ $h^{-1}$ animal$^{-1}$), of *E. andrei* specimens.

The lack of works found in the literature about respiration in earthworms, and the variety of measurement methods, as well as differences in experimental designs and data analysis, make the information difficult to compare. In an attempt to make our data comparable to results found in literature, we have standardised oxygen consumption as mass specific metabolic rate, by dividing oxygen uptake by earthworm live weight ($\mu L$ $O_2$ $h^{-1}$ $mg^{-1}$). In works where $CO_2$ production was reported, it was converted to the equivalent oxygen consumption value using an assumed respiratory quotient of 0.82 (Meehan 2006).

In our experiment, measurements from earthworms cultured individually in horse manure (optimal conditions) were 0.118 and 0.0817 $\mu L$ $O_2$ $h^{-1}$ $mg^{-1}$ for immature (94.41 to 370,05 mg live weight) and mature (474.31 to 1867.4 mg live weight) individuals respectively. These figures match those provided by Uvarov (1998) for *Dendrobaena octaedra* worms with live weights ranging from 67.6 to 276 mg (0.129 $\mu L$ $O_2$ $h^{-1}$ $mg^{-1}$), and also data for single cultured *Lumbricus rubellus* specimens with an average live weight of 600 mg (0.1067 $\mu L$ $O_2$ $h^{-1}$ $mg^{-1}$) reported by Uvarov & Scheu (2004). In an early work performed on *Octochaetona serrata* individuals (1000 mg live weight), Saroja (1964) reported a respiratory rate of 0.08 $\mu L$ $O_2$ $h^{-1}$

mg $^{-1}$, in coincidence with our measurements in *E. andrei* individuals of that body size. The higher metabolic rates found by Moment & Haberman (1979) in *Eisenia foetida* individuals (0.20 μL $O_2$ h$^{-1}$ mg$^{-1}$) can be attributed to the smaller size of the specimens (100-250 mg live weight), as can be the case in a study with small immatures of *Allolobophora rosea* with sizes below 100 mg, performed by Phillipson & Bolton (1976), where average respiratory rate was slightly higher than our data for immature specimens (0.141 *vs.* 0.118 μL $O_2$ h$^{-1}$ mg$^{-1}$). In contrast, Šustr & Pižl (2010) reported respiration rates for juveniles and adults of *Dendrobaena mrazeki* of 0.067 and 0.064 μL $O_2$ h$^{-1}$ mg$^{-1}$ respectively, which were below our figures for the same sizes. Abe & Buck (1985) also reported lower metabolic rates for *Glossoscolex paulistas* individuals than those found in our work (0.0462 *vs.* 0.089 μL $O_2$ h$^{-1}$ mg$^{-1}$), but in this case, the difference can be explained by the larger size of the studied individuals (9500 mg live weight).

## 4.1. Effects of body size

The effect of size on metabolic rate has been thoroughly studied since the 19th century. This dependence can be expressed by the equation $M = aW^b$, where "M" is the metabolic rate, in our case μL $O_2$ h$^{-1}$, "W" is the body weight, "b" is the value at which metabolic rate scales to weight and "a" is a constant. This is a special case of the so called allometric formula (Huxley 1932), which expresses the dependence on body size for an enormous amount of morphological, biochemical, physiological and evolutionary data (von Bertalanffy 1957).

According to Rubner (1883), metabolic rate is proportional to the animal surface or the $^2/_3$ power of weight. However, Kleiber (1932) found that the weight exponent was more similar to $^3/_4$ rather than $^2/_3$ . The value of this exponent and its meaning has been discussed for 150 years and there is still no universally accepted theory (Hulbert & Else 2000; Glazier 2005; White *et al.* 2007). Due to the high interdependence between metabolism and size, in this work metabolic responses were analysed allometrically, and the influence of the culture conditions (grouped or individualized) on metabolic level of the specimens were tested using ANCOVA procedures, that allow for the inclusion of weight as a covariate. In addition, in order to preclude any effect

due to differences in hydration level between animals, and as body water does not contribute to the total respiration of the specimens, dry weight was chosen as the best predictor.

In our experiment, results for the exponent value demonstrated that allometry was not altered by differences in size ranges attained by worms reared in group or individually. In fact, a common exponent of 0.66 was obtained in accordance with the theory proposed by Rubner (1883). Similar values have been found in a work performed with *D. octaedra* (Uvarov 1998), and in a global study of the metabolic rate of litter and soil invertebrates (Meehan 2006), the exponent being 0.71 in both cases. Krüger (1952), in an early work performed with *E. foetida,* reported an exponent of 0.75, and Barnes *et al.* (1993) reported an exponent of 0.76 for adults belonging to the family Lumbricidae. Overall, values ranged from $^2/_3$, as found in our experiment, to ¾, matching the theoretical values explained above. Allometric growth of tissues, and differences among them in growth rates and in biochemical composition, have been mentioned as possible reasons of b < 1 (Hulbert & Else 2000; Glazier 2005). Earthworms have no specialized respiratory organs, and oxygen diffuses through the cuticle and epidermal tissue into the blood, so respiration and excretion are surface related functions. During ontogeny, surface/volume ratio decreases and thus would limit the ability to serve a metabolism directly proportional to body mass (Hulbert & Else 2000). Moreover, the emergence during growth of metabolically less active components, such as granular storage material associated with the development of gonadal tissue, may account for a relative global depletion of metabolic rate in mature individuals. In fact, studies with the snail *Cornu aspersum* (Rodriguez 2013) and with the slug *Arion ater* (Bizarro 2010) reported lower weight specific metabolic rates in gonadal tissue than those shown by somatic tissues, contributing to a global reduction of metabolic costs per weight in mature animals.

## 4.2. Influence of isolation on metabolic expenditure

As described by several authors, and in a previous chapter of the present work, population density affects growth and reproduction of earthworms. With this experiment, our aim was to determine whether the respiration of animals was affected by the presence of co-specific

individuals. Although no variation in the relationship between oxygen consumption ($\mu L\ O_2\ h^{-1}$) and dry weight (mg) was observed for both culture conditions, the elevation remained different, being 2.63 and 3.191 in earthworms cultured in group or isolated, respectively. This indicates that metabolic level was higher in isolated individuals by a factor of 1.21.

In the experiment, the development of reproductive organs (clitellum appearance) occurs at statistically significant (*p-value* < 0.01) smaller sizes in earthworms reared in group (423.68 ± 21.76 *vs.* 530.28 ± 21.92 mg live weight). This might explain, partially, that even though metabolism scales similarly to weight in both culture conditions, isolation causes a metabolic level increase in individually cultured earthworms due to their late (in terms of body size) maturation. Moreover, oxygen consumption is known to correlate positively with food availability in many species of animals (Koehn & Bayne 1989; Barnes *et al.* 1993), and in particular in a study with *E. fetida*, the oxygen consumption was significantly affected by food availability (Diehl & Williams 1992). In this line, although for the present study horse manure was added every 20 to 25 days in order to preclude food limitation, the quantity of foodstuff available per mg of earthworm tissue is lower in group-cultured organisms than in isolated ones, so intraspecific competition for food and space, together with the earlier gonadal development, can explain the depletion of metabolic level in crowded environments. In fact, Uvarov & Scheu (2004) found that respiration rate was significantly lower in *D.octaedra* worms kept in groups as compared to specimens kept individually; nevertheless, they found no influence of density in the metabolic rate of *Lumbricus rubellus* specimens.

## 4.3. Effect of the developmental stage

Changes in the value for the exponent of the weight during ontogeny have been previously described in different species. At this respect, Glazier (2005) proposed four animal models depending on the variation of this exponent during developmental stages. Animals reared isolated can be associated with Group III described by Glazier (2005), characterized by a metabolism directly proportional to weight (b=1) up to a critical size, and from that point on, a negative allometry is observed. In our experiment, a homogeneous population in isolated

immature individuals was obtained, being the shift point (point when b < 1) associated with the detention of clitellum. This fact supports the hypothesis proposed above in which the allometry of metabolism was related to growth and development of reproductive structures.

In contrast with the results for isolated animals, in group-cultured organisms an heterogeneous population was observed in immature worms, and three different age classes were observed (see Table 3 of results), indicating that the presence of co-specific individuals does alter the development pattern of a given organism.

# 5. Conclusions

The effect of body mass on metabolic expenditure has been thoroughly exposed in this work and a common exponent of 0.66 was obtained irrespective of the culture conditions (grouped or isolated), observing lower metabolic rates with increasing in size. The study also demonstrates that the presence of co-specific individuals produces a decrement in metabolism (elevation for group-cultured 2.63; elevation for isolated 3.19), explained by the earlier development of reproductive structures and by the intraspecific competition for food and space that take place in group-cultured organisms.

# Chapter 5

**5. Life cycle traits of *Eisenia andrei* dwelling in aging grass: An approach to food quality of litter for epigeic earthworms**

# Life cycle traits of *Eisenia andrei* dwelling in aging grass:

# An approach to food quality of litter for epigeic earthworms

## Abstract

Epigeic earthworms play a crucial role in maintaining soil fertility due to their ability to process both spontaneous inputs of plant material and remnants from grass management practises in agro-ecosystems. We have analysed eclosion, survival and individual growth performance of new-born *Eisenia andrei* in grass submitted to different composting periods, to understand long-term recruitment success and colonization capacities of epigeic lumbricids foraging on grass litter. For this purpose, 5 grass substrates in a range of aging periods from 1 to 13 weeks were selected and a horse manure substrate was added as reference. In this scheme, cocoons and hatchlings (mean live weight 3.19 ± 1.19 mg) were individually cultured in darkness under controlled temperature and humidity (20ºC and 80 % RH) and eclosion success, mortality and individual growth were computed. Organic matter and biochemical analyses of substrates were undertaken to provide an approach to food quality: increased mineralization associated to decreasing fibre percentages along aging resulted in reduced viability in terms of eclosion, survival and growth. Mortality appeared after 3 weeks (11.11 %) raising lineally to 33.33 % after 13 weeks aging while average maximal live weights (MLW) declined from ~120 mg (Horse manure, 1 and 3 weeks) to ~60 mg (7 to 13 weeks). Growth patterns along aging and organic matter (OM:% dry weight) can be portrayed by a sigmoid function explaining 90 % of variability and the shift point between high and low growth rates is set at 80 % OM. Reduction of both survival and growth rates when organic matter content remains high (62.02 %-77.7 %) is explained by lower food quality understood as limited supply of labile carbohydrates with increased digestive costs and poor absorption efficiencies.

# 1. Introduction

Preservation of soil health has become a main concern along the second half of the 20th century and, in this context, definition of soil quality becomes a key factor (Nortcliff 2002). Under natural or semi-natural conditions, soil generation and amendment relays basically on the activity of microbiota and epigeic earthworms, their mutualistic relation contributing to litter and coarse organic matter degradation, providing plants with ready available nutrients. Although mineral fertilizers are widely employed, studies carried out mainly in unmanaged ecosystem find a reduction in soil microbial biomass concomitant to Nitrogen increase with potential modifications in community composition (Geisseler & Scow 2014). So, in sustainable agriculture practices, soil fertility should be preferably linked to maintaining and enhancing activity of these ecosystem engineers since formation of new soil layers and soil structure are dependent on population yield of worms and microorganisms (Brown *et al.* 2000). Regarding epigeic earthworms, preferred habitats include litter rich deciduous forest sites (Curry 1998) and grassland areas, where they aggregate beneath cattle dung (Curry & Schmidt 2007) showing highest densities in manure and pastures (Edwards & Lofty 1977). European semi-natural pastures and meadows, claimed as high ecological value landscapes (White *et al.* 2000) are submitted to a variety of management practices (grazed, mowed and/ or cut but not ploughed or tilled), being dominated by grasses or herbs mainly self-seeded. Nutritive value as cattle fodder is both regionally and seasonally variable (Crampton & Harris 1969) and inversely related to fibre content: from around 25 % in spring green grass to 40 % in mature pasture. In fact, surface feeding earthworms forage preferably on dung, followed by succulent herbage litter (Edwards & Lofty 1977).

In Northern Atlantic Spain herbivore feces and grass litter enter the chain of composting and soil generation through low intensity familiar eco-agro-systems exploitations either by leaving cut grass in the open air or by composting of green wastes material in order to obtain natural fertilizer. In our study, suitability of these substrates to support active populations of the epigeic earthworm *Eisenia andrei* BOUCHÉ 1972 has been tested using horse manure and

spontaneously decaying mowed grass (in the open air) at different periods of aging. Since the aim of this work involved gaining insights on success in long-term recruitment and colonization capacities deriving in patchy distribution of epigeic earthworms, most labile stages of the vital cycle (from cocoon to juvenile pre-clitellated stage) have been chosen. For this purpose, individual responses have been analyzed in terms of added output of physiological mechanisms: eclosion, survival and growth.

# 2. Material and methods

## 2.1. Experimental design

Cut-grass, submitted to variable periods of aging, was used as incubating and growing substrate in cocoons and hatchlings of *E. andrei*. Mowed grass from an Atlantic pasture (43°35´ N, -2°86´ E) was collected in May 2012 and spontaneously composted in the open air in heaps less than half a metre height. Samples of grass were collected initially and after 1, 3, 7, 11 and 13 weeks and horse manure, obtained from horses grazing the same pasture, was taken as a control.

Experiments performed with freshly cut grass (1 day) resulted in total eclosion failure and absolute mortality of newly hatched worms precluding further treatment of data.

### 2.1.1. Parental selection

Mature clitellated individuals of homogenous size (LW ~600 mg) were extracted from an on-going culture of *E. andrei* reared under constant conditions (darkness; $20 \pm 2$ °C; $80 \pm 3$ % RH) in horse manure (86 % organic matter; pH = $7.25 \pm 0.04$). Collection took place by hand shorting (Edwards & Bohlen 1996) and selected adults (n = 60) were placed in a box (10.4 dm$^3$) containing 5 dm$^3$ of horse manure where conditions were kept similar to the origin culture. In order to prevent the initiation of incubating period in the shedding tank, cocoon gathering involved intensive inspection guarantying extraction closest possible to shedding (< than 24 h).

### 2.1.2. Eclosion experiment

A number of 40 cocoons were individually incubated in 25 cm$^3$ boxes (identical controlled conditions of parental) filled alternatively with horse manure (20 boxes) and aging grass

belonging to the initial (1 week) and final (13 weeks) stages of composting (10 cocoons per stage). Eclosion dynamics were daily monitored and measurements of eclosion success (%), incubation time (days), number of hatchlings and individual biomass per cocoon were recorded.

### 2.1.3. Survival and growth experiment

A group of 65 cocoons were individually incubated in 25 $cm^3$ boxes filled with reference substrate (horse manure) and, immediately after eclosion, one hatchling per cocoon was randomly selected and individually placed in identical containers filled with horse manure (20 boxes) and grass-composted for 1,3,7,11 or 13 weeks (9 boxes per substrate). Both cocoons and newly hatched worms were kept under identical controlled conditions of parental regarding temperature, humidity and light regime.

## 2.2. Biomass determination

Individual biomass was determined by the photogrammetric, non-destructive, method (see chapter 2) developed for tubular organisms (Bernardini *et al.* 2000; Brea 2009) where area of pictures taken every 3 days, is transformed in live weight using a scale of live weight (mg) to body surface ($mm^2$) obtained from simultaneous recordings of both parameters.

## 2.3. Biochemical analysis of substrates

Dry weight was measured after 24 h oven exposure at 90 $^o$C and organic matter by ashing previously dried samples at 450 $^o$C for 24 hours. Colorimetric methods were undertaken to obtain total protein content (Lowry *et al.* 1951) and total soluble carbohydrates (Dubois *et al.* 1956). Total lipids were isolated according to Bligh & Dyer (1956) and crude fiber extracted with an acetic-nitric acid mixture described by Sloneker (1971), both compounds being quantified by weight determination ($10^{-6}$ balance). Potentiometrical analysis of pH was performed in samples suspended in bidistilled water (1:2, vol:vol) with a soil pH-meter (Hanna Instruments, S.L. ; Model: HI 99121).

## 2.4. Statistical analysis

Basic statistics (Frequency distribution, ANOVA procedures and non-parametric Kruskall-Wallis test) were employed to describe and compare samples of animals and substrates (STATVIEW 5.0.). Non-linear regression procedures were performed with SYSTAT version 13, from Systat Software, Inc., San Jose California USA, www.sigmaplot.com.

# 3. Results

## 3.1. Chemical analyses of substrates

Initial characteristics of biochemical composition and pH of grass substrates are summarized in Table 1. ANOVA analysis found statistical differences in pH between horse manure and grass but no differences were evident between grass substrates (*p-value* = 0.126). No effects of aging were found as regards lipid (*p-value* = 0.426) or soluble carbohydrates (*p-value* = 0.825) proportions, being the latter significantly different from horse manure regardless composting period (*p-value* < 0.0001). Conversely, both fibre and protein percentages differed significantly among substrates: fibre content varied for every substrate (*p-value* < 0.05) whereas protein content showed differences between grass composted for 1 or 7 weeks (*p-value* = 0.0038) and between the latter and the set of substrates over 7 weeks (*p-value* < 0.001). Mineral matter increased continuously along composting time as shown in Fig. 1. An exponential equation has been chosen to analyze time effects: *InorganicMatter%* = 15.421*$e^{(0.009*Time)}$; $R^2$ = 0.952.
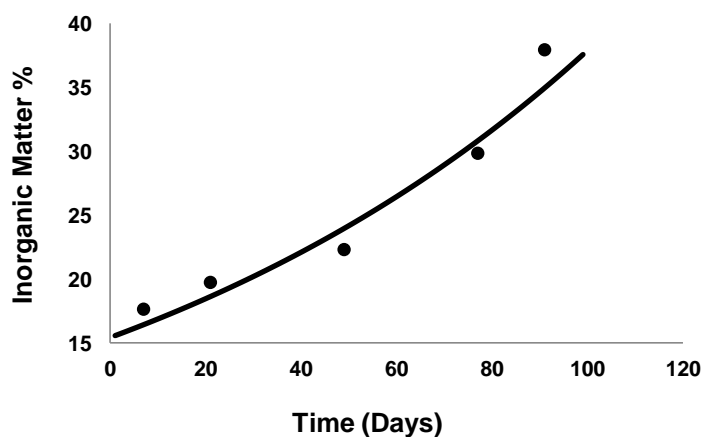


**Fig. 1** Evolution of Inorganic matter (% of dry matter) along grass aging (days). *InorganicMatter%* = 15.421*$e^{(0.009*Time)}$, $R^2$ = 0.952.

**Table 1.** Chemical composition of horse manure and grass aged along different periods (weeks). Means with standard errors are shown. SCH % = Soluble Carbohydrate.

| Substrate | pH | Ash % | Lipid % | Protein % | SCH % | Fibre % |
|---|---|---|---|---|---|---|
| **Horse manure** | 7.25 ± 0.04 | 13.11 ± 0.07 | 4.32 ± 0.14 | 21.09 ± 0.30 | 25.11 ± 0.06 | 36.36 ± 0.07 |
| **Grass 1 Week** | 6.39 ± 0.04 | 17.63 ± 0.80 | 3.99 ± 0.06 | 22.86 ± 0.63 | 10.81 ± 1.32 | 44.68 ± 0.51 |
| **Grass 3 Week** | 6.48 ± 0.05 | 19.73 ± 0.06 | 4.49 ± 0.13 | 24.12 ± 0.68 | 10.08 ± 0.04 | 41.56 ± 0.30 |
| **Grass 7 Week** | 6.35 ± 0.05 | 22.30 ± 0.71 | 4.50 ± 0.75 | 25.12 ± 0.35 | 9.62 ± 1.42 | 38.43 ± 0.80 |
| **Grass 11 Week** | 6.51 ± 0.06 | 29.84 ± 0.41 | 3.62 ± 0.05 | 20.06 ± 0.26 | 9.61 ± 0.82 | 36.84 ± 0.31 |
| **Grass 13 Week** | 6.53 ± 0.07 | 37.94 ± 0.63 | 3.47 ± 0.16 | 19.57 ± 0.22 | 9.49 ± 0.04 | 29.51 ± 0.68 |

**Table 2**. Means for eclosion parameters of cocoons of *Eisenia andrei* reared in the different substrates used in the experiment. Standard errors are shown. MET=Mean eclosion time (days); MIC=Mean individuals per cocoon (n); MIB=Mean initial individual biomass at eclosion (mg); MBC=Mean biomass per cocoon (mg).

| Substrate | Eclosion % | MET (days) | MIC (n) | MIB (mg) | MBC (mg) |
|---|---|---|---|---|---|
| **Horse Manure** | 90 | 21.56 ± 0.40 | 3.44 ± 0.33 | 3.16 ± 0.22 | 10.88 ± 0.99 |
| **Grass 1 Week** | 60 | 32.00 ± 1.59 | 3.83 ± 0.48 | 3.74 ± 0.49 | 14.34 ± 2.87 |
| **Grass 13 Week** | 40 | 27.75 ± 1.32 | 3.75 ± 0.95 | 7.47 ± 1.13 | 28.03 ± 5.49 |

On a second experiment, we determined water retention potential of increasingly aged pasture grass along 14 days in terms of field capacity (water content / wet substrate, by weight) measuring as well inorganic content (% on dry weight basis). Similarly, inorganic matter increased along composting period and concomitantly field capacity decreased (Fig. 2).
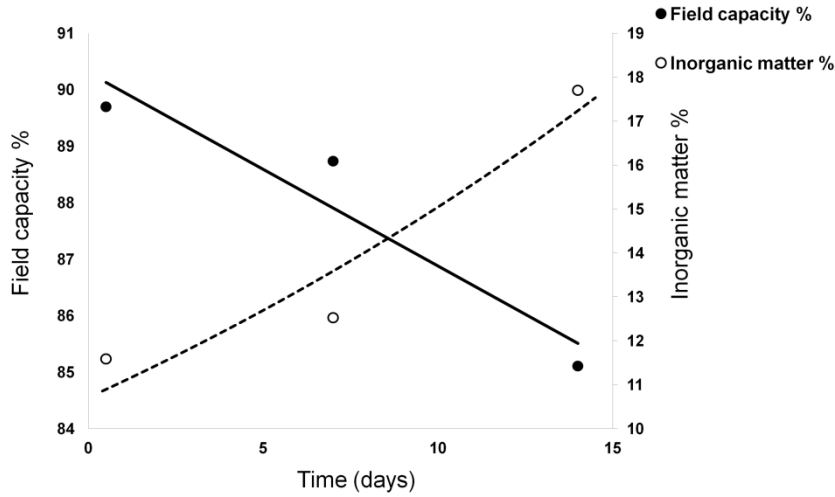


**Fig. 2** Evolution of Field capacity (water content/ wet substrate*100, by weight) and Inorganic matter (% of dry matter) of grass along 15 days aging. Black circles: *FieldCapacity%* = 90.3 - 0.3422*Time*, $R^2$ = 0.911 Open circles: *InorganicMatter%* = 10.707*e$^{(0.034*Time)}$, $R^2$ = 0.8959.

In the course of mineralization, protein content experienced a gradual raise until inorganic matter reached ~22 % declining after that level (Fig. 3 A). Total fibre on its turn, decayed at a rate described by the equation: *TotalFibre %* = 60.114*e$^{(-0.018*InorganicMatter\ \%)}$; $R^2$ = 0.939 (Fig. 3 B).
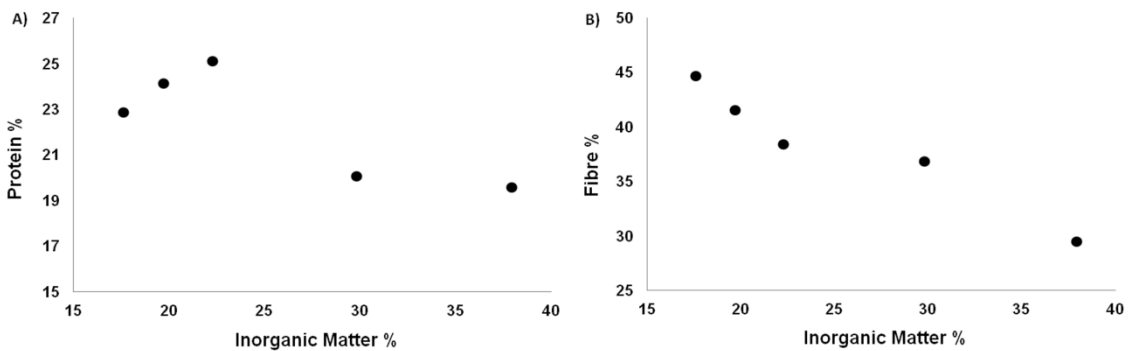


**Fig. 3** Evolution of biochemical components of grass along the mineralization process. A) protein (% of dry matter); B) fibre (% of dry matter).

## 3.2. Eclosion

Results of the eclosion experiment performed in reference medium (horse manure) and initial (1 week) and final (13 weeks) periods of aging are shown in Table 2. Eclosion success decreased from 90 % in horse manure to 60 % and 40 % in aging grass during 1 or 13 weeks respectively. Differences between substrates have been tested by means of Tukey-Kramer test (Table 3). Mean eclosion time was significantly different for every substrate: from lowest for horse manure (21.56 days) to highest for 1 week grass (32 days). No effect of incubating medium on number of individuals per cocoon (3-4) was evident whereas, on the contrary, both mean biomass per cocoon (mg) and mean individual biomass (mg) revealed the dissimilarity between both horse manure and 1 week substrates and most aged grass with highest figures for the latter: MIB =3.31 ± 0.21 mg or 7.47 ± 1.13 mg and MBC = 11.74 ± 1.04 mg or 28.03 ± 5.49 mg.

**Table 3.** Tukey-Karmer analysis for eclosion parameters of cocoons of *Eisenia andrei* incubated in horse manure and grass 1 and 13 weeks aged. MET=Mean eclosion time (days); MIC=Mean individuals per cocoon (n); MIB=Mean individual biomass (mg); MBC=Mean biomass per cocoon (mg).

**TUKEY-KRAMER: MET; MIC; MIB; MBC.**

|  | Horse manure | Grass 1 Week | Grass 13 Week |
|---|---|---|---|
| **MET (days)** | 21.56 | 32 | 27.75 |
| **MIC (n)** |  | 3-4 |  |
| **MIB (mg)** | 3.31 | | 7.47 |
| **MBC (mg)** | 11.74 | | 28.88 |

## 3.3. Survival and growth

Viability of newly hatched worms (n = 65) within an initial size ranging from 0.65 to 11.74 mg live weight was tested in pre-composted grass at different maturing periods of time. Size frequencies distribution of hatchlings used in this experiment (live weight in mg) appear in Fig. 4. Initial weights below 4.9 mg (Mean = 3.19; SD = 1.09) are exhibited by 75 % of the off-springs, being the remaining 25 % uniformly distributed between 5 to 12 mg (Mean = 7.54; SD

= 1.98). Groups of worms (n = 9) selected for each substrate were homogeneous as regards initial live weight: Kruskal-Wallis non parametric test resulted in *p-value* of 0.1435.



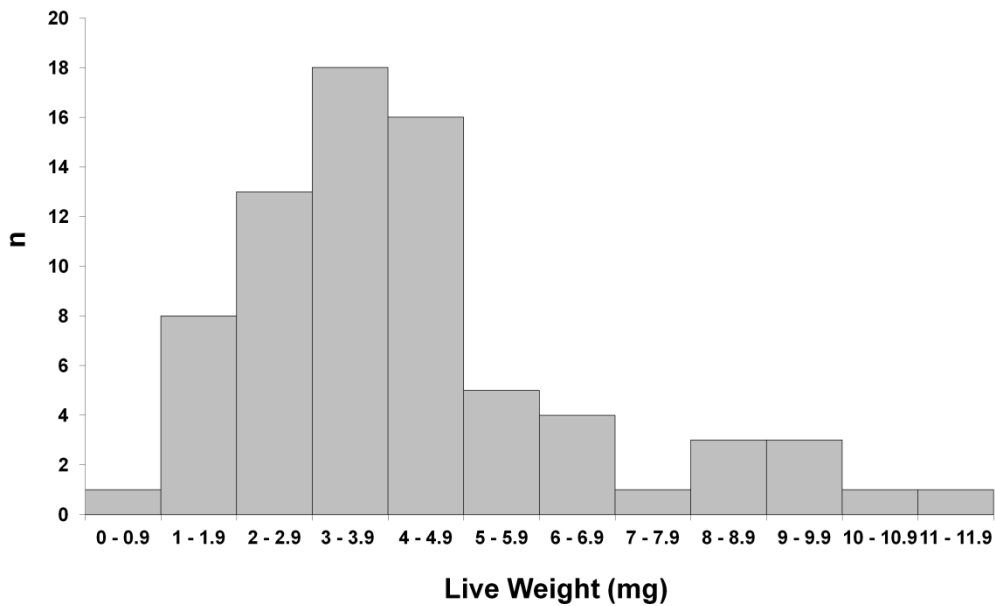**Fig. 4** Histogram for frequency distribution of live weight (mg) for specimens of *Eisenia andrei* used in the study

### 3.3.1. Survival

A relationship between survival (%) of *E. andrei* individuals and organic matter (%) is illustrated in Fig. 5.
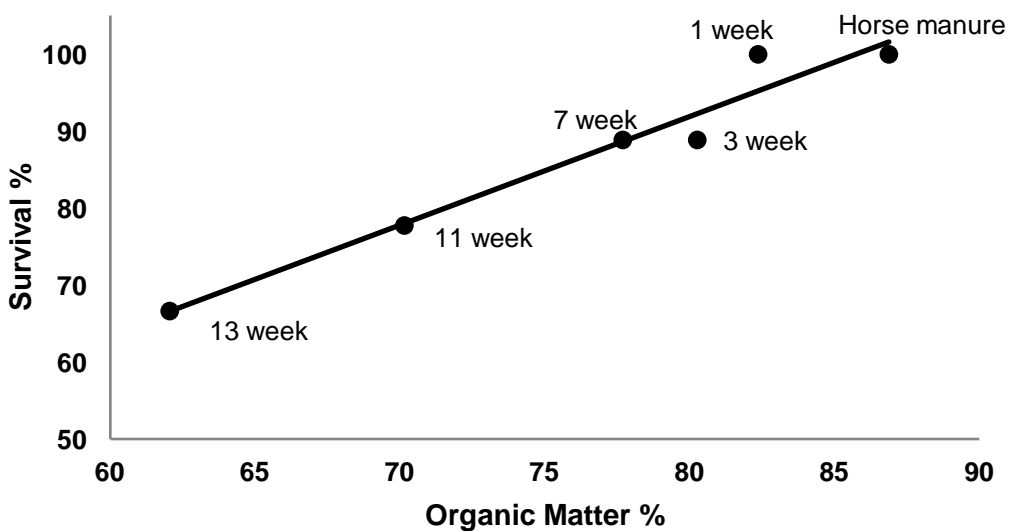


**Fig. 5** Survival (%) of *Eisenia andrei* hatchlings as related to organic matter (% of dry matter). *Survival% = 1.4085\*OrganicMatter% - 20.821*, $R^2 = 0.9563$

Hatchlings grown on substrates where organic matter exceeded 82 % showed no mortality. Nevertheless, the analysis of the overall influence of organic matter on survival can be undertaken in terms of linear regression, explaining over 95 % of variability: *Survival % = 1.4085\*OrganicMatter% - 20.821.*

This relation predicts 100 % mortality in aging grass for organic contents $\leq$ 14.78 %. So, survival (%) decreased linearly with composting time (weeks) according to the equation: *Survival % = 100.9 - 2.35\*Time*, $R^2 = 0.895$.

### 3.3.2. Growth

Growth patterns of newly hatched *E. andrei* in the substrate matrix hitherto described are shown in Fig. 6.



**Fig. 6** Biomass (live weight in mg) evolution along time (days) of *Eisenia andrei* hatchlings in horse manure and aging grass.

Mean initial live weight value for the whole batch was 4.37 mg (SD = 2.40) differences among substrates being non significant. Analyses of individual growth equations of the 38 and 20 specimens for every grass media and horse manure respectively, have allowed to pool data for each substrate in a single equation relating biomass (Live weight = LW, mg) to time (t, days):

Horse manure: $LW = 13.174 + (17.150*e^{(0.071*t)})$, $R^2 = 0.952$.

Grass 1 week: $LW = (148.623*t^{3.238}) / (19.956^{3.238} + t^{3.238})$, $R^2 = 0.985$.

Grass 3 week: $LW = (124.787*t^{3.152}) / (17.327^{3.152} + t^{3.152})$, $R^2 = 0.998$.

Grass 7 week: $LW = 78.813*(1 - e^{(-0.054*t)})$, $R^2 = 0.976$.

Grass 11 week: $LW = 59.206*(1 - e^{(-0.071*t)})$, $R^2 = 0.977$.

Grass 13 week: $LW = 2.3132*t - 0.8748$, $R^2 = 0.968$.

Four different models (exponential for horse manure, sigmoid for 1 and 3 weeks aging, asymptotic for 7 and 11 weeks periods and linear for 13 weeks) have been used to analyze biomass evolution along the initial 30 days of the life cycle ($R^2$ ranging from 0.952 to 0.998). Maximal live weights achieved during the experiment can be distributed into two groups: hatchlings living in horse manure and grass pre-composted for 1 and 3 weeks reached ~120 mg, while in the remaining substrates arrived to ~ 60 mg. At this respect, ANOVA analysis showed statistical differences between the former groups ($p < 0.0001$; $F$-value$_{5,51} = 16.148$).

Influence of organic matter content on growth was analyzed confronting with maximal live weight achieved at the end of the experiment (Fig. 7).
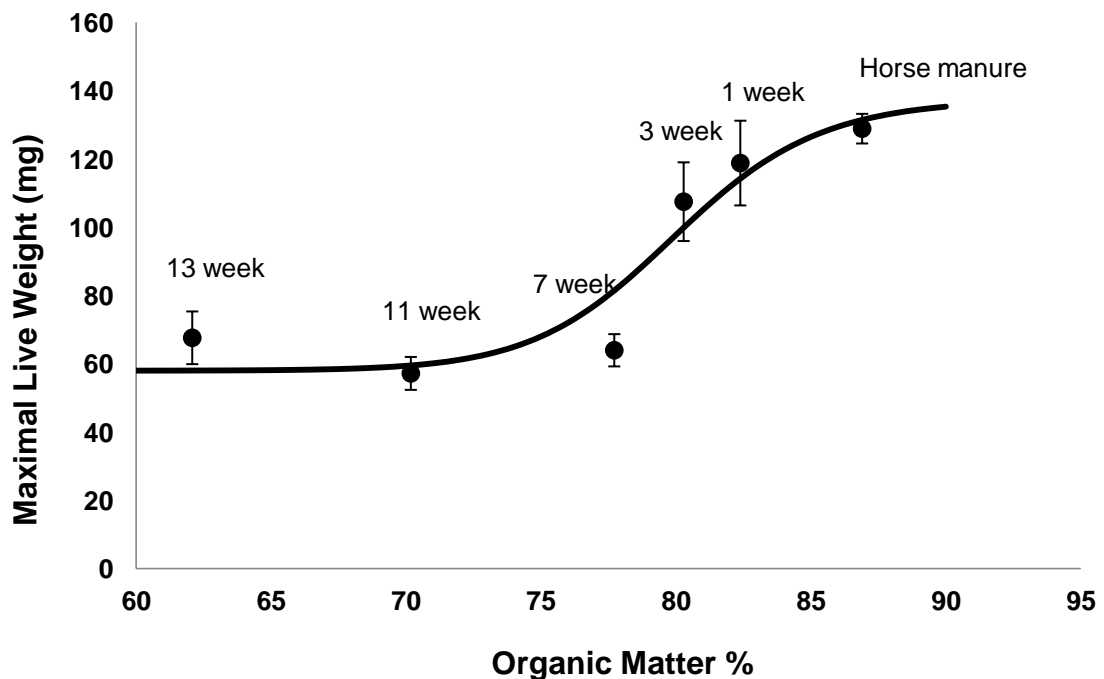


**Fig. 7** Maximal average live weight (mg) of *Eisenia andrei* hatchlings as related to organic matter (% of dry matter). Bars for standard errors are shown
$MaximalLiveWeight = 58.015 + (79.585*OrganicMatter\%^{30}) / (80^{30} + OrganicMatter\%^{30})$, $R^2 = 0.900$.

Two groups were evident: 80 % of organic content represented a boundary between substrates associated to lower growth rates (maximal live weights~60 mg) and those rendering higher rates (maximal live weights~120 mg). Tukey-test resulted in significant differences between horse manure and initial aging periods (one & three weeks) and the remaining aging times (from 7 to 13 weeks). This information is summarized in Table 4. Nonetheless MLW related to aging time of grass: MLW (mg) = $123.59*t^{-0.266}$ ($R^2$ = 0.876).

**Table 4.** Tukey-Kramer analysis for maximal live weight of *Eisenia andrei* individuals hatched from cocoons incubated in horse manure and grass 1, 3, 7, 11 and 13 weeks aging. MLW=Maximal Live Weight (mg).

**TUKEY-KRAMER: MLW**

| Horse manure | Grass 1 Week | Grass 3 Week | Grass 7 Week | Grass 11 Week | Grass 13 Week |
|---|---|---|---|---|---|
| 129.19 | 117.91 | 106.99 | 61.59 | 53.33 | 67.99 |

| Mean | Mean |
|---|---|
| 121.65 | 60.58 |

# 4. Discussion

Grass is a generic term for a variety of plants found in poliphylic pastures consisting basically in Gramineae and Leguminosae. In agro-ecosystems habitats such as those in North Atlantic Iberian Peninsula (78 % of Spanish prairies) traditional management of forages involves leaving cut grass drying in the open air to tolerate compacting and packing. This practice provides a continuously evolving substrate available to soil communities. Our results indicate that no changes in pH associated to aging occur within a period of 1 to 13 weeks (mean pH = 6.45 ± 0.05), in contrast with freshly mowed grass that exhibits an acid pH ~ 4.6 (Calsamiglia *et al.* 2004). Analysis of nutritional value in terms of biochemical composition results in figures ranging from 19.57 % to 25.12 % of dry matter for proteins, 3.47 % to 4.50 % for lipids and from 29.51 to 44.68 % for total fibre, in good agreement with reported data for Iberian Atlantic pastures (Roza *et al.* 1992; Calsamiglia *et al.* 2004). As commonly found in composting literature, we have detected increased mineralization associated to composting time. Our data

reveals an exponential decreasing pattern for organic proportion in soil as it was also the case in Vinceslas-Akpa & Loquet (1997) and Frederickson *et al.* (1997) being this mineralization process mediated by soil microorganism (Tripathi & Bhardwaj 2004).

Water availability is a key factor determining foraging behavior of soil organisms and although earthworms show a considerable ability to survive adverse moisture conditions enduring loss of a large part of total body water, activity is lower under dry conditions (Edwards & Lofty 1977). Our data for *E. andrei* indicates that water constitutes 90 % of the live weight of a fed worm (Ortega-Hidalgo and Iparraguirre-Bolaños, unplb. data) and Domínguez & Edwards (1997) reported increasing growth and reproduction associated to environments characterized by high moisture conditions. In our experiment, water retention capacity is directly related to organic matter content in coincidence with previous results from top soil analysis from a variety of landscapes in the Basque Country including prairies (Orrantia 2006). So, dehydration is associated to composting and could derive in lethal and/or sub-lethal (i.e. feeding restrictions) effects in worms related to water loss by suction pressure of the media (Kretzschmar & Bruchou 1991). In order to focus on the influence of simultaneous changes in quantity and quality of food derived from aging, excluding concomitant influence of water content, we have maintained every substrate near water saturation (80 % RH).

## 4.1. Eclosion experiment

Sensibility of cocoons to both environmental conditions and elements present in soils, has led to propose this type of experiments as a tool to ascertain the suitability of substrates, suggesting earthworm reproductive fitness as a soil quality indicator (Robidoux *et al.* 2001; Nortcliff 2002; Nunes *et al.* 2016).

In our work, extreme stages of grass evolution (initial and final) were chosen as substrates, with horse manure as a reference, with the aim of achieving maximal differences as regards the effect of grass maturation on eclosion parameters. Earthworm cocoons are extremely permeable to water and metals (Holmstrup & Westh 1994; Holmstrup *et al.* 1998) and different authors mention their ability to acquire bacteria from the environment: Zachman & Molina (1993) and

Daane & Häggblom (1999) reported uptake of specific bacteria added to the soil by egg capsules in *Eisenia fetida* (Savigni, 1826). So, they are presumably sensitive to changes in the environmental, biological and physicochemical conditions. At this respect, total eclosion failure of cocoons incubated in freshly cut grass can be attributed to acid pH (pH ~4.6) since *Eisenia* sp. specimens are most commonly found in environments with neutral pH (7 to 8) (Edwards & Lofty 1977) not tolerating pH values below 5 (Edwards & Arancon 2004). In the remaining substrates analysed, eclosion success (%) changed from 100 % in reference substrate (horse manure) to 60 % and 40 % in grass composted for 1 and 13 weeks respectively. These figures are in concordance with earthworm substrate preferences reported by Edwards & Lofty (1977) where dung presence determines the distribution of epigeic earthworms in prairies with reduced livestock uses. Maturation time of cocoons reveals that in substrates with lower eclosion success, newly hatched earthworms spent longer periods in the cocoon, probably exhausting nutritional resources before hatching. So, while organic degradation takes place, appearance of metabolites may have restrictive effects on developing hatchlings.

Although there was no clear pattern of the effect of pre-composting on the number of hatchlings produced, in accordance with results obtained by Gunadi *et al.* (2002) individual live weight (mg) of newly hatched specimens and total earthworm biomass (mg) per cocoon were higher (3.31 mg *vs.* 7.47 mg for MIB and 11.74 mg *vs.* 28.88 for MBC) in the older substrate (13 week grass) indicating covariation with grass mineralization. At this respect, reproduction strategies predict larger offspring in adverse environments to survive post-hatching restrictive conditions (Sibly & Calow 1986).

## 4.2. Survival and growth experiment

Size distribution in our sample constitutes a representative selection of the range of sizes in an eclosion batch. Most offspring (75 %) ranged from 0.5 to 4.9 mg on live weight basis, clustered at the lowest end of frequency distribution histogram, whereas the remaining 25 % were homogeneously located in sizes categories between 5 and 12 mg. In any case, selected hatchlings attributed to the different experimental substrates showed no significant differences

in mean initial biomass. Results of the experiment for the control group (horse manure) displayed no mortality and maximal live weights achived were similar to those reported by Neuhauser *et al.* (1980) for newly hatched specimens of *E. fetida* during the first month of experiment.

## 4.2.1. Influence of food quantity: organic matter availability

*E. andrei* is an epigeic species, dwelling at the upper part of the soil (topsoil) feeding preferably in surface litter (rich in organic matter) and in our experiments we found a linear decay (a function explaining 95 % of variability) of earthworm viability related to soil organic loss. In fact, organic matter richness is known to correlate directly to abundance of earthworms (Edwards & Lofty 1977; Edwards 2004; van Vliet *et al.* 2007). No mortality was found at the upper limit where organic matter content was over 82 %. These results match data from Gunadi *et al.* (2002) who reported total survival for earthworms cultivated in cattle solids pre-composted for 1 week.

Differences in biomass evolution along time relate as well to organic matter content of soil and two groups become significantly different regarding MLW attained (120 mg vs. 60 mg). Frederickson *et al.* (1997) in a study of vermiculture with traditional green wastes reported decreasing individual biomass of *E. andrei* worms as organic matter content decreased. Similarly, Neuhauser *et al.* (1988) found a negative relation between age of media and biomass reached by earthworms, indicating that maturation of the material reduces food availability. Likewise, storage of cattle foodstuffs redound in impoverishment of organic matter reducing digestibility and growth expectancies of livestock (Roza *et al.* 1992).

Although it was not the objective of this work, products resulting from decomposition of vegetal tissues are known to have a potential toxic effect on soil macrofauna. Being after carbohydrates a major component of plant cells (Pridham 1965), phenolic compounds have received particular attention: associated to glycosil residues in living plant tissues, potential toxicity would appear after hydrolysis following plant necrosis (see Min *et al.* 2015 for a review). In fact, and although adult earthworms have relative tolerance to phenolic compounds (Krishna *et al.* 2011), Hrushikesh *et al.* (2012) found pronounced effects on a variety of soil worms including *E. fetida*

and Widarto *et al.* (2004) described a negative effect on growth rate of hatclings of *Dendrobaena* (initial weight ≥ 5 mg) during the earliest stages (100 days) of life cycle. Hence, we cannot discard toxicity as a side effect covariating with food quantity and quality. At this respect, Calow (1991) suggested that presence of stressors determine allocation of energy obtained from food to detoxification processes of deleterious elements, at the expense of somatic growth.

## 4.2.2. Influence of food quality: organic matter composition

Transforming potential chemical energy contained in foodstuffs in net (available) energy to sustain growth, repair and reproduction depends both on the structure of a given nutrient and digestive capacity of the organism. Additionally, metabolic investments in feeding, digestion and absorption depend on the chemical nature of food, determining ultimately the foraging behaviour of an animal. In this context, food quality relates directly to absorption efficiency and regarding nutritive value of pastures, the extensive literature existing for grazing mammals reports decrease in digestibility and/or metabolizable energy associated to increased proportions of non-soluble cell wall constituents (Van Soest 1967), with considerable variation within and among species.

In our experiment, comparison of general biochemical profile of aging grass with horse manure reveals differences only in the case of soluble carbohydrates with higher figures (9.92 % vs. 25.11 %) for fecal matter. Metabolic investments in horse digestion, especially mucus, could account for the enrichment in free sugars. From our data, time-course evolution of aging grass indicates that both lipids and soluble carbohydrates remained constant (4.01 % and 9.92 % respectively) while total organic matter decreased noticeably (~20 %). Such decrease would only be slightly explained by protein dynamics which increased initially (7 weeks) reversing the trend later. Thus, main organic losses are attributable to total fibre (15.7 %, representing 75 % of OM decline) which would account for cell wall residues composed by lignin, cellulose and hemicelluloses, mentioned in diminishing order of hydration and, consequently, of digestibility for most fauna. Regarding pastures, data for Atlantic prairies (Calsamiglia *et al.* 2004) and Virginian common pasture weeds (Abaye *et al.* 2009) evidence that readily digested

hemicelluloses constitutes 30 % of the total fibre content, being most refractory lignin the minor constituent (~10 %).

Focusing on the fate of fiber in the context of soil microbiota activity, Vinceslas-Akpa & Loquet (1997) referred no degradation of lignin along the first month of composting of pruning wastes, whereas ~ 30 % of total content was lost in the following two months. These authors found as well an exponential reduction in cellulosic materials, representing ~ 40 % of initial values after 3 mo composting. In accordance with our data for composting grass along a similar period of time (13 weeks), their added losses (lignin + celluloses) would account for major organic decrease (2 thirds of total OM ). In fact, Sinsabaugh & Linkins (1993) modeling litter decomposition, reported a functional linear relationship between cumulative mass loss and cumulative cellulase activity. Fiber processing by edafic macrofauna, particularly epigeic earthworms, implies further degradation (Vinceslas-Akpa & Loquet 1997) pointing out their role in humification of plant litter. At this respect, Zhang *et al.* (2000) reported high cellulolytic activity in the gut of *E. fetida* while data for the same species in a composting reactor indicated that both cellulose and hemicelullose are degraded either by intrinsic activity or by interactions with microorganisms (Elvira *et al.* 1996b; Aira *et al.* 2006). Cellulose in nature appears heteromorphic including both crystalline and amorphous regions. The latter presents pores and capillaries partially hydrated, being readily available to cellulolytic enzymes: depletion of this labile pool along earlier stages of composting increases the proportion of crystalline cellulose reducing decomposition rate achieved by both earthworms and microorganisms (Lynd *et al.* 2002).

Protein content in freshly cut Atlantic grass in Northern Spain is ~ 15.5 % (Calsamiglia *et al.* 2004) while data for aging grass included in this study stands well over such figures (20-25 %). Although no attempt was made to identify microbial communities appearing along composting, the ample set of indirect methods to estimate microbial biomass (see Harris & Steer 2003 for a critical review) includes nitrogen transformations and respiration as predictors of microbial activity. In fact, we have found an exponential relationship between soil respiration and total protein percentage (unpubl. data) and Zhang *et al.* (2013) referred that, among several markers

of microbial presence, protein level of soil behaved as the most sensitive parameter in a gradient of contamination stress. We have recorded an increase in total protein percentages during the first 7 weeks of composting followed by decay along the latest periods of mineralization. If, as assumed, protein level can be considered a reliable estimator of microbial biomass, evolution of fiber pool previously discussed could explain, at least partially, these dynamics: easily digested fraction of fiber, which allows initial rapid microbial growth, would tend to disappear along the later stages, redounding in reduced capacity of the substrate to sustain biomass. At this respect Aira *et al.* (2006; 2007), in experiments with *E. fetida* in composting and vermicomposting layers, reported a stable microbial biomass during the first 4 weeks decreasing later. Similarly, Bansal & Kapoor (2000) found a decrement in microbial activity after 1 month of vermicomposting.

Grass litter decomposition involves thus the co-evolution of two different pools: remnants of vegetal origin and microbiota. Considering the latter, diet of earthworms is known to include soil bacteria and fungi (Edwards & Fletcher 1988; Edwards 2004; Curry & Schmidt 2007) and there is some evidence of digestive abilities to process microorganisms (Schönholzer *et al.* 1999; Brown *et al.* 2000). Additionally, mutualistic relations between worms and soil microbiota (Trigo *et al.* 1999; Brown & Doube 2004) emphasize its role in enhancing digestive abilities of annelids whereas gut mucus production would provide a suitable environment for microbial proliferation (Brown *et al.* 2000) buffering losses associated to fecal voidance.

Within this frame, an approach to understanding biochemical food quality of aging grass for *E. andrei* can be undertaken analyzing the holistic response of hatchlings in terms of survival and growth to the evolving composition of substrates hitherto exposed. As already discussed when dealing with organic matter richness, mortality and reduced growth (~ -50 %) appear at organic matter percentages between 62.02 % and 77.7 % which cannot be considered limiting on terms of purely food availability. In consequence, deleterious effects of substrate or/and reduced digestibility of foodstuff should be invoked. The last assert could well be explained by gradual foraging of carbohydrate pool of vegetal origin by microorganisms, increasing the fraction of sugars available to hatchlings while providing replacement of gut microbiota involved in

digestion. As a consequence, along initial stages, both food quality and digestive efficiency would be enhanced, allowing survival and growth rates comparable to those obtained in horse manure, characterized by high levels of soluble carbohydrates. Later ($\geq$ 7 weeks), depletion of labile components of fibre would reduce both the population of microorganisms and biochemical quality of nutrients accessible to young earthworms (i.e higher digestive costs) redounding in lower life expectancies and growth.

### 4.2.3. Final Remarks

Along earliest stages of life cycle in *E. andrei*, higher Surface/Volume ratio would derive simultaneously in increased mucus production and metabolic costs per weight as compared to adults and, presumably, limited digestive capacity. Under these circumstances, any stress factor would exert a stronger effect in young earthworms compromising a positive energy balance compatible with both survival and growth. If, as proposed by Sibly & Calow (1989) a trade-off between energy allocation to defense mechanisms and growth can be assumed in order to guarantee survival at increased environmental stress, defense costs would limit growth investments. This seems to be the case in our experiment (Fig. 8) for aging periods over ~ 6-7 weeks, where decay in food quality would be requiring higher metabolic investments in nutritional activities (increased foraging periods, lower transit times and net absorption efficiencies, etc.) uncoupled though, presumably, with maintenance of net absorption rates. If, as inferred from the outcome of the eclosion experiment, appearance of toxic metabolites associated to humification processes cannot be discarded, deleterious effects in terms of mortality and growth would be offset only when high quality foodstuffs allow surplus net energy in terms of energy budget. Our results agree with the strong negative correlation between the duration of pre-composting and growth and reproduction of *E. andrei* specimens during vermicomposting described by Frederickson *et al.* (1997), indicating that extensive maturation of the grass interferes with the viability of epigeic earthworms.
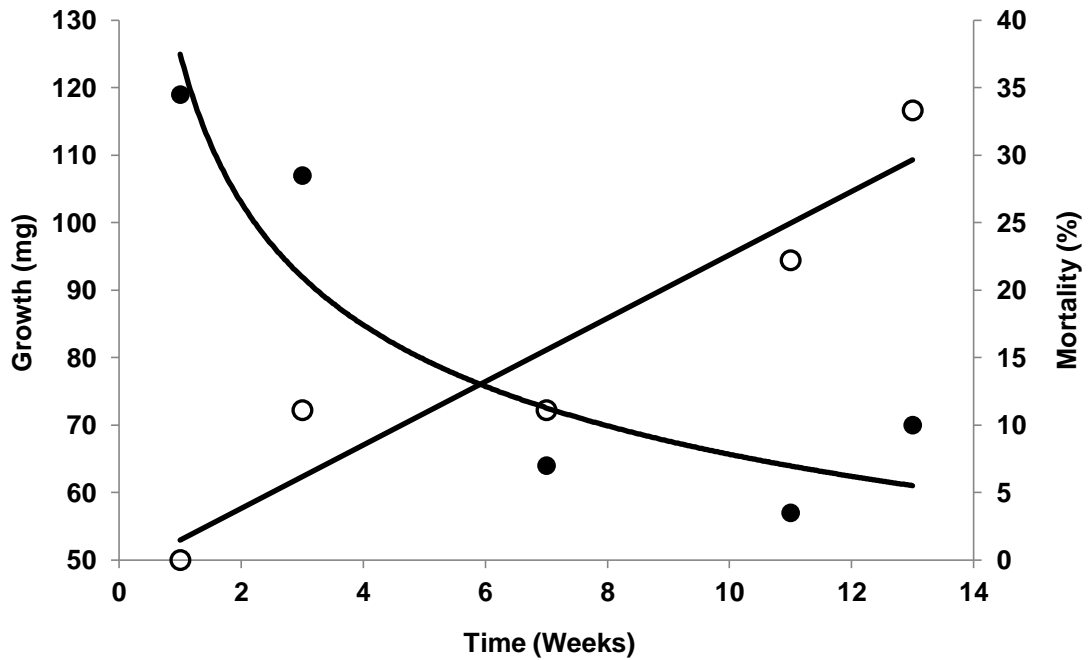
**Fig. 8** Growth (Maximal live weight in mg) and Mortality (%) of *Eisenia andrei* hatchlings as related to grass aging (weeks). Black circles: *Growth* = 123.59*Time$^{-0.266}$, $R^2$= 0.876. Open circles: *Mortality* = 2.350*Time - 0.897, $R^2$= 0.895.

# 5. Conclusions

In the context of management of semi-natural agro-ecosystem habitats, maintaining adequate densities of detritivorous communities guarantees recycling of plant material preventing soil degradation and contributing to generation of new soil. In this work, we have focused on the initial, more labile phases of life cycle of *E. andrei*, and eclosion, survival and growth of hatchlings, as representative of physiological performance, have been analyzed in a matrix of covariating food quantity and quality using Atlantic freshly cut aging grass. From our results, standing crop of *E. andrei* in grass litter appears determined by the proportion of readily available carbohydrates extracted from plant cells by the assemblage between earthworms and soil microbiota within a mutualistic relationship. Impoverishment of quality associated to aging appears linked to the decline of hydrated, readily extractable sugars within the fibre pool redounding in decreasing survival and growth.

# Chapter 6

**6. Eclosion, survival and individual growth of**

***Eisenia andrei* in soil from three different**

**vegetation units**

# Eclosion, survival and individual growth of *Eisenia andrei* in soil from three different vegetation units

## Abstract

Edaphic organisms are considered keystone species in the maintenance of soil health and, among them, earthworms cause special interest for their role in the conservation of edaphic properties, helping to maintain soil structure, and for their positive interactions with soil microbiota and plants. For this reason, in this work our aim has been to analyse the suitability of soils from different vegetation units to support active populations of the earthworm *Eisenia andrei* using soil from *Eucalyptus globulus* plantation (EP), forest dominated by *Quercus robur* (FQR) and Scrub dominated by *Crataegus monogyna* (SC) focusing on the nutritional value of their litter layer for epigeic earthworms. Two control substrates were employed: Horse manure (HM) and Coconut coir (CC). Eclosion success, survival and growth of newly hatched and juvenile *Eisenia andrei* specimens were analysed. Initial characteristics of substrates displayed the highest organic matter percentage in CC, followed by HM and the lowest organic content was obtained in SC; however, CC displayed the lowest figures for soil respiration (virtually zero). A negative relation was established between substrate respiration ($\mu LO_2\ h^{-1}\ g^{-1}$) and fibre (% of organic matter), with the exception of HM. Regarding eclosion success, a significant influence of the substrate was found in the initial biomass and the total biomass per cocoon, observing the highest figures in CC and SC, which is explained by the increment in the rendering time in the cocoon associated with an unfavourable environment. Survival percentage differed highly between substrates being 100 %, 90 %, 80 %, 67 % and 60 % in HM, EP, FQR, SC and CC respectively. Growth patterns also differed largely among substrates and we obtained average maximal live weights (MLW) of 247, 117, 56, 19 and 10 mg in HM, EP, FQR, SC and CC respectively for hatchlings and 1473, 881, 479, 282 and 100 in HM, EP, FQR, SC and CC respectively for juveniles. Survival of newly hatched specimens and growth of hatchlings and juvenile individuals were positively related to substrate organic matter percentage (except CC) and to substrate respiration ($\mu LO_2\ h^{-1}\ g^{-1}$). Overall, a decrease of the non-fibre organic content (% of dry matter) of substrates and an increase in the proportion of their fiber content (% of organic matter), would account for the lower digestibility of the organic matter for both soil microorganisms and earthworms, redounding in lower live expectancy and growth for epigeic earthworms.

# 1. Introduction

Soil is one of the most important natural resources in the world. Complex, with a slow formation rate, and difficult to replace, it is a highly variable element dependent of geo-climatic characteristics and the vegetation and fauna it contains. Currently soil degradation, together with water shortage and littoral ecosystem disappearance, is one of the critical problems in the context of the Iberian Peninsula (Antipolis 2003; De Luis *et al.* 2010).

During the last part of XX century, soil quality concept has gained importance in both the scientific and socioeconomic fields, and has been defined as the capacity of a specific kind of soil to function within natural or managed ecosystem boundaries, to sustain biological productivity, maintain environmental quality, and promote plant and animal health (Herrick 2000). In this context, edaphic organisms are considered keystone species in the maintenance of soil health, and, among them, earthworms cause special interest for their role in the conservation of edaphic properties, thus helping to maintain soil structure, and for their positive interactions with soil microbiota and plants (Brown *et al.* 2000; Scheu *et al.* 2002). The ecological category of epigeic earthworms in particular, which dwell in the upper part of soil feeding on litter, are responsible, together with microorganisms, for the organic matter degradation, contributing to the formation of new soil layers and soil fertilization by providing soil with cast containing nutrients easily assimilable by plants (Edwards & Lofty 1977). Under these circumstances, earthworms are considered soil ecosystems engineers (Jouquet *et al.* 2006) and maintenance and enhancement of both earthworms and soil microorganisms is of vital importance in the context of sustainable agriculture, forestry management and soil conservation. Besides, soil organisms, due to their sensibility to changes in environmental conditions and their relation with ecological processes in soil (Doran & Zeiss, 2000), have become a tool in soil ecosystem management (Baguer *et al.* 2000; Nortcliff 2002). The variety of works analysing the effects of contaminants, soil acidification and land uses on epigeic earthworms (Chan 2001; Ma 2005; Eriksen-Hamel *et al.* 2009; Ernst & Emmerling 2009) emphasises their capacity to evaluate soil health and highlight their use as bioindicators (Paoletti 1999; Muys & Granval 1997).

The Iberian Peninsula is characterized by the presence of a variety of landscapes (forest, meadows, scrubs etc.) submitted to different management practices (e.g. forest plantation), and in a context in which soil degradation is a global problem, especially in managed ecosystems where the disappearance of soil fertility results in multimillion losses, an evaluation of the nutritional resources exploited by these ecosystems engineers is of great interest, in order to gain insight on success in long-term recruitment and colonization capacities of epigeic earthworms. Although food quantity in terms of organic matter covariate with abundance of Oligochaeta, food quality associated to the presence of labile organic carbon (glucose) correlates positively with high growth rates in detritivorous lumbricids (Tiunov & Scheu 2004), suggesting that nitrogen availability may not be the key limiting element of soil animals. For these reasons, our aim has been to analyse the suitability of soils from different vegetation types to support active populations of the earthworm *Eisenia andrei* using soil from *Eucalyptus globulus* plantations, forest dominated by *Quercus robur* and Scrub dominated by *Crataegus monogyna,* focusing on the nutritional value of their litter layer for epigeic earthworms.

# 2. Material and methods

Three vegetation types were tested on their suitability to support active populations of *Eisenia andrei* specimens: *Eucalyptus globulus* plantation (EP), Forest dominated by *Quercus robur* (FQR) and Scrub dominated by *Crataegus monogyna* (SC). Two control substrates were employed: horse manure (HM) and coconut coir (CC), that despite presenting similar organic matter quantities (86 % and 93% in HM and CC respectively), differed in the structural components and in the actual nutrient availability. In sum, a matrix of 5 substrates was built, in which HM was understood as the positive control and CC as the negative.

## 2.1. Sampling area and substrate selection

Substrate sampling area was the Alonsotegi municipality (43°24´ N, -2°99´ E), situated in the East Atlantic Iberian Peninsula, within the Cantabroatlantic sector of the Atlantic European biogeographic province. Substrates were collected in October 2013. After a study of soil characteristics of the different vegetation units present in the area (Orrantia 2016), the highest

differences regarding organic matter were found between *Eucalyptus* plantation (*Eucalyptus globulus*) and Scrub (dominated by *Crataegus monogyna*), so the selection of substrates was made following this criterion. Forest dominated by *Quercus robur* was chosen for being the potential forest in our sampling area (Loidi *et al.* 2011). 1 m$^2$ squares were placed in each vegetation unit, and samples were taken from the upper 3 cm of the soil (topsoil), due to the epigeic character of the species, thus sampling the litter layer and the soil.

Coconut coir (CC) was an industrial plant support supplied by Ikea group, and horse manure (HM) was collected from horses maintained in an Atlantic meadow under semi-extensive conditions (43°35´ N, -2°86´ E).

## 2.2. Experimental design

Two experiments took place. In the first part we tested eclosion, survival and growth of *Eisenia andrei* newborn specimens reared in the above described substrates; in order to ascertain the effect of the culture media on the whole vital cycle, a second trial, using juvenile animals, was undertaken.

### 2.2.1. Trial 1: Newly hatched specimens

### 2.2.1.1. Parental selection

Mature clitellated individuals of homogenous size (live weight ~600 mg) were extracted from an ongoing culture of *E. andrei* reared under constant conditions (darkness; $20 \pm 2$ ºC; $80 \pm 3$ % RH) in horse manure (86 % organic matter; pH = $7.25 \pm 0.04$). Collection took place by hand shorting (Edwards & Bohlen 1996) and selected adults (n = 100) were placed in a box (10.4 dm$^3$) containing 7 dm$^3$ of horse manure where conditions were kept similar to the origin culture. In order to prevent the initiation of incubating period in the shedding tank, cocoon gathering involved intensive inspection guarantying extraction closest possible to shedding (less than 24 h).

### 2.2.1.2. Eclosion experiment

A total of 100 cocoons, 20 per substrate, were collected within 24 hours after shedding and individually incubated until eclosion in 25 cm$^3$ containers (identical controlled conditions of

parental) filled with substrates described above. Eclosion dynamics were daily monitored and measurements of eclosion success (%), incubation time (days), number of hatchlings and individual and total biomass per cocoon were recorded.

### 2.2.1.3. Survival and growth experiment

A number of 100 cocoons, generated by parental, were individually incubated in 25 cm$^3$ boxes filled with reference substrate (horse manure), and immediately after eclosion, one hatchling per cocoon was randomly selected and individually placed in identical containers filled with substrates described above (20 per substrate). Analogous to the incubation set up, a total of 100 newborn individuals were used, comprising a size range of 0.9 to 9.22 mg. Survival and growth of hatchlings generated by cocoons were monitored along five weeks permanence in incubation substrates described above.

Both cocoons and newly-hatched worms were kept under identical controlled conditions of parental regarding temperature, humidity and light regime.

### 2.2.2. Trial 2: Juvenile specimens

Individual growth of juvenile specimens extracted from an ongoing culture of *E. andrei* reared in horse manure (see above for details) was monitored for a three-months permanence in substrates described above (see: sampling area and substrate selection). For this purpose, 10 juvenile per substrate, 18 in the case of horse manure, were individually placed in 500cm$^3$ containers and were maintained in darkness, under controlled conditions of temperature ($20 \pm 2$ ºC) and humidity ($80 \pm 3$ % RH) (identical conditions of origin culture).

## 2.3. Biomass determination and growth modelling

Individual biomass was determined by the photogrammetric, non-destructive, method (see chapter 2) developed for tubular organisms (Bernardini *et al.* 2000; Brea 2009) where area of pictures taken every 3 days, is transformed in live weight using a scale of live weight (mg) to body surface (mm$^2$) obtained from simultaneous recordings of both parameters.

Standard equations commonly seen in growth curve modelling were used and are summarized in Table 1.

**Table 1**. Equations for the five growth curves used in the experiment.

| Model | Expression | References |
|---|---|---|
| Linear | $Y = a + bX$ | Walford *et al.* 1946 |
| Exponential | $Y = ae^{bX}$ | Kaufman 1981 |
| Power | $Y = aX^b$ | Kaufman 1981 |
| Asymptotic | $Y = (a-c)(1 - e^{(-bX)}) + c$ | Kaufman 1981 |
| Logistic (Sigmoid) | $Y = a - (1/(c/a + (1/(a - d) - c/a)e^{bX}))$ | Wan *et al.* 2000 |

## 2.4. Biochemical analysis of the substrates

Dry weight was measured after 24 h oven exposure at 90 $^{o}$C and organic matter by ashing previously dried samples at 450 $^{o}$C for 24 hours. Colorimetric methods were used to obtain total protein content (Lowry *et al.* 1951) and total soluble carbohydrates (Dubois *et al.* 1956). Total lipids were isolated according to Bligh & Dyer (1956) and crude fiber extracted with an acetic-nitric acid mixture described by Sloneker (1971), both compounds being quantified by weight determination ($10^{-6}$ balance). Potentiometrical analysis of pH was performed in samples suspended in bidistilled water (1:2, vol:vol) with a soil ph-meter (Hanna Instruments, S.L. ; Model: HI 99121).

Substrate oxygen consumption ($VO_2$), understood as an indirect measurement of aerobic microbial activity, was measured using the Warburg manometric method (Chapter 4), based in the ideal gas law, in which pressure change in the respiratory chamber relative to the atmosphere is measured under constant temperature and volume. Warburg apparatus (Model: V 166) was supplied by B. Braun Melsungen AG company.

## 2.5. Statistical analysis

Basic statistics (Frequency distribution, one-way ANOVA procedures, and non-parametric Kruskall-Wallis test) were employed to describe and compare samples of animals and substrates (STATVIEW 5.0.).

ANCOVA tests were employed to determine the effect of the substrate on MLW excluding the effect of the initial live weight (STATVIEW 5.0).

Growth rates (mg mg$^{-1}$ day$^{-1}$) were related to initial live weight (mg) by means of the equation $Y = a*X^{b}$. Linear regression procedures (after logarithmic$_{10}$ transformation of data), followed by ANCOVA test, have been used to ascertain the significance of differences between both regression coefficients and intercepts.

Linear and non-linear regression procedures were performed with nlme R package (Pinheiro *et al.* 2015), and SYSTAT version 13, from Systat Software, Inc., San Jose California USA, www.sigmaplot.com.

# 3. Results

## 3.1. Chemical analyses of substrates

Initial characteristics of biochemical composition of the substrates are summarized in Table 2. pH remained similar in every substrate, ranging from 6.54 in EP to 7.25 in HM, although statistical differences were found between HM and the rest of substrates. One-way ANOVA displayed differences (*p-value* < 0.001) regarding ash content, obtaining the lowest values in CC (6.78 %) followed by HM (13.11 %) being mineralization higher in soils (70.18 %, 78.13 % and 79.42% in EP, FQR and SC respectively). No differences were found in SCH percentage when comparing FQR to SC (*p-value* = 0.1303), meanwhile *p-value* from one-way ANOVA was significant among the remaining substrates. HM and CC showed the highest SCH%, whereas in the rest of substrates it ranged from 6.45 to 3.79. Protein content also differed in every substrate (*p-value* < 0.001) and such was the case for the lipid content (*p-value* < 0.001) except between SC and CC (*p-value* = 0.2154). Both biochemical components (proteins and lipids) decreased with mineralization in all substrates, with the exception of CC. Maximal fibre percentage corresponded to CC (71.41 % of dry weight) and HM (36.36 % of dry weight), and similar fibre percentages (around 10 % of dry weight) were found in EP, FQR and SC, although statistical differences were found among all substrates.

**Table 2.** Initial chemical composition of substrates used in the experiment. Means with standard error are shown.

| Substrate | pH | Ash % | Lipid % | Protein % | SCH % | Fibre % | VO$_2$ |
|---|---|---|---|---|---|---|---|
| Horse Manure | $7.25 \pm 0.02$ | $13.11 \pm 0.68$ | $4.32 \pm 0.14$ | $21.09 \pm 0.30$ | $25.11 \pm 0.06$ | $36.36 \pm 0.07$ | $279.18 \pm 21.71$ |
| Eucalyptus Plantation | $6.54 \pm 0.07$ | $70.87 \pm 0.72$ | $2.84 \pm 0.04$ | $8.49 \pm 0.04$ | $6.45 \pm 0.06$ | $11.34 \pm 0.09$ | $32.43 \pm 4.56$ |
| Forest *Quercus robur* | $6.61 \pm 0.03$ | $78.13 \pm 0.56$ | $1.23 \pm 0.11$ | $7.03 \pm 0.23$ | $3.79 \pm 0.04$ | $9.82 \pm 0.23$ | $21.31 \pm 2.28$ |
| Scrub | $6.68 \pm 0.03$ | $79.42 \pm 0.42$ | $0.82 \pm 0.05$ | $5.05 \pm 0.04$ | $4.63 \pm 0.03$ | $10.08 \pm 0.16$ | $9.66 \pm 0.39$ |
| Coconut Coir | $6.58 \pm 0.23$ | $6.78 \pm 0.37$ | $0.60 \pm 0.12$ | $6.01 \pm 0.28$ | $15.20 \pm 0.47$ | $71.41 \pm 0.46$ | $0.02 \pm 0.01$ |

**SCH %= Soluble Carbohydrates %; VO$_2$=Substrate Oxygen Consumption Rate ($\mu$L O$_2$ h$^{-1}$ g$^{-1}$).**

**Table 3**. Eclosion parameters of cocoons of *Eisenia andrei* reared in the different substrates used in the experiment. Means with standard errors are present.

| Substrate | Eclosion % | MIC (n) | MBC (mg) | MIB (mg) | MET (days) |
|---|---|---|---|---|---|
| Horse Manure | 90 | $3.44 \pm 0.33$ | $10.88 \pm 0.99$ | $3.16 \pm 0.22$ | $21.56 \pm 0.40$ |
| Eucalyptus Plantation | 90 | $3.00 \pm 0.27$ | $7.84 \pm 0.76$ | $2.61 \pm 0.23$ | $22.72 \pm 0.40$ |
| *Quercus robur* Forest | 85 | $2.82 \pm 0.29$ | $6.66 \pm 0.97$ | $2.36 \pm 0.21$ | $21.59 \pm 0.53$ |
| Scrub | 95 | $2.84 \pm 0.29$ | $9.38 \pm 0.89$ | $3.30 \pm 0.24$ | $23.00 \pm 0.74$ |
| Coconut Coir | 95 | $3.10 \pm 0.31$ | $12.67 \pm 0.79$ | $4.08 \pm 0.30$ | $23.11 \pm 0.94$ |

**MIC=Mean individuals per cocoon; MBC=Mean biomass per cocoon; MIB=Mean initial individual biomass; MET=Mean eclosion time.**

Statistical differences were found among all substrates (one-way ANOVA after log transformation: p-value < 0.0001) for the oxygen consumption rate ($VO_2$ in $\mu$L $O_2$ h$^{-1}$ g DW$^{-1}$), finding the highest figures in horse manure, and no $VO_2$ was obtained in CC. $VO_2$ for the soil ranged from 32.35 (EP) to 9.66 (SC) $\mu$L $O_2$ h$^{-1}$ g $^{-1}$.

A positive relation was found, in all but CC, when confronting $VO_2$ ($\mu$L $O_2$ h$^{-1}$) to both organic content (OM in g) and the non-fibre pool of organic matter (NFO in g):

$VO_2 = 821.47 OM - 314.14$; $R^2 = 0.949$

$VO_2 = 1217.1 NFO - 250.72$; $R^2 = 0.92$

This indicates that metabolic activity on substrates ($VO_2$ $\mu$L $O_2$ h$^{-1}$) depends on the nutrient availability. In fact, no relation was found between fibre content (F in g) and $VO_2$, although a slightly decreasing pattern was observed while removing HM from the equation ($VO_2 = 166.71 - 689.72F$; $R^2 = 0.472$). Hence, the number of microorganisms probably decreased with the decay of the proportion of easily metabolizable components of OM. In line with this, a relation was established between respiratory rate ($VO_2$ $\mu$L$O_2$ h$^{-1}$ g $^{-1}$) and fibre content (FC as % of organic matter) of substrates, from which HM was excluded ($VO_2 = 264916*e^{-0.217FC}$, $R^2 = 0.97$).

## 3.2. Eclosion

The results of the eclosion experiment performed in the different substrates are summarized in Table 3. Eclosion success (%) ranges from 85 to 95 %, obtaining the highest values in SC and CC. Kruskal-Wallis non-parametric test displayed no effect of the incubation medium on number of individuals per cocoon, which varied from 3.5 in HM to 2.82 in FQR . Analysis of variance (one-way ANOVA) of the initial live weight of newly hatched specimens and of the total biomass per cocoon displayed differences between substrates (*p-value* < 0.001), and these differences have been tested by means of Tukey-Kramer test (Table. 4).Regarding initial live weight, two groups became discernible: FQR and EP with MIC= 2.49 ± 0.153 mg and HM, SC and CC with MIC = 3.513 ± 0.14 mg. Nevertheless, there was an intermediate set formed by EP and HM which shared similarities with both groups mentioned above. Similarly, results for total biomass per cocoon displayed differences when comparing CC to FQR and EP, and also

differences were found between FQR and HM. Overall, the eclosion time produced the expected

differences between substrates, obtaining the highest values in SC and CC.

**Table 4**. Tukey-Kramer test for the mean initial biomass (MIB) and mean biomass per cocoon (MBC) found in the different substrates. HM = Horse manure; EP=*Eucalyptus* plantation; FQR= *Quercus robur* Forest; SC=Scrub; CC=Coconut coir.

**TUKEY-KRAMER: MIB**

| FQR | EP | HM | SC | CC |
|-----|-----|-----|-----|-----|
| 2.495 | | | | |
| | 2.906 | | | |
| | | 3.513 | | |

**TUKEY-KRAMER: MBC**

| FQR | EP | SC | HM | CC |
|-----|-----|-----|-----|-----|
| 8.011 | | | | |
| | 9.367 | | | |
| | | 10.979 | | |

Observation of the mean incubation times let us establish a relation between eclosion time and

the initial live weight of newly hatched specimens. ANCOVA of the initial live weight among

different substrates, with eclosion time as covariant and an interaction term for the product of

substrate and eclosion time is represented in Table 5.

**Table 5.** Results of ANCOVA of the initial live weight of *Eisenia andrei* individuals, including eclosion time (days) as covariate and an interaction term for the product of eclosion time and substrate.

| ANCOVA | F-Value $_{4,66}$ | P-Value |
|-----|-----|-----|
| **Substrate** | 0.712 | 0.5845 |
| **Eclosion Time** | 17.535 | <0.0001 |
| **Substrate*Eclosion Time** | 1.209 | 0.3073 |

Maturation period significantly influenced the initial live weight of hatchlings, as *p-value* is

lower than 0.05. ANCOVA indicated a covariation between substrate and eclosion time, so,

differences in initial live weight are attributed to the incubation period, meaning that slight differences in eclosion time from one substrate to another caused significant change in initial live weight of newly hatched specimens.

## 3.3. Survival and growth in newly hatched specimens

### 3.3.1. Size structure of samples

Newly hatched earthworms (n = 100), within an initial size range of 0.905 to 9.22 mg live weight, were used to test their viability in the experimental substrates. Distribution of size frequencies (live weight in mg) of newly hatched specimens of *E. andrei* used in the experiment is shown in Fig. 1. The histogram reveals that the initial weight of 85 % of the offspring was under 5 mg (Mean = 3.14; SD = 1.09), the remaining 15 % being distributed in weights from 5.34 to 9.22mg (Mean = 6.51; SD = 1.10).
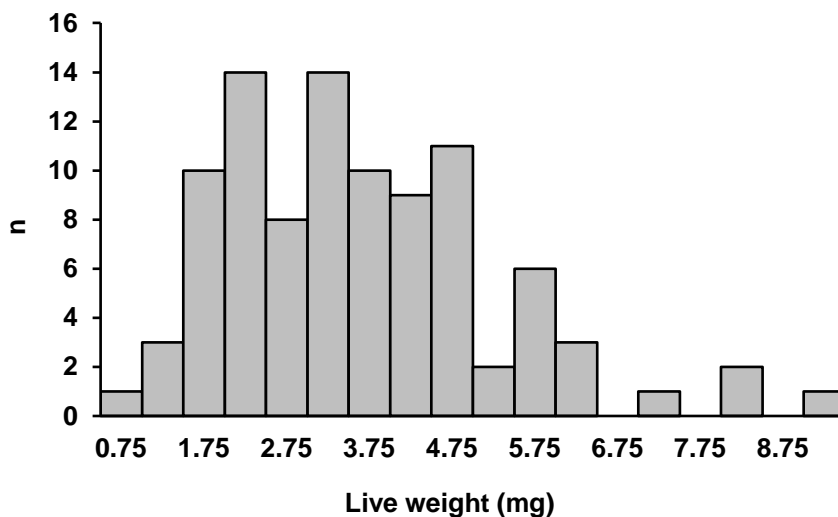


**Fig.1.** Histogram for frequency distribution of Live Weight (mg) of specimens of *E. andrei* used in the experiment.

### 3.3.2. Survival

Survival of newly hatched specimens largely differed among substrates. The relationship between the mortality percentage of *E. andrei* individuals and organic matter % of the media is illustrated in Fig. 2. With the exception of coconut coir, which despite having an organic matter content of 92 % experienced a mortality of 40 %, results for mortality in the rest of substrates adjusted primarily to an exponential pattern, displaying decreasing mortality as organic matter percentage increased.
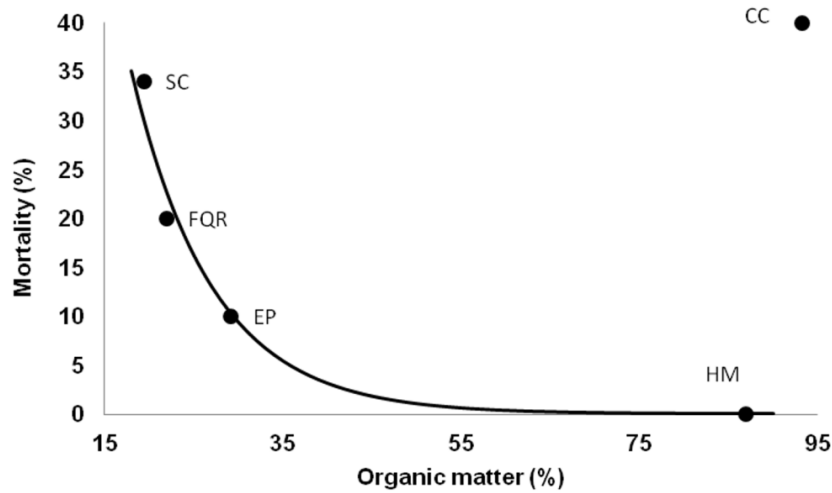
**Fig. 2.** Mortality % of newly hatched specimens of *Eisenia andrei* related to the organic matter % of the substrate by an exponential regression.
*Mortality%* = 253.608*e $^{(-0.11*OrganicMatter\%)}$, $R^2$ = 0.934.

In the next step, mortality was related to substrate respiration rate by a sigmoid Michaelis-Menten equation, accounting for 96 % of the variance: *Mortality* = 39.76*$VO_2^{-2.22}$/ (20.653$^{-2.22}$+ $VO_2^{-2.22}$) Fig. 3.
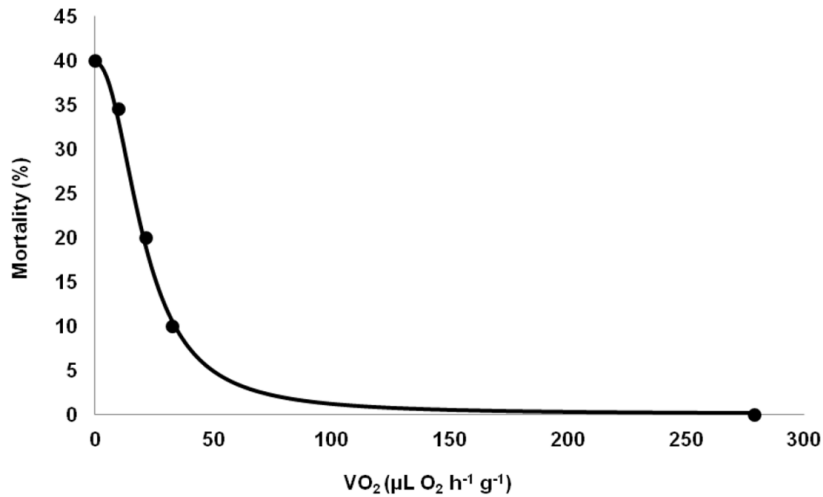


**Fig. 3.** Mortaliy (%) of *Eisenia andrei* newly hatched individuals related to substrate oxygen consumption rate ($VO_2$ in µL $O_2$ h$^{-1}$ g$^{-1}$).
*Mortality* = 39.76*$VO_2^{-2.22}$/ (20.653$^{-2.22}$+ $VO_2^{-2.22}$); $R^2$ = 0.96.

### 3.3.3. Growth

Monitoring of newly hatched worms for 40 days let us model the individual growth of *E. andrei* over time. We have established the equation of each specimen's growth pattern, unifying those with similar parameters in a single equation. Table 6 summarises the equations and growth parameters found in our experiment in the different substrates. In the same way, growth curves

are represented in Fig. 4. Individuals with a biomass increase of less than 8 mg are not considered to have continuously grown and are excluded from the analysis (100 % in CC and 60 % in SC of the surviving earthworms).

Most of the newly hatched specimens presented an exponential growth pattern, which appeared in 100 %, 55.5 %, 100 % and 60 % of survivors with a positive growth, reared in HM, EP, FQR and SC respectively. In this line, individual growth rate analysis was performed in animals exhibiting an exponential growth. Instantaneous growth rates (r), in terms of mg of biomass gain per mg of tissue per time, were obtained by dividing the difference between the initial and final sizes (after natural log transformations) by the elapsed time: r = (Ln final size - Ln initial size)/time.
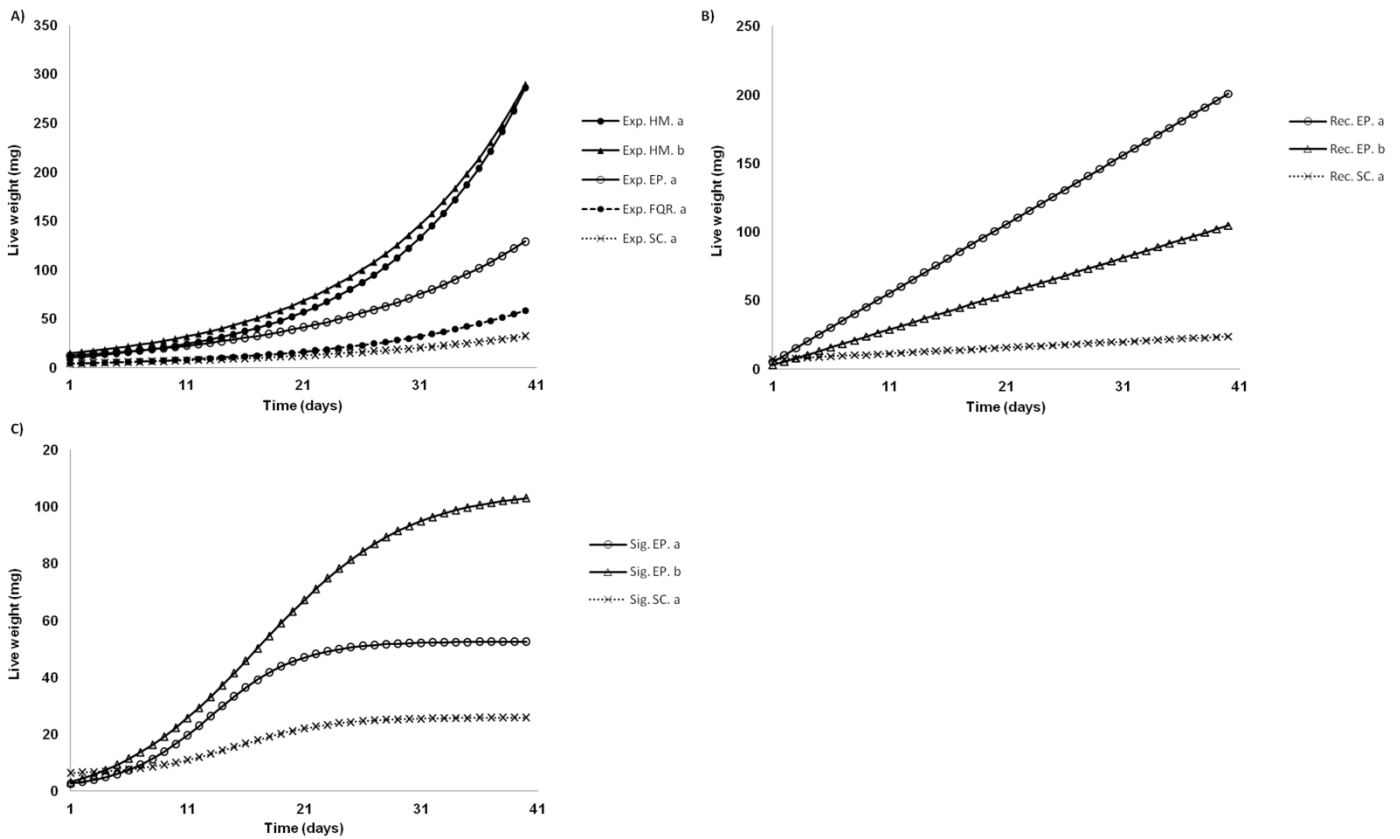


**Fig. 4.** Growth patterns for individuals of *Eisenia andrei* growing in different substrates. A: Exponential $Y=a*(e^{Xb})$; B: Rectilinear $Y= a + b*X$; C: Sigmoid $Y= a-(1/(b/a+(1/(a-c)-b/a)*e^{dX}))$. Black symbols: Horse manure (HM); White symbols: *Eucalyptus globulus* Plantation (EP); Dashed line: *Quercus robur* Forest (FQR); Doted line: Scrub (SC).

**Table 6**. Growth equations and parameters of *Eisenia andrei* newly hatched individuals reared in the different substrates. HM = Horse manure; EP=*Eucalyptus* plantation; FQR= *Quercus robur* Forest; SC=Scrub.

| Substrate | Type of growth | Equation | $R^2$ | n (%)* | ISR (mg) | MIS (mg) | MMS (mg) | TMS (days) |
|---|---|---|---|---|---|---|---|---|
| HM | Exponential a | $LW = 9.533*e^{0.085*t}$ | 0.96 | 70 | 2.31-6.22 | 3.93 | 249.87 | 41 |
| HM | Exponential b | $LW = 13.836*e^{0.076*t}$ | 0.94 | 30 | 3.55-9.22 | 5.78 | 241.24 | 41 |
| EP | Exponential a | $LW = 11.708*e^{0.06*t}$ | 0.82 | 50 | 1.95-4.77 | 3.07 | 114.17 | 41 |
| EP | Exponential b | $LW = 5.282*e^{0.093*t}$ | 0.98 | 5 | - | 3.17 | 99.67 | 41 |
| EP | Rectilinear a | $LW = 5.025*t$ | 0.90 | 10 | 6.38-6.49 | 6.44 | 158.02 | 41 |
| EP | Rectilinear b | $LW= 2.613*t$ | 0.89 | 10 | 2.33-3.24 | 2.78 | 100.10 | 41 |
| EP | Power a | $LW = 3.204*t^{0.7519}$ | 0.95 | 5 | - | 3.90 | 48.66 | 41 |
| EP | Sigmoid a | $LW=52.566-(1/(1.013/52.566+(1/(52.566-2.199)-1.013/52.566)*e^{(0.269*t)}))$ | 0.99 | 5 | - | 2.45 | 97.54 | 41 |
| EP | Sigmoid b | $LW=105.755-(1/(0.965/105.755+(1/(105.755-1.920)-0.965/105.755)*e^{(0.159*t)}))$ | 0.99 | 5 | - | 2.99 | 52.72 | 41 |
| FQR | Exponential a | $LW = 4.02*e^{0.067*t}$ | 0.84 | 80 | 1.32-3.09 | 1.97 | 55.69 | 41 |
| SC | Exponential a | $LW = 4.366*e^{0.05*t}$ | 0.81 | 15 | 4.49-8.00 | 5.72 | 30.13 | 41 |
| SC | Rectilinear a | $LW = 6.607*t$ | 0.83 | 5 | - | 4.96 | 24.11 | 41 |
| SC | Sigmoid a | $LW=25.91-(1/(1.292/25.91+(1/(25.91-6.284)-1.292/25.91)*e^{(0.25*t)}))$ | 0.93 | 5 | - | 7.30 | 26.21 | 41 |

**ISR=Initial Size Range; MIS = Mean Initial Size; MMS = Mean Maximal Size; TMS = Time Maximal Size; LW = Live weight; T = Time.**
**\*Percentage from the initial sample size.**

Results of the analysis of variance of growth rates showed high differences (*p-value* < 0.0001) among substrates, obtaining the greatest figures in HM (0.107 mg mg$^{-1}$day$^{-1}$), followed by EP (0.09.1 mg mg$^{-1}$day$^{-1}$) and FQR (0.084 mg mg$^{-1}$day$^{-1}$), and finding the lowest values in SC (0.042 mg mg$^{-1}$day$^{-1}$). The influence of initial live weight on growth was evident, observing higher instantaneous growth rates in animals with lower initial sizes, the two variables being related by a power law, so an ANCOVA test was performed after variable log-log transformation. In order to avoid negative values in logarithmic transformations, growth rate (r) was normalized prior to log transformation: r*100. Results of the linear regression treatments are shown in Table 7. No statistical differences were evident regarding slope, whereas values for elevation differed between substrates, indicating that the relationship between growth rate and initial live weight was the same in all substrates, but growth rate figures were uniformly larger in worms cultured in HM, followed by EP, FQR and SC. These results matched previous one-way ANOVA analyses for the growth rate.

Consequently, the highest maximal live weights (MLW in mg) were obtained by individuals cultured in HM (Mean = 247.288; SD = 32.389), followed by EP (Mean = 117.008; SD = 30.922) and FQR (Mean = 56.721; SD = 14.443) cultured earthworms. *E. andrei* specimens reared in SC showed lower maximal size (Mean = 19.984; SD = 10.546) than the previous substrates, and in CC, maximal sizes hardly differ from the initial ones (Mean = 10.220; SD = 2.721), indicating no growth during the experiment. These differences were statistically significant (Log transformed ANOVA *p-value* < 0.0001).

In order to control for the effect of the initial size on growth, an analysis of covariance was performed after log-log transformation of the variables. ANCOVA of the maximal live weight (mg) achieved by individuals reared in the different substrates, excluding dead worms, with initial live weight (mg) as covariant and an interaction term for the product of initial live weight and substrate, is shown in Table 8. Significant influence of the initial size was found. Nevertheless, the highest influence regarding MLW was attributed to rearing conditions which accounted for 53 % of the variability.

**Table 7**. Coefficients of the Log-Log regression equations of Growth rate (mg/mg/day*100) vs. Live Weight (mg) of *Eisenia andrei* for different substrates. Differences in individual equations for growth rate between substrates are analyzed through ANCOVA. HM= Horse manure; EP= *Eucalyptus globulus* Plantation; FQR= *Quercus robur* Forest; SC= Scrub; DF= degrees of freedom; $CI_{95\%}$= Confidence intervals. Intercept r = Recalculated elevation value for the common slope of -0.18.

| Substrate | n | Size range (mg) | Intercept ($CL_{95\%}$) | Slope ($CL_{95\%}$) | slope p-value | Intercept r |
|---|---|---|---|---|---|---|
| HM | 20 | 2.03 - 9.22 | $1.155 \pm 0.038$ | $(-0.201) \pm 0.059$ | $< 0.05$ | 1.140 |
| EP | 10 | 1.96 - 4.77 | $1.032 \pm 0.081$ | $(-0.154) \pm 0.165$ | $< 0.05$ | 1.044 |
| FQR | 16 | 1.32 - 3.93 | $0.968 \pm 0.049$ | $(-0.166) \pm 0.162$ | $< 0.05$ | 0.972 |
| SC | 3 | 4.49 - 8.00 | $0.715 \pm 3.596$ | $(-0.126) \pm 0.365$ | 0.79 | 0.755 |
| **ANCOVA** | **DF** | **F-Value** | **P-Value** | **Common values** | | |
| Slope | 3 , 41 | 0.179 | 0.9102 | -0.18 | | |
| Intercept | 3 , 44 | 221.665 | <0.0001 | | | |

**Table 8**. Results of the ANCOVA of the Log Maximal Live Weight achieved by *Eisenia andrei* individuals in the different substrates used in the experiment, including Log Initial Live Weight (mg) as a covariant and an interaction term for the product of Log Initial Live Weight and Substrate.

| ANCOVA | F-Value $_{4,66}$ | P-Value |
|---|---|---|
| **Substrate** | 26.991 | <0.0001 |
| **Initial Live Weight** | 24.830 | <0.0001 |
| **Substrate*Initial Live Weight** | 1.840 | 0.132 |

The influence of organic content on growth was analysed by confronting it to the maximal live weight achieved at the end of the experiment (Fig. 5). Differences regarding maximal live weight (MLW in mg) are related to substrate organic matter content (in %), in all substrates except CC, by an asymptotic equation explaining 91 % of the variability: $MLW=251.44*(1-e^{(-0.059*(OrganicMatter-18.33))})$.
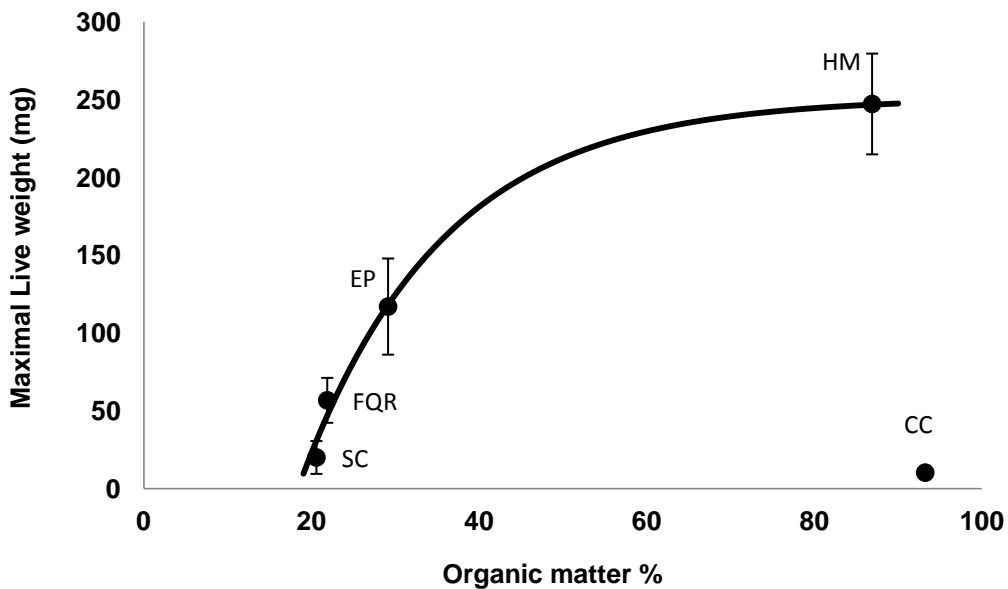


**Fig. 5.** Average maximal live weight achieved by newly hatched specimens of *Eisenia andrei* in the experiment related to the organic matter percentage of each substrate.
$MLW=251.44*(1-e^{(-0.059*(OrganicMatter-18.33))})$.

In the next step, we related biomass gain ($\Delta$LW in mg) by newborn individuals to substrate respiration ($VO_2$ in $\mu L\ O_2\ h^{-1}\ g^{-1}$). The response of individuals to differences in respiration rate, in terms of biomass gain, is illustrated in Fig. 6 A by a sigmoid equation that accounts for 94 % of the variability. $\Delta$LW increased exponentially with values of $VO_2$ ($\mu L\ O_2\ h^{-1}\ g^{-1}$) from 0 to 40 $\mu L\ O_2\ h^{-1}\ g^{-1}$ (Fig. 6 B), arriving at a maximum for $VO_2$ levels over 100.
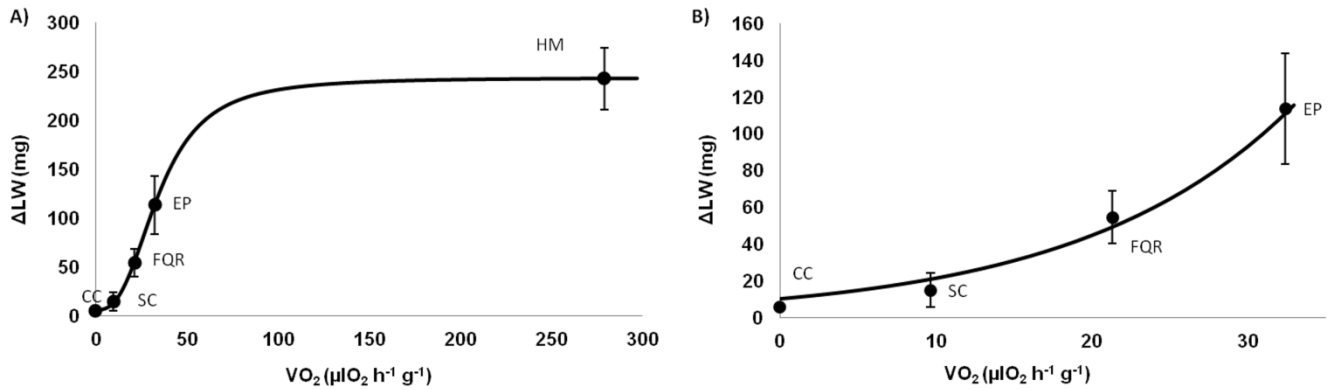
**Fig. 6.** Biomass variation (ΔLW in mg) of *Eisenia andrei* newly hatched specimens along the experiment in relation to the substrate respiration rate (VO$_2$ in µLO$_2$ h$^{-1}$ g$^{-1}$). A: Sigmoid equation relating ΔLW to VO$_2$ in every substrate ($VO_2 = 6.55 + (((246.59\text{-}6.548)*ΔLW^{2.75}) / (34.83^{2.75} + ΔLW^{2.75}))$; $R^2 = 0.94$. B: Exponential equation relating ΔLW to VO$_2$ in every substrate except Horse Manure ($VO_2 = 10.394*e^{0.07ΔLW}$; $R^2 = 0.87$).

## 3.4. Growth of juvenile specimens

### 3.4.1. Size structure of samples

Juveniles (n = 58) within an initial size range of 34.43 to 187 mg live weight were used to test their viability in experimental substrates. The distribution of size frequencies (live weight in mg) of juveniles of *Eisenia andrei* used in the experiment is shown in Fig. 7. The histogram reveals that 75 % of the individuals had an initial weight below 100 mg (Mean = 70.52; SD = 18.96), the other 25 % being distributed from 106 to 187 mg (Mean = 131.22; SD = 26.26). Groups of worms selected for each substrate were homogeneous regarding initial live weight (ANOVA after log-transformations: *p-value* > 0.05).
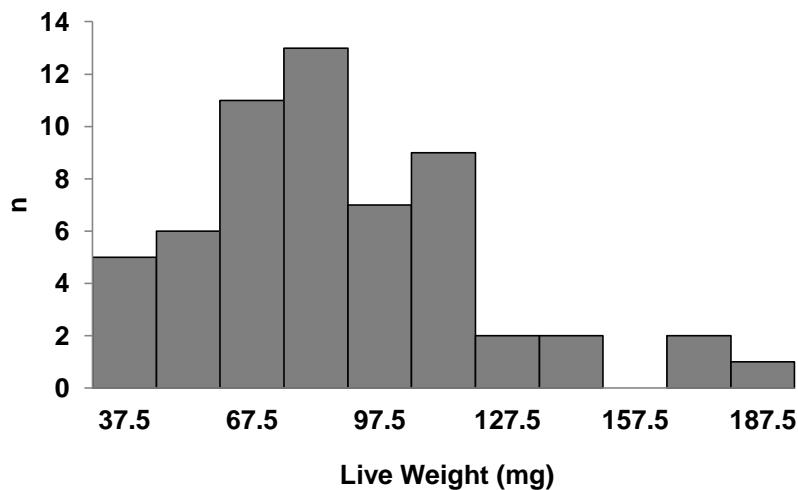


**Fig.7.** Histogram for frequency distribution of initial Live Weight (mg) of specimens of *E. andrei* used in the experiment.

### 3.4.2. Growth

Monitoring of juveniles for 100 days let us model the individual growth of *E. andrei* over time. In a similar way to newly hatched specimens, we have established the equation of the growth pattern for each specimen, joining those with similar parameters in a single equation. Table 9 summarises the equations and growth parameters found in our experiment with different substrates. In the same way, growth curves are represented in Fig. 8. Analysis of the growth patterns reveals that most of the juveniles reared in HM displayed an exponential or rectilinear model, characterized by a continuous growth along the experiment, and higher maximal sizes were reached compared to the rest of substrates (1000 to 2000 mg). Individuals incubated in EP mostly showed a sigmoid model, in which a short initial lag stage is experienced, and an asymptote is reached at a weight of 600 to 900 mg. Analogous asymptotic pattern was found in juveniles reared in FQR, achieving maximal live weights from 300 to 500 mg. Finally, although power law models were described by juveniles cultured in SC, their growth rates were lower compared to juveniles with homologous growing patterns reared in HM, EP and FQR, resulting in smaller individuals at the end of the experiment. Specimens reared in CC showed a constant or decreasing live weight, hence, no growth pattern was assigned to these animals.

Different growth patterns preclude growth rate comparison between substrates. For this reason, the first month of growth was analysed separately; a period that permits exponential modelling of growth in every substrate except CC (growth rate approached to 0). As in the case of newly hatched specimens, instantaneous growth rates (r) were analysed. Results of the analysis of variance of instantaneous growth rates showed high differences (*p-value* < 0.0001) among substrates, obtaining the greatest figures in horse manure (0.062 $\pm$ 0.003 mg mg$^{-1}$ day$^{-1}$), followed by EP (0.0531 $\pm$ 0.002 mg mg$^{-1}$ day$^{-1}$) and FQR (0.04611 $\pm$ 0.002 mg mg$^{-1}$ day$^{-1}$), and finding the lowest values in SC (0.0289 $\pm$ 0.001 mg mg$^{-1}$ day$^{-1}$).

**Table 9**.Growth equations and parameters of *Eisenia andrei* juveniles reared in the different substrates. HM = Horse manure; EP=*Eucalyptus* plantation; FQR= *Quercus robur* Forest; SC=Scrub.

| Substrate | Type of growth | Equation | $R^2$ | n (%)* | ISR (mg) | MIS (mg) | MMS (mg) | TMS (days) |
|---|---|---|---|---|---|---|---|---|
| HM | Exponential a | LW= 190.991*e $^{0.021t}$ | 0.92 | 22.22 | 40.22-108.55 | 73.29 | 1453.71 | 100 |
| HM | Exponential b | LW= 104.215*e $^{0.026t}$ | 0.95 | 5.5 | - | 34.43 | 1084.78 | 100 |
| HM | Rectilinear a | LW=216.97+16.56*t | 0.95 | 27.77 | 111.34-187.00 | 141.34 | 1874.50 | 100 |
| HM | Rectilinear b | LW=95.21+16.38*t | 0.98 | 5.5 | - | 141.72 | 1697.40 | 96 |
| HM | Rectilinear c | LW=164.69+12.38*t | 0.95 | 11.11 | 94.41-98.72 | 96.56 | 1385.67 | 96 |
| HM | Rectilinear d | LW=163.54+8.756*t | 0.96 | 5.5 | - | 117.51 | 991.15 | 96 |
| HM | Power a | LW= 47.75*t $^{0.705}$ | 0.96 | 11.11 | 47.49-86.75 | 67.12 | 1283.25 | 98 |
| HM | Power b | LW= 128.26*t $^{0.447}$ | 0.93 | 5.5 | - | 168.79 | 1091.44 | 98 |
| HM | Asymptotic a | LW=(640.39-105.8)*(1-e $^{(-0.028t)}$)+105.8 | 0.87 | 5.5 | - | 106.32 | 718.78 | 88 |
| EP | Power a | LW=50.205*t $^{0.627}$ | 0.96 | 10 | - | 116.15 | 885.64 | 85 |
| EP | Sigmoid a | LW=880.02-(1/(0.94/880.02+(1/(880.02-56.8)-0.94/880.02)*e $^{(0.063*t)}$)) | 0.92 | 70 | 44.84-97.51 | 74.56 | 938.81 | 90 |
| EP | Sigmoid b | LW=625.01-(1/(1.01/625.01+(1/(625.01-72.04)-1.01/625.01)*e $^{(0.067*t)}$)) | 0.97 | 20 | 60.77-76.99 | 68.88 | 678.92 | 89 |
| FQR | Power a | LW= 21.378*t $^{0.634}$ | 0.85 | 50 | 42.11-71.49 | 55.29 | 400.49 | 86 |
| FQR | Power b | LW= 37.38*t $^{0.595}$ | 0.92 | 10 | - | 68.65 | 449.52 | 97 |
| FQR | Asymptotic a | LW=(548.8-56.06)*(1-e $^{(-0.027t)}$)+56.06 | 0.80 | 30 | 71.16-132.17 | 93.43 | 606.94 | 92 |
| FQR | Asymptotic b | LW=(385.528-53.42)*(1-e $^{(-0.03t)}$)+53.42 | 0.94 | 10 | - | 75.30 | 408.08 | 93 |
| SC | Power a | LW=35.546*t $^{0.455}$ | 0.85 | 50 | 68.97-88.46 | 79.29 | 320.105 | 92 |
| SC | Power b | LW=47.951*t $^{0.331}$ | 0.80 | 30 | 68.78-82.82 | 76.37 | 244.13 | 90 |
| SC | Power c | LW=19.08*t $^{0.57}$ | 0.89 | 10 | - | 45.63 | 305.93 | 90 |
| SC | Power d | LW=11.659*t $^{0.58}$ | 0.86 | 10 | - | 39.52 | 187.99 | 94 |

ISR=Initial Size Range; MIS = Mean Initial Size; MMS = Mean Maximal Size; TMS = Time Maximal Size; LW = Live weight; T = Time. *Percentage from the initial sample size.
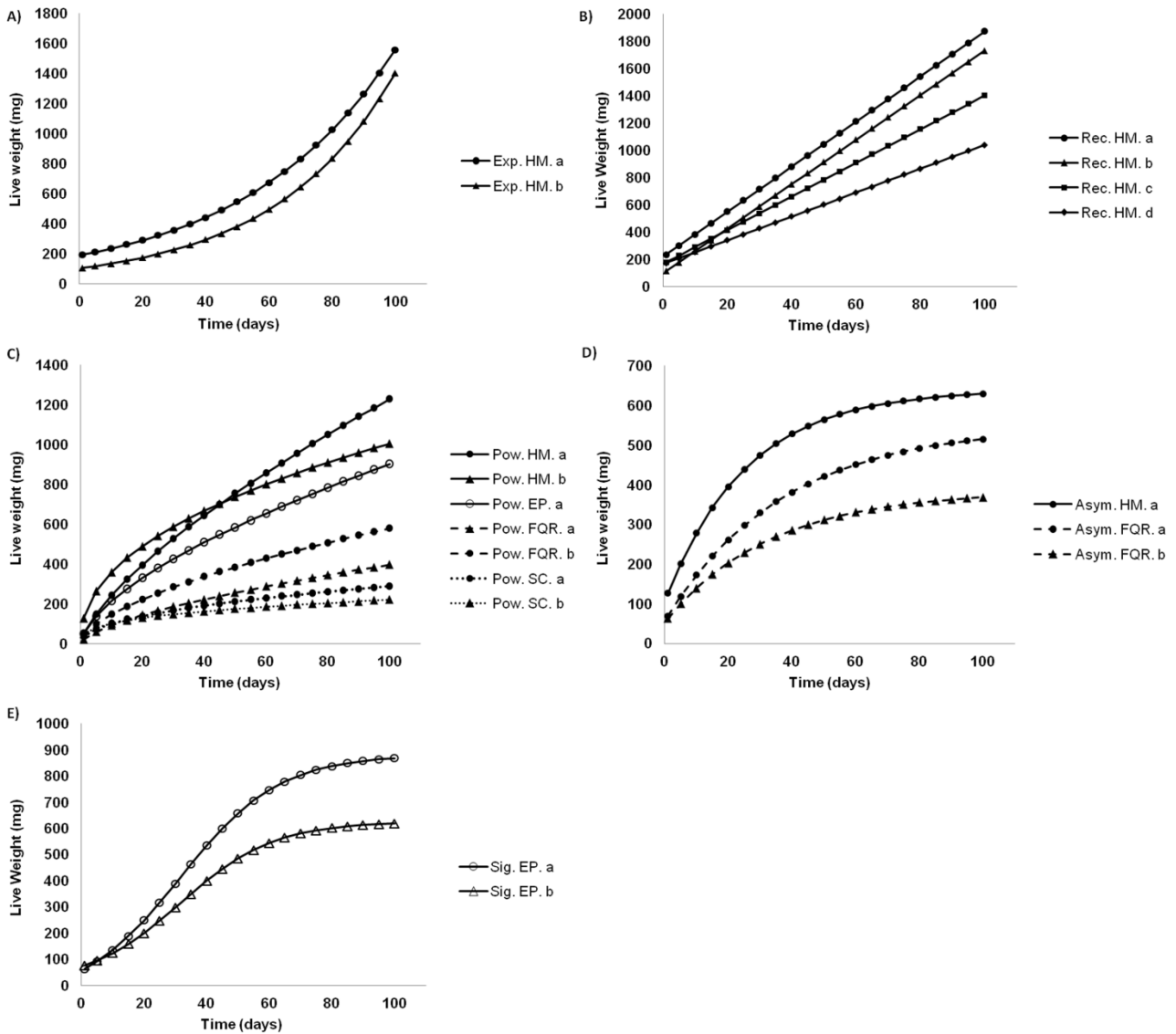
**Fig. 8.** . Growth patterns for individuals of *Eisenia andrei* growing in different substrates. A: Exponential $Y=a*(e^{Xb})$; B: Rectilinear $Y= a + b*X$; C: Power $Y=a*X^b$; D: Asymptotic $Y =( a-c)*(1- e^{Xb})+c$, E: Sigmoid $Y= a-(1/(b/a+(1/(a-c)-b/a)*e^{dX}))$. Black symbols: Horse manure (HM); White symbols: *Eucalyptus* Plantation (EP); Dashed line: *Quercus robur* Forest (FQR); Doted line: Scrub (SC).

As in the case of newborn specimens, notable influence was found by the initial live weight on the growth rate, obtaining higher r values in animals with lower initial size, being the two variables related by a power law within the size range used in the test, so an ANCOVA was performed after variable log-log transformation. Once again, growth rate (r) was normalized to r*100 in order to avoid negative values in the log transformation. Results of the linear regression treatments are shown in Table 10.

**Table 10**. Coefficients of Log-Log regression equations of Growth rate (mg/mg/day*100) *vs.* Live Weight (mg) of *Eisenia andrei* juveniles for different substrates. Differences between individual growth rate equations of the different substrates are analysed through ANCOVA. HM= Horse manure; EP= *Eucalyptus* Plantation; FQR= *Quercus robur* Forest; SC= scrub; DF= degrees of freedom; $CI_{95\%}$= Confidence intervals. Intercept r = Recalculated intercept for the common slope -0.320.

| Substrate | n | Size range (mg) | Intercept ($CL_{95\%}$) | Slope ($CL_{95\%}$) | Slope p-value | Intercept r |
|---|---|---|---|---|---|---|
| HM | 18 | 34.43 - 187.00 | $1.49 \pm 0.19$ | $-0.355 \pm 0.09$ | < 0.05 | 1.418 |
| EP | 10 | 44.84 - 116.15 | $1.29 \pm 0.55$ | $-0.304 \pm 0.29$ | < 0.05 | 1.321 |
| FQR | 10 | 40.74 - 132.18 | $1.10 \pm 0.23$ | $-0.246 \pm 0.23$ | < 0.05 | 1.236 |
| SC | 10 | 39.46 - 98.46 | $0.92 \pm 0.75$ | $-0.250 \pm 0.41$ | < 0.05 | 1.045 |
| **ANCOVA** | **DF** | **F-Value** | **P-Value** | **Common values** | | |
| Slope | 3 , 40 | 0.394 | 0.7579 | -0.320 | | |
| Intercept | 3 , 43 | 122.128 | < 0.0001 | | | |

**Table 11**. Results of ANCOVA analysis of the Log maximal live weight (mg) by *Eisenia andrei* individuals in different substrates used in the experiment, including Log initial live weight (mg) as covariant and an interaction term for the product of Log initial live weight and substrate.

| ANCOVA | F-Value $_{4,48}$ | P-Value |
|---|---|---|
| **Substrate** | 9.406 | <0.0001 |
| **Log Initial Live Weight** | 9.608 | 0.0033 |
| **Substrate*Log Initial Live Weight** | 3.647 | 0.0516 |

No interaction was found. Nonetheless, values for elevation differed between substrates, indicating that the relationship between growth rate and initial live weight was the same for all substrates but values for the growth rate were uniformly larger in worms cultured in HM, followed by EP, FQR and SC. These results matched previous ANOVA analyses for the growth rate.

Maximal live weights (MLW) in the various substrates were statistically different (Log transformed ANOVA *p-value* < 0.0001), the highest values being reached in HM (Mean = 1453.09; SD = 362.35), followed by earthworms cultured in EP (Mean = 881.52; SD = 126.202) and FQR (Mean = 479.97; SD = 117.52) cultured earthworms. *E. andrei* specimens reared in SC showed lower maximal size (Mean = 282.69; SD = 52.99) than the three previous substrates, and in CC, maximal sizes do not differ from the initial ones (Mean = 100.36; SD = 28.317) indicating no growth during the experiment. In order to control for the effect of the initial size on growth, an analysis of covariance after log transformations of the variables was performed. ANCOVA of the Log MLW (mg) achieved by individuals in the different substrates used in the experiment with Log initial live weight (mg) as covariant and an interaction term for the product of Log initial live weight and substrate is shown in Table 11.

In accordance with the analysis for newborn specimens, in juvenile earthworms influence of the initial live weight on MLW was observed, and explained 8 % of the variance. Nevertheless, rearing condition was the factor that explained most of the variability (34 %).

The influence of organic content on growth was analyzed by confronting it to biomass variation detected in the experiment (Fig. 9). Differences regarding $\Delta$ Live weight ($\Delta$LW in mg) are related to substrate organic matter content (in %), in all substrates except CC, by an asymptotic equation explaining 90 % of the variability: $\Delta LW = 1392.09 * (1 - e^{(-0.069 * (OrganicMatter - 18.33))})$.
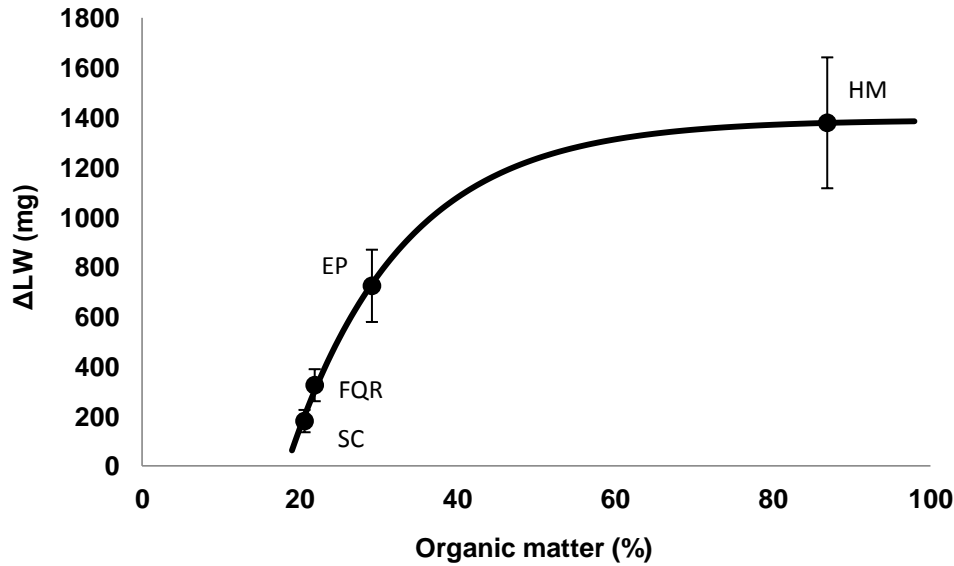
**Fig. 9.** Biomass variation (ΔLW) of *Eisenia andrei* juveniles along the experimental time related to the organic matter percentage of each substrate.
$\Delta LW = 1392.09*(1-e^{(-0.069*(OrganicMatter-18.33))})$.

In the next step we related biomass gain (ΔLW in mg) by juvenile individuals to the substrate oxygen consumption rate ($VO_2$, in $\mu LO_2$ $h^{-1}$ $g^{-1}$). The response of the specimens, in terms of biomass gain, to differences in respiration rate is described by an asymptotic equation explaining 87 % of the variability (Fig. 10 A). ΔLW increased linearly with respect to $VO_2$ ($\mu LO_2$ $h^{-1}$ $g^{-1}$) from 0 to 40 $\mu LO_2$ $h^{-1}$ $g^{-1}$ (Fig. X B), reaching a maximum ΔLW for values of $VO_2$ over 100 (Fig. 10 A).
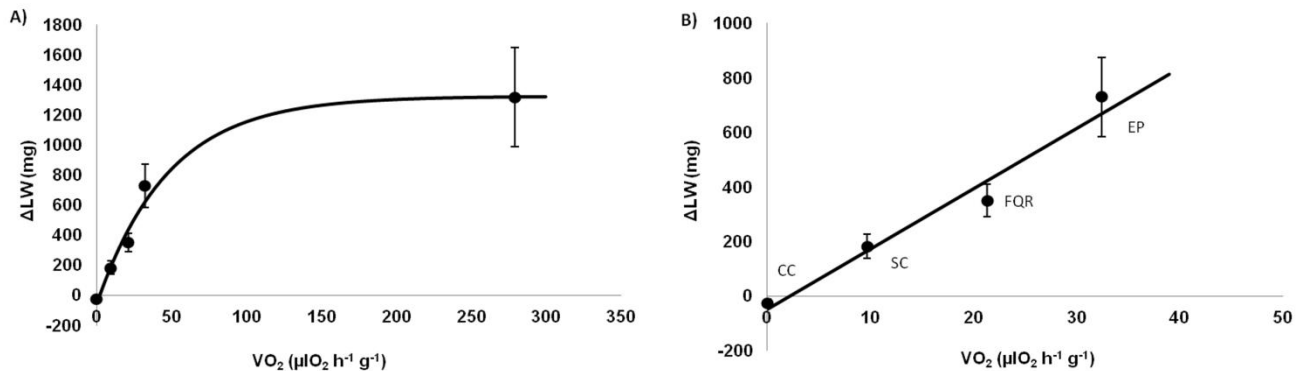


**Fig. 10.** Biomass variation (ΔLW in mg) of *Eisenia andrei* juveniles along the experiment in relation to the respiration rate ($VO_2$ in $\mu LO_2$ $h^{-1}$ $g^{-1}$) of each substrate. A: Asymptotic equation relating ΔLW to $VO_2$ in every substrate ($VO_2 = (1323.48+59.15)*(1-e^{(-0.021*\Delta LW)})-59.15$; $R^2 = 0.87$). B: Rectilinear equation relating ΔLW to $VO_2$ in every substrate except Horse Manure ($VO_2 = 22.043*\Delta LW - 47.916$; $R^2 = 0.88$).

# 4. Discussion

The Basque country is characterized by the presence of a wide variety of landscapes (deciduous and evergreen forest, plantations and scrubs, meadows etc.), and the preservation of these vegetation types is directly linked to climate changes and physicochemical characteristics of soil. In this context, the role of earthworms, associated with soil microorganisms, on organic matter cycles is well known (Brown *et al.* 2000; Ojha & Devkota 2014; Lemtiri *et al.* 2014). Maintenance and enhancement of these ecological engineers is of great interest to maintain soil properties and fertility (Edwards 2004), so, from this point of view it is essential to study the nutritional resources for epigeic earthworms that would enable the prevalence of earthworm active populations, whose abundance often correlates with organic matter availability (Edwards & Lofty 1977; Tiunov & Scheu 2004 ).

In a preliminary analysis of top soil characteristics in a variety of landscapes in the Basque Country, we found significant differences regarding organic matter content between EP and SC, the selection of substrates being made according to this criterion (Orrantia 2016). Forest environments dominated by *Quercus robur* are the potential vegetation type in our sampling area (Loidi *et al.* 2011) and in order to understand the effect of a natural, unmanaged environment on worms, this substrate has been included in the experiment. Applying the perspective brought about by the study with aging grass (Chapter 5), which addressed the question of organic quality of the substrate, this work employs a matrix of substrates including two controls; which, despite their similar organic content (86 % and 93 % in HM and CC, respectively), differ greatly in the actual nutrient availability. Regarding organic content of selected soils, high differences were found compared to the control groups, ranging from 29.13% in EP to 20.58 % in SC.

Since the objective of this study was to test the suitability of substrates, in terms of nutritional resources, to sustain earthworm populations, and to understand the actual probability of recruitment of reproductive individuals, we have worked with pre-mature stages of the ontogeny. In the first part eclosion, survival and growth of newly hatched specimens with initial

sizes ranging from 0.90 to 9.22 mg was tested. A second experiment was performed with juvenile worms with starting sizes within 34.43 to 187 mg.

## 4.1. Eclosion experiment

Recruitment of new specimens in a population depends, first of all, on the proportion of the lay that is able to hatch. Reproductive tests on earthworms have been broadly used as indicator of soil quality (Schaefer 2004; Robidoux *et al.* 2001; Yasmin & D´Souza 2007) due to the sensibility of cocoons and newly hatched earthworms to adverse environmental conditions.

In our experiment, no effect of the substrate was observed in terms of the number of hatchlings per cocoon. In contrast, initial biomass of hatchlings and total biomass per cocoon differed among incubation media. ANCOVA of the initial live weight of newly hatched specimens, with eclosion time as a covariate and substrate as a factor, demonstrated a covariation between substrate and cocoon maturation time, the latter being the contributing factor of the variability in initial live weight. The highest figures for eclosion time were obtained in earthworms cultured in SC and CC, suggesting that cocoons reared in these substrates spent longer periods in the cocoon, probably exhausting nutritional resources before hatching. Cocoons of *Eisenia* sp. contain a diverse population of symbiotic bacteria taken from the surrounding soil or passed directly from the parents. In fact, Zachmann & Molina (1993) reported uptake of specific bacteria by egg capsules of *Eisenia fetida*. In our experiment, the lowest aerobic microbial metabolic rate ($\mu L$ $O_2$ $h^{-1}$ $g^{-1}$), was obtained in SC ($9.66 \pm 0.39$ $\mu L$ $O_2$ $h^{-1}$ $g^{-1}$), while an absence of respiratory activity (virtually zero) was recorded in CC. The notion that developing progeny react to adverse environmental conditions by lengthening their permanence inside the cocoon agrees with previous results obtained by us with aging grass (Chapter 5).

## 4.2. Survival and growth experiment

Size distribution of newly hatched specimens in our sample constitutes a representative selection of the range of sizes in an eclosion batch, and matched results for offspring used in the study with aged grass (Chapter 5). Most hatchlings (85 %) ranged from 0.905 to 5 mg on a live

weight basis, clustered at the lowest end of the frequency distribution histogram, whereas the remaining 15 % were homogeneously distributed in size categories between 5 and 9.22 mg. An analogous size distribution was observed in juvenile specimens, where 75 % of the population was constrained to sizes below 100 mg (Mean = 70.52; SD = 18.96), the remaining 25 % of the animals being distributed at the highest end of the frequency distribution histogram (Mean = 131.22; SD = 26.26). In any case, worms attributed to the different experimental substrates showed no significant differences in mean initial biomass.

Results of the experiment with the positive control group (horse manure) displayed no mortality, and the maximal live weights achieved were similar to those reported by Neuhauser *et al.* (1980) for newly hatched specimens of *Eisenia fetida* during the first month of experiment. Maximal live weights reached by juvenile specimens (initial live weight 100 mg) individually reared in pig manure for 50 days (Domínguez & Edwards 1997) were similar to our results for the same time period (930 mg in pig manure to 816 mg in our experiment).

## 4.2.1. Influence of food quantity: organic matter availability

One of the main factors affecting earthworm abundance in soil, excluding temperature and humidity, is organic matter availability. In fact, Hendrix *et al.* (1992) reported significant correlation between earthworm density and soil organic C content over a range of sites in Georgia, USA. Moreover, studies in a variety of landscapes demonstrated an increase in earthworm populations after the application of high-quality organic materials such as animal manure (Curry 1998; Marhan & Scheu 2005a; Riley *et al.* 2008). In our experiment, in all substrates except CC, mortality decreased at an exponential rate as organic content increased. Similarly, growth patterns of newly hatched and juvenile specimens, excluding CC, also relate to organic matter availability. An asymptotic relation was observed between ΔLW and organic matter percentage in both developing stages.

When analysing the influence of the substrate in the relation between growth rate (mg mg$^{-1}$ day$^{-1}$) and initial live weight (mg), a significant effect of the culture medium was observed in both developing stages. In this line, maximal live weights (MLW) reached by newly hatched worms differed largely between substrates, especially in the extremes of organic content, the highest

figures being found in HM (247.288 mg) and the lowest, by a factor of 12, in SC incubated worms. The same pattern was observed in juveniles, where an average MLW of 1453.09 mg (SD=362.35) was obtained in HM, 881.52 mg (SD = 126.2) in EP, 479.97 mg (SD = 117.5) in FQR and 282.69 mg (SD= 52.99) in SC. ANCOVA of the biomass evolution revealed that the maximal live weights attained by newly hatched organisms were affected by their initial size. Nevertheless, rearing conditions still explained 53 % of the variability found in the analysis, having the highest influence of the culture media. Same results were obtained for juvenile individuals, where rearing conditions explained 34 % of the variability.

Individuals reared in coconut coir presented the highest mortality percentage (40 %) and no growth was observed in newly hatched specimens, whereas in juveniles a decreasing pattern was recorded. In contrast with the results explained above, organic matter in coconut does not explain the growth performance of organisms, so further analysis of organic matter composition is necessary to understand the physiological responses of earthworms.

## 4.2.2. Influence of food quality: organic matter composition

From results obtained in this work, it appears to be the quality of the food, rather than the actual quantity of organic matter, that most often limits the earthworm population. Conversion of organic nutrients into energy implies metabolic investments in digestion, and digestibility of the organic matter depends on both the structure of a given component and the digestive capacity of the organism. Fibre is the organic component that is most difficult to degrade; however, as the study with aging grass addressed (Chapter 5), major differences appeared among fibre components regarding digestibility. Fibre is composed of three elements: hemicelluloses, celluloses and lignin, mentioned in diminishing order of digestibility for most fauna, the latter being a group of aromatic polymers deposited predominantly in the walls of secondarily thickened cells, making them rigid and impervious, providing protection from microbial degradation (Vanholme *et al.* 2010). In fact, in a study of carbon cycling in arid and semi-arid soils after the addition of ^{13}C-labeled cellulose and lignin (Torres *et al.* 2014), and comparing these results to those found by Bastida *et al.* (2013) after the addition of glucose, Torres *et al.* (2014) concluded that lignin derived carbon lasted longer in soils than carbon derived from

glucose or cellulose. In the present study, high proportion of fibre (75 % of organic matter) was recorded in CC, meanwhile the proportion found in horse manure is comparable to that of soil substrates (41 for HM and 39, 44, 48 for EP, FQR and SC respectively, every figures as % of organic matter). Regarding fibre composition, in a study of physicochemical properties of CC of different origins (Abad *et al.* 2002), lignin represented ~50 % of the total fibre, the easily digestible hemicelluloses being the minor constituent of the fiber pool (~10 % of fibre). In contrast, data from a study of biochemical composition carried out on 43 leaf types (Fourty *et al.* 1996), including deciduous plants, displayed an average lignin content of 23 (% of fibre content), against values of 33.5 % for hemicelluloses. So, data from literature point out the differences regarding fibre composition in the substrates studied in this work.

Aerobic metabolism of a substrate, measured in terms of oxygen consumption rate ($\mu L\ O_2\ h^{-1}\ g^{-1}$) or $CO_2$ efflux, is in fact a measure of microbial activity, and is used as an indirect method to estimate microbial biomass (Harris & Steer 2003). In our study, $VO_2$ ($\mu L\ O_2\ h^{-1}$) was positively related to both organic content (g) and to the non-fibre pool of organic matter (NFO in g), in all substrates but CC which, although exhibiting a high proportion of NFO (~20 % of dry matter), has a high proportion of lignin (Abad *et al.* 2002), conforming a matrix around the plant cell, that shields labile cellular compounds from degradation by microorganisms (Vanholme *et al.* 2010). In fact, lignin content is often negatively related to microbial biomass and activity (Schutter & Dick 2001), as it presumably hinders microbial colonization. DeAngelist *et al.* (2011) affirmed that lignin can create a non-favorable chemical environment for the development of microbial biomass, with the exception of certain populations that are able to use lignin. At this respect, Vinceslas-Akpa & Loquet (1997) found no degradation of lignin along the first month of composting.

 In contrast with the regression obtained for $VO_2$ vs. NFO, no relation was observed between $VO_2$ ($\mu L\ O_2\ h^{-1}$) and fibre content (g) of the substrates indicating that substrate respiration (microbial activity) depends on the proportion of the more easily assimilable organic matter components in soil. This fact may explain the decreasing exponential pattern found in the relation between the respiration rate of substrates ($\mu L\ O_2\ h{-}1\ g^{-1}$) and their fibre content (% of

organic matter), which suggests that when fiber represents an increasing fraction of the organic weight, the proportion of readily extractable pool of food decreases, resulting in a lower microbial activity (Fontaine *et al.* 2003). This relation is amplified in the case of CC due to its high proportion of lignin. Horse manure represents an exception and was excluded from this equation: although fiber content in organics (%) was comparable to that found in soils, digestive processes in horses involve priming transient foodstuffs with microorganisms, resulting in microbial enrichment of faeces, which are finally discharged inside mucus packs, so that both free sugars (25 % of dry matter) and microbial content appear enhanced.

In the context of soil macrofauna, many studies on the palatability of different litter had demonstrated that earthworms are able to select the food, and earthworm abundance is in most cases affected by the carbohydrate content (Curry *et al.* 2004; Edwards & Lofty 1977), polyphenolic compounds, C/N ratio and lignin proportion (Satchell & Lowe 1967; Hendriksen 1990; Korboulewsky *et al.* 2016).

Although no effort has been made in this study to quantify total polyphenolic content (TPC), from results obtained in the eclosion experiment, where an eclosion success over 85 % was recorded in every substrate tested, we cannot presume any toxic effect of the media operating on earthworms. Moreover, in a revision of works in which these compounds were analysed, higher figures for TPC were obtained in *Eucalyptus globulus* (Dezsi *et al.* 2015) than in *Quercus robur* leaves measured by Scalbert *et al.* (1988). In our experiment, EP represents, after HM, the substrate with the highest survival percentage and the highest growth of earthworms, so, although we cannot discard toxic effects derived from polyphenolic compounds, results suggest that other factors might be of greater relevance in our experiment.

Focusing on the fate of fiber in earthworms, cellulolytic activity in the gut of *Eisenia fetida* has been reported (Zhang *et al.* 2000) and data for the same species in a composting reactor indicated degradation of cellulose and hemicelluloses mediated by intrinsic activity or by interactions with microorganisms (Aira *et al.* 2006; Elvira *et al.* 1996b). In contrast, degradation of lignin, mediated by microorganisms, happens at a very low rate and, in the presence of more easily degraded compounds, lignin mineralization decreases (Scheu 1992). Hence, lignin is the

most recalcitrant product of the fiber pool, and since it protects cell wall polysaccharides from microbial degradation, increasing its proportion (% of OM) reduces degradability of the easily digestible organic pool. In fact, in a study of leaf litter selection by detritivore and geophagous earthworms, a negative correlation was found between earthworm abundance and lignin content (Hendriksen 1990). Nonetheless, the correlation was not statistically significant.

Mutualistic relationships between earthworms and microorganisms, detailed accurately by Trigo *et al.* (1999), promote the digestion of organic compounds. Optimization of this relation occurs in the gut of earthworms, where mucus secretion provides optimal physiological conditions for microbial proliferation, whereas microorganisms enhance the digestive abilities of annelids (Brown *et al.* 2000) at a rate that is consistent with the metabolic demands of the earthworms (Lavelle *et al.* 1997). In this context, in substrates with high microbial biomass, degradation of the most refractory pool of organic matter would be enhanced either in the gut of earthworms or by microbial processing of organic matter before the ingesta. Moreover, diet of earthworms is known to include soil bacteria and fungi (Curry & Schmidt 2007; Zirbes *et al.* 2012) and there is evidence of digestive abilities to process microorganisms (Schönholzer *et al.* 1999; Edwards 2004). In this context, results obtained in this work are consistent with microbial activity found in substrates, since lower mortality (%) and higher growth rates were found in substrates with increasing proportion of microbiota: microbial proliferation could account for the increasing digestibility of the fiber proportion and provides a suitable source of food in the form of microorganisms.

In this context, an approach to understand the food quality of soil of different vegetation types could be undertaken by analysing the holistic response of *Eisenia andrei* individuals in terms of eclosion, survival and growth to the substrates analysed. In our experiment, organic matter explained only partially the survival and the growth performance of earthworms, since individuals reared in CC, characterized by having the highest organic content, displayed the lowest survival and growth figures. In consequence, quality of organic matter, in terms of digestibility of foodstuff, should be invoked. A decrease of the non-fibre organic content (% of dry matter) of substrates and an increase of the proportion of their fiber content (% of organic

matter) would account for lower digestibility of the organic matter for both soil microorganism and earthworms, this relation being enhanced in lignin enriched substrates. Besides, due to their mutualistic relationship, the decrement of soil microbial activity reduces the digestive capacity of earthworms, and since soil microbial activity could be an indirect measurement of biomass, the decay of the microbial pool reduces the available food for earthworms in the form of soil microbiota, redounding in lower live expectancy and growth for epigeic earthworms.

# 5. Conclusions

Substrate respiration was negatively related to the proportion of fibre (% of organic matter), and survival and growth of *E. andrei* individuals were positively related to substrate respiration ($\mu LO_2$ $h^{-1}$ $g^{-}1$). A decrease of the non-fibre organic content (% of dry matter) of substrates and an increase of the proportion of their fiber content (% of organic matter) would account for the lower digestibility of the organic matter for both soil microorganism and earthworms, this relation being enhanced in lignin enriched substrates. Due to the mutualistic relation between earthworms and microbiota, in substrates with high microbial biomass, degradation of the most refractory pool of organic matter would be enhanced and also provides food in the form of microorganisms. So, a decrease of the proportion of easily metabolizable components in soil, with a concomitant reduction in soil microbial content, would reduce live expectancy and growth of these epigeic earthworms.

# Chapter 7

## 7. General discussion

# General discussion

This chapter is divided in three sections. In the first one, a discussion about the techniques developed in this thesis is made. The second part of the discussion is focused on the effects of isolation and population density on growth traits and metabolic level. The last section deals with the concept of organic matter quantity and quality in the sense of the effect of nutritive substrates on life cycle traits of *Eisenia andrei* individuals.

## 1. Techniques

### 1.1. Photogrammetric method

A photogrammetric method suitable for every stages of the vital cycle has been developed and is explained in the second chapter of this thesis. We observed that the relation between Photographic surface (PS) and Live weight (LW) was irrespective of the position of the animal in the photo or the number of pictures taken. Nonetheless, feeding conditions altered this relation, yielding higher PS in starved individuals due to the flattening effect observed in fasted earthworms.

This technique allows non invasive biomass measurements compared with gravimetric methods. Earthworms have a cutaneous respiration, which requires an aqueous layer on the respiratory surface in which $O_2$ is dissolved and diffuses through the cuticle and epidermal tissue. In gravimetric techniques, it is necessary to remove surface water in order to obtain the actual live weight of the animal. This manipulation could affect the viability of individuals and, without a carefully manipulation, live weight measurements could be sub-estimated by and over exposure to the suction pressure of the cloth.

As earthworms exhibit a cylindrical shape, photographs permit estimation of the volume (view Chapter 2), and also estimations of the radio, total surface and length, that give extra information to the weight measurements. This method also provides a recorded picture data,

allowing the correction of suspicious values and the compilation of information that could not be of interest in sampling time but could be important in further analysis. Besides, photographs also provide information of the health status of the animal in a given time.

Another advantage of the method is the time expended to obtain the measurements. We calculated that the time used taking the photo was 1/3 of that expended in gravimetric techniques, reducing the exposure of specimens to unfavourable conditions.

Apart from photogrammetric method, in the second chapter of the thesis a scale was set between live and dry weight permitting estimations of dry weight without killing the animal. This was of vital importance in the $4^{th}$ chapter of the thesis in which metabolic measurements of individuals were recorded during three month exposure (monthly determinations) to different culture conditions and dry weight was used as a time varying covariate.

## 1.2. Fluorescent elastomer tagging method

The second technique that has been standardized in this work is tagging earthworms with fluorescent elastomers. This method has been broadly used in marine species, not only in fishes but also in soft-bodied invertebrates (Replinger & Wood 2007; Zeeh & Wood 2009; Brewer & Norcross 2012). Although in terrestrial earthworms this technique has already been employed (Gonzalez *et al.* 2006; Butt & Lowe 2007; Butt *et al.* 2009), we could not find any work that used earthworms with sizes below 100 mg. As earthworms in the initial stages of the vital cycle are more sensitive to manipulation, in this work we obtained an asymptotic pattern while confronting survival (1- mortality associated to elastomer inoculation) to the size of the individuals, observing a survival percentage of 100 in sizes over 270 mg. This allows having an idea of the initial sample necessary to carry out an experiment depending on the stage of the vital cycle of individuals in which the study is performed.

Retaining time was surprisingly high since after 3 months 75 % of individuals remained labeled. Therefore, this method allows individual monitoring of earthworms within a population and has been used in the $3^{th}$ and $4^{th}$ chapters of this thesis in which a study of the effects of isolation and population density on physiological parameters of *Eisenia andrei* was performed.

# 2. Effect of isolation and population density

## 2.1 Growth traits

Population density is an important parameter determining the growth performance of an animal in a given environment, to which the competition for food and space and the cost of reproduction limit the energy available for new tissue formation (somatic growth). The variety of works carried out in Oligochaeta analyzing the effects of the presence of co-specific individuals on growth and reproduction have been made on a population average basis due to the difficulty to distinguish individuals growing in the same culture tank (Reinecke & Viljoen 1993; Butt *et al.* 1994; Domínguez & Edwards 1997; Klok 2007; Giraddi 2008, among others). One of the major contributions of the present work is the individualized analysis of physiological responses of specimens growing in group, allowing the detection of inter-individual differences, using for this purpose fluorescent elastomers.

As has been demonstrated in the present work (Chapter 3), there is a high influence of the presence of co-specific individuals on growth traits. In group-cultured organisms only 89.65 % of specimens showed a positive growth ($\Delta$ live weight > 0) and a change in growth patterns has been observed between both culture methods: isolated worms adjusted primarily to a linear pattern, whereas grouped organisms displayed primarily asymptotic growth curves. In this line, maximal live weights (MLW, in mg) and growth rates (r, mg day$^{-1}$) were higher in individualized organisms: 1453.08 *vs.* 598.52 mg for MLW and 16.53 *vs.* 7.39 mg day$^{-1}$ for growth rate.

Many authors have reported an increment of total biomass together with a decrement in earthworm individual biomass as the population density increased (Domínguez & Edwards 1997; Klok 2007; Giraddi 2008). This finding can be partially explained by an increment of the total ingestion rate of the population but, as the food availability per individual is lower, individual ingestion rates decrease. However, a loss in total biomass when achieving the maximal stocking rate has been reported by many authors (Neuhauser *et al.* 1980; Vorsters *et al.* 1997; Ndegwa *et al.* 2000; Unuofin & Mnkeni 2014) and has also been observed in the present

work. This is of interest in industrial productive systems, such as vermicomposting, where maintaining the earthworm population density in levels of optimal or sub-optimal production rates can lead to important economic gains, favouring economical sustainability of the vermicomposting industry, with the consequent benefits for the environment.

## 2.2 Metabolism

In our research no variation in the relationship between oxygen consumption ($\mu L \ O_2 \ h^{-1}$) and dry weight (mg) has been observed for both culture conditions. Nevertheless, the elevation remained different being 2.63 and 3.19 in earthworms cultured in group or isolated respectively. This indicates that metabolic level was increased by a factor of 1.21 in isolated individuals.

Animals growing in group mature at smaller sizes (clitellum evidence) than isolated worms and weight specific oxygen consumption is higher in single cultured organisms. Weight specific metabolic rates for gonadal tissue are lower than those shown by somatic tissues in the snail *Cornu aspersum* (Rodriguez 2013) an the slug *Arion ater* (Bizarro 2010) contributing to a global reduction of metabolic cost per weight in mature *mollusca* in reproductive state. This pattern may be well extensive to *E. andrei* explaining a mass specific decay in oxygen consumption in smaller size mature worms (grouped cultured) when compared to worms kept individually. In addition, the quantity of food available per mg of earthworm tissue is lower in organisms cultured in group and can explain the depletion of metabolic level in crowded environments.

## 2.3 Scope for growth

The energy available for growth (scope for growth, P) depends on the energy derived to reproduction ($P_r$), absorbed energy (A) and metabolic cost ($R_m$): $P = A - P_r - R_m$ (Koehn & Bayne 1989).

Part of the ingested energy is sent to reproduction (matting cost, formation of cocoons, etc.) and in this work the asymptotic point of the growth curve (maximal live weight) for group-cultured organisms was related to the detention of clitellum, observing a halt in somatic tissue production when arriving to the reproductive stage. Although metabolic level of grouped specimens was

lower, the allocation of absorbed energy to reproduction and the lower food availability per individual associated with crowded environments explain the differences in growth rates and patterns between animals kept individualized or in group.

# 3. Effect of the diet

This section is divided in two parts. The first one is a global discussion about eclosion, survival and growth of hatchlings cultured in the different natural substrates used in the thesis (grass and soil from vegetation units) with the aim of having a global idea about the effects of the different diets in physiological responses of *E. andrei*. The second part of the discussion is focused only in substrates belonging to the vegetation units in an attempt to integrate results obtained for newly hatched and juvenile specimens.

## 3.1. Life cycle traits of *Eisenia andrei* in grass and soil

### 3.1.1. Recruitment

Recruitment of new individuals has been calculated as the product of the proportion of the seeding able to hatch (eclosion success) and the proportion of surviving hatchlings. As shown in Table 1, the lowest recruitment figures correspond to those incubated and cultured in grass composted for 13 weeks. The highest values are attained in Horse manure and *Eucalyptus* plantation, being the recruitment around 60 % in the rest of substrates. Eclosion failure can be principally attributed to the possible presence of toxic substances, such as polyphenolic compounds, whereas mortality, apart from the toxicity of the media, could be more linked to the lack of foodstuff in the nutritive substrates.

**Table 1**. Recruitment of *Eisenia andrei* individuals in different natural substrates.

| Substrate | Eclosion % | Survival % | Recruitment % |
|---|---|---|---|
| Horse Manure | 90 | 100 | 90 |
| Grass 1 week | 60 | 100 | 60 |
| Grass 13 weeks | 40 | 66.6 | 26.5 |
| *Eucalyptus* Plantation | 90 | 90 | 81 |
| *Quercus robur* Forest | 85 | 80 | 68 |
| Scrub | 95 | 67 | 63.6 |
| Coconut Coir | 95 | 60 | 57 |

## 3.1.2. Growth

Maximal live weights obtained by earthworms in the different substrates were analyzed by means of Tukey-Kramer test after logarithmic transformation of variables (Fig.1). No statistical differences were obtained among grass composted for 1 to 3 weeks and *Eucalyptus* plantation (a), and among grass composted for more than 7 weeks (b).
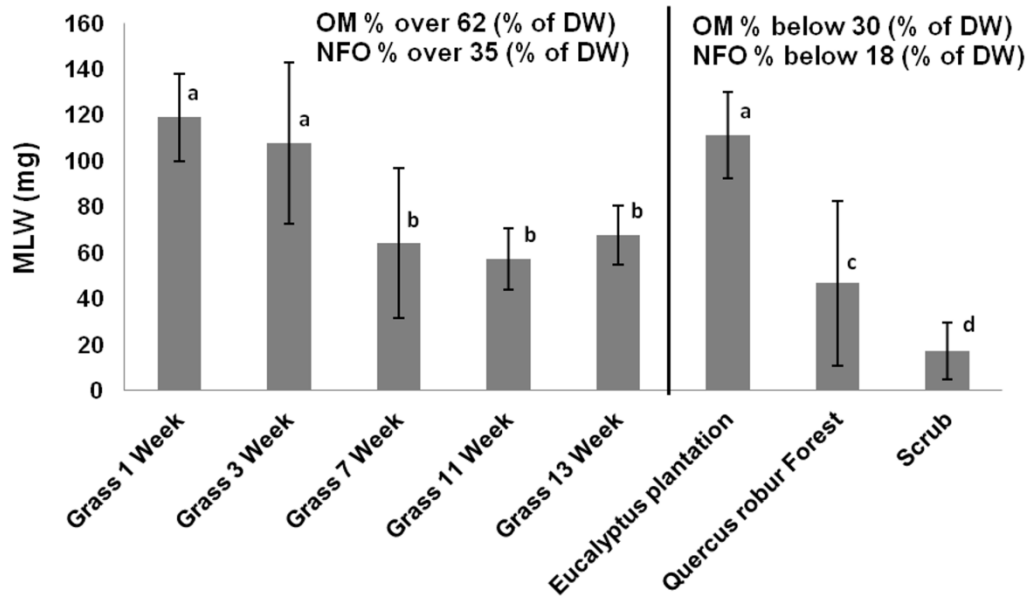


**Fig. 1**. Maximal live weights (MLW, in mg) attained by newly hatched earthworms cultured in different substrates. Left: grass composted for 1 to 13 weeks. Right: soil from broadleaved vegetation units. Bars with standard deviation are shown. OM = Organic Matter; NFO = Non-fibre Organic Matter; DW = Dry weight.

In Fig. 1 maximal live weight, as an index of growth performance, has been represented for every substrate choosing total organic matter (OM) as an indicator of the gross amount of energy available upon ingestion (quantity) and the proportion of non-fibre organic matter (NFO) to represent the amount of more readily available energy regarding digestive processes. We can see that similar growth rates have occurred for early aging grass (1 and 3 weeks) and *Eucalyptus* soil although both OM and NFO are reduced to one half in the latter.

In an attempt to understand growth differences among the two sources of substrates (grass and soil + litter) as well as the wide variations within each them, we have analyzed the chemical properties of diets considering energy intake as submitted basically to the simultaneous influence of quantity (gross availability) and quality. As above mentioned, the proportion of

total organic matter (% dry weight) has often been taken as a good approach to food quantity (Koehn & Bayne 1989). The concept of quality implying increased rates of energy absorbed at lower costs (gross absorption efficiency) appears dependent on digestive abilities of the different designs of guts in the animal kingdom. In our case, and although some benefits from the mutualistic relationship between earthworms and microbiota are evident, a considerable proportion of carbohydrates grouped under the term of fibre remains largely refractory. So, we have tentatively used the ratio between non-fibre and fibre content as an indicator of food quality. Implicitly, and as explained in chapters 5 and 6, high heterogeneity within the fibre pool with some compounds becoming more digestible than others, confers some limits to this indicator. Additionally, compensatory responses related to guarantee the intake of essential nutrients (both organic and inorganic) may be adding some noise to this simplification of guidelines to understand optimal foraging behaviour.

In Table 2 substrates have been ranked regarding maximal live weight attained by earthworms during the first month of life. Lowest growth rates registered in coconut coir substrate relates to highest quantity and lowest quality in our experiment, and absorbed energy merely satisfies costs (scope for growth approaching zero). In the upper end horse manure ranks high both in quantity and quality and mean growth rate was 4.16 mg live weight per day. In SC, FQR and EP, the organic matter availability is much lower than that found in the rest of substrates, but the quality is medium-high, observing in EP the highest quality figures. According to that indicator, quality in grass do not differs substantially among composting period (discussed later), but the organic matter availability decreased with grass mineralization.

The interaction between quality and quantity has been calculated and was confronted to the maximal size attained by individuals in the experiment (Fig. 2). Although this relation only explains partially growth patterns in our experiment, we observe a positive relation between the binomial quantity-quality and the maximal live weight achived by individuals reared in the experimental substrates.

**Table 2**. Ranking of the substrates used in the experiment regarding the mean maximal live weight (MLW, in mg) attained by individuals during one month of experiment. Substrate organic matter quantity (OM) and quality (NFO/F) are exposed. OM = Organic matter; NFO = Non-fibre organic matter; F = Fibre. Every figure calculated as proportion of dry matter.

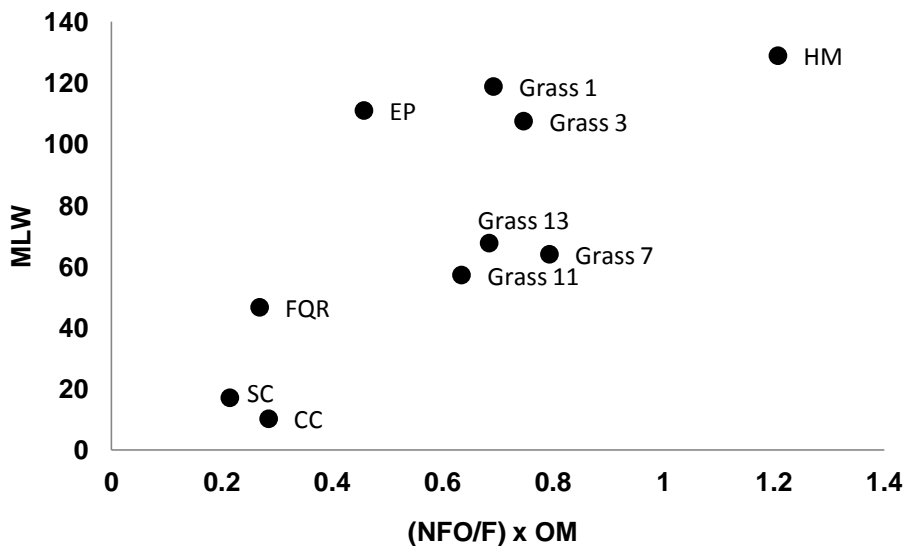| Substrate | OM | NFO/F | MLW |
|---|---|---|---|
| **CC** | 0.93 | 0.31 | 10.22 |
| **SC** | 0.21 | 1.04 | 17.09 |
| **FQR** | 0.22 | 1.23 | 46.68 |
| **Grass 11 Week** | 0.70 | 0.90 | 57.25 |
| **Grass 7 Week** | 0.78 | 1.02 | 64.04 |
| **Grass 13 Week** | 0.62 | 1.10 | 67.69 |
| **Grass 3 Week** | 0.80 | 0.93 | 107.59 |
| **EP** | 0.29 | 1.57 | 111.07 |
| **Grass 1 Week** | 0.82 | 0.84 | 118.91 |
| **HM** | 0.87 | 1.39 | 129.00 |



**Fig.2.** Maximal live weight (MLW, in mg) attained by *E. andrei* newly hatched specimens during one month permanence in experimental substrates related to the interaction term of organic matter quantity (OM) and quality (NFO/F), both as proportion of dry weight. NFO = Non-fibre organic matter; F = Fibre.

In substrates where the quantity or/and the quality of organic matter is too low, as is the case of CC and SC, growth rate of animals decreases (SC) or becomes virtually 0 (CC). In contrast, in HM in which the highest growth is attained, is characterized by presenting both high quantity and quality of foodstuff. In the case of EP, although the quantity of organic material is lower compared to grass substrates, the high quality of the litter compensates the scarcity of foodstuff, and animals grow as fast as in the initial periods of grass decomposition (1 to 3 weeks). To explain these data, we have to invoke the optimal foraging behaviour theory. In CC and SC, the absorbed energy is similar to that expended in metabolism, and the increase in the ingestion rate

would not presumably compensate the metabolic costs associated with the digestion of foodstuff, and as a consequence, the growth rate falls. In contrast, in EP, due to the high quality of the organic material, animals compensate the scarcity of organic matter probably increasing the ingestion rate, since the digestive cost are lower and the absorption efficiency is probably high. In the case of FQR, the situation is intermediate between SC and EP, with a relatively high quality of organic material that presumably allows the increment of the ingestion rate.

In grass substrates, although our indicator of litter quality displayed medium to high figures in every composting time, the quantity is reduced along grass mineralization. Moreover, as is explained in chapter 5, during grass decomposition, the easily digestible fibre components (hemicelluloses and amorphous cellulose) probably decreased, deriving in substrates increasingly enriched in lignin and crystalline cellulose, reducing digestibility of nutritive substrates for *E. andrei*. Consequently, in the grass mineralization process, the proportion of recalcitrant fibre components (% of fibre content) is higher in grass composted for longer periods of time, redounding in lower growth rates in *E. andrei* specimens. Besides, grass composted for longer periods is probably less palatable and could be responsible for the presumably lower ingestion rates.

A part from the organic matter quality and quantity of substrates, the ingestion, together with litter, of a mixture of organic and mineral material in substrates belonging to the vegetation units could be an explanation for the differences in growth rates. The presence of sand grains is known to facilitate assimilation of nutrients from organic matter in the case of litter-feeding earthworms by enhancing the grinding action of the gizzard (Marhan & Scheu 2005b). In fact, preference for substrates containing organic-mineral mixtures rather than pure organic materials has been reported, not only for anecic and endogeic but also for epigeic species of earthworms (Doube *et al.* 1997), and data from a study with *E. fetida* performed by Flack & Hartenstein (1984) suggest that grit is necessary if optimal growth is to be achieved.

In addition to the effects of nutrient bioavailability and the ingestion of mineral soil, differences in microbial community could also account for the growth divergences among substrates. Fungi are known to be a good source of food for epigeic and anecic earthworms (Curry & Schmidt

2007; Huang *et al.* 2013) and their presence in the growing medium increases the bioavailability of carbohydrates and nitrogenous compounds. Nevertheless, differences among species have been found, and in a study with different types of fungi inoculation to the growing medium, higher growth rates for *E. andrei* specimens were obtained in cultures with *Aspergillus flavus* (Pižl & Nováková 2003). Bonkowski *et al.* (2000) in an study with worms belonging to all ecological groups reported food preferences for the fungi *Fusarium nivale* and *Cladosporium cladosporioides*, concluding that this type of fungi are indicators of the presence of fresh organic resources since they are the first colonizers in organic matter decomposition processes. Nevertheless, other authors reported that fungi such as *Aspergillus* spp. and *Fusarium* spp. appear to be detrimental for earthworms (Edwards & Fletcher 1988; Morgan 1988). Overall, earthworms show food preferences for fungi that exploit carbohydrates and cellulose, rather that lignolytic species that appear later in the mineralization processes (Edwards 2004). Protozoa are considered of major importance for the epiegeic earthworms *Eisenia fetida* and are thought to be essential for their diet (Edwards & Fletcher 1988). The role of bacteria in the diet of earthworms and the extent of species-specific feeding patterns and digestion are largely unknown. In a study with the epigeic earthworm *E. fetida* Flack & Harstenstein (1984) found a significant increase in earthworms' growth rate with the addition to the culture of 22 different species of bacteria, obtaining the highest values in cultures with *Mycobacterium smegmatis* and *Proteus vulgaris*. However, Morgan (1988) found that of 12 bacterial species tested, only two allowed *E. fetida* to maintain weight. As we have exposed, mutualism relationships (see introduction chapter) between microbiota and earthworms are strongly dependent on the microbial community composition and earthworm species. Therefore, differences between both types of substrates in microbial community could be responsible for the differences in growth rates found in our work.

Further analyses are needed to confirm the discussion explained above, where a more exhaustive analysis of fibre and a characterization of the microbial community present in the growing media have to be performed. Concerning the latter, although many studies have been made, there is still too much information unknown. In this line, due to the high influence of

earthworms and soil microbiota in organic matter cycles and soil fertility, my aim, if it is possible, is to continue the research in that direction.

## 3.2. Growth of newly hatched and juvenile individuals in soil

Finally, in Chapter 6 an analysis of physiological performance of newly hatched and juvenile individuals incubated and cultured in substrates from different vegetation units (*Eucalyptus globulus* plantation, Forest dominated by *Quercus robur* and Scrub dominated by *Crataegus monogyna*) has been performed. In that chapter, influence of the rearing substrate in the relation between growth rate and initial live weight was ascertained through ANCOVA. In this part of the general discussion we want to join this information in a single analysis that covers the whole size range used in the thesis.

As the procedure followed in chapter 6, in order to avoid negative values in logarithmic transformation of data, growth rate (r) was normalized previous log transformation: r*100. As it is shown in Table 3, no differences in the slope are observed allowing the evaluation of the influence of the substrates. Intercepts remained different among substrates and growth rates followed the same pattern observed in chapter 6, with the highest growth rates for horse manure and the lowest for scrub (Fig. 3). So, irrespective of the developmental stage, growth reduction is associated with a lower quality of nutritive material present in the nutritive substrates.
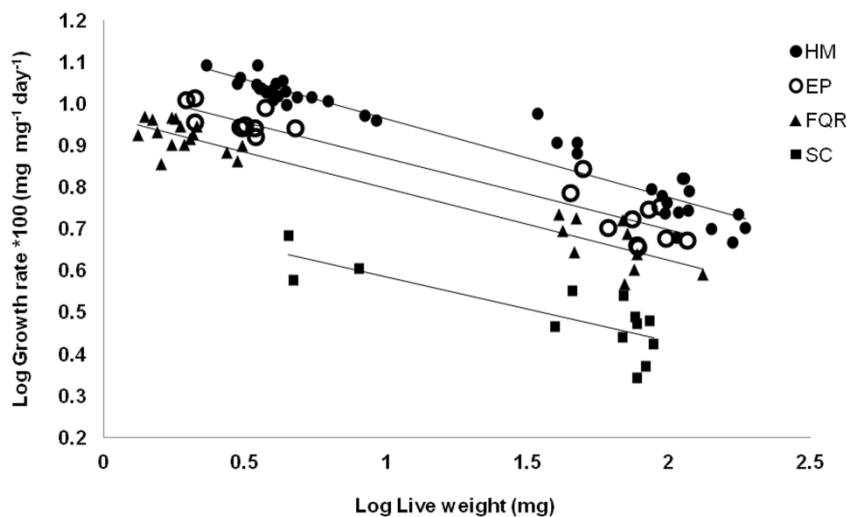


**Fig. 3**. Log growth rate *100 (mg mg$^{-1}$ day $^{-1}$) *vs.* Log initial live weight (mg) of *Eisenia andrei* individuals reared in different substrates. HM = Horse manure; EP = *Eucalyptus* plantation; FQR = *Quercus robur* Forest; SC = *Crataegus monogyna* Scrub.

**Table 3.** Coefficients of Log-Log regression equations of growth rate (mg/mg/day*100) *vs*. Live Weight (mg) of *Eisenia andrei* for different substrates. Differences in individual equation for growth rate between substrates are analyzed through ANCOVA. HM= Horse manure; EP= *Eucalyptus* Plantation; FQR= *Quercus robur* Forest; SC= *Crataegus monogyna* Scrub; DF= degrees of freedom; $CI_{95\%}$= Confidence intervals. Size range in mg of live weight. Intercept $_r$ = Recalculated value of the elevation for the common slope.

| Substrate | n | Size range (mg) | $R^2$ | Intercept ($CI_{95\%}$) | Slope ($CI_{95\%}$) | Intercept $_r$ |
|---|---|---|---|---|---|---|
| **HM** | 38 | 2.30 - 187.00 | 0.92 | $1.152 \pm 0.02$ | $-0.189 \pm 0.02$ | 1.139 |
| **EP** | 20 | 1.95 - 116.15 | 0.92 | $1.041 \pm 0.03$ | $-0.172 \pm 0.03$ | 1.049 |
| **FQR** | 26 | 1.32 - 132.17 | 0.92 | $0.971 \pm 0.02$ | $-0.173 \pm 0.02$ | 0.975 |
| **SC** | 13 | 4.40 - 88.46 | 0.65 | $0.739 \pm 0.10$ | $-0.154 \pm 0.08$ | 0.777 |
| **ANCOVA** | DF | F-value | P-value | Common values | | |
| **Slope** | 3,89 | 0.8680 | 0.4607 | -0.178 | | |
| **Intercept** | 3,92 | 260.59 | < 0.0001 | | | |

# Chapter 8

## 8. General conclusions

# General conclusions

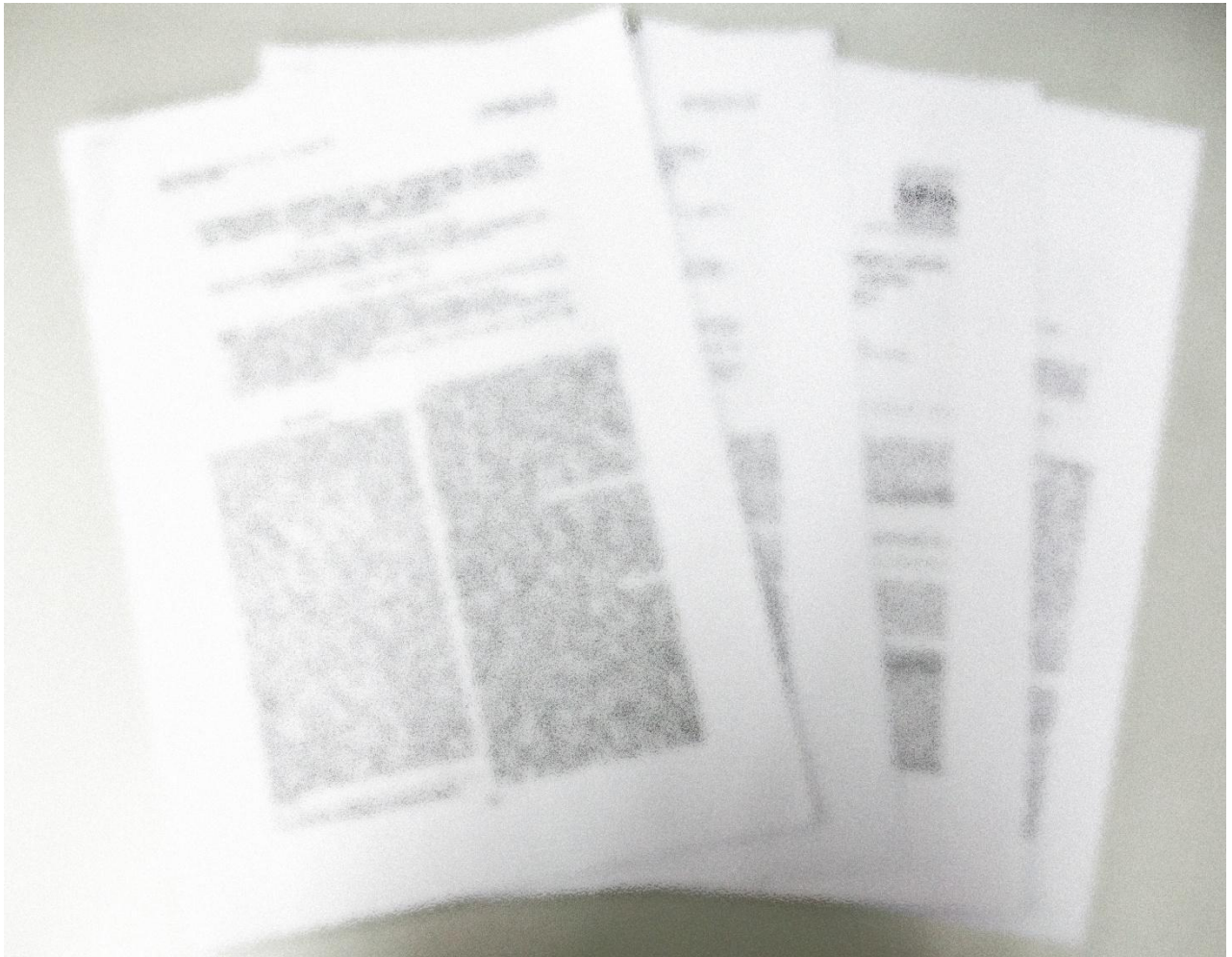**1.** Images taken without special requirements can be a good tool to assess biomass of soft-bodied tubular organisms within a wide size range (starting on eight mg live weight) and this regardless of shape (body position) and number of pictures taken. Water loss derived from starvation implies loss of turgidity and flattening and, consequently, larger proportion of surface exposure to camera lens (a factor of 1.136).

No differences between laboratory or field method appear when size exceeds 8 mg live weight and a common linear function relating area to live weight with a weight exponent of 0.681 and an elevation of 3.27 results. Below 8 mg weight exponent remains unchanged but the value for the elevation increases to 4.21 (factor increase 1.287).

**2.** Concerning tagging method, survival to elastomer inoculation was related to the size of the individuals by an asymptotic equation explaining 93 % of the variability and total survival was reported in individuals larger than 270 mg. No effect of number of punctures on survival was detected and, after 3 months 75 % of individuals remained labeled.

**3.** There is a high influence of the presence of co-specific individuals on growth traits of *E. andrei*. In group-cultured organisms only 89.65 % of specimens showed a positive growth (Δ live weight > 0) and a change in growth patterns has been observed between both culture methods: isolated worms adjusted primarily to a linear pattern, whereas grouped organisms displayed primarily asymptotic growth curves. In this line, maximal live weights (MLW, in mg) and growth rates (r, mg day$^{-1}$) were higher in individualized organisms: 1 453.08 *vs.* 598.52 mg for MLW and 16.53 *vs.* 7.39 mg day$^{-1}$ for growth rate. In group-cultured organisms, asymptotic point was related to detection of clitellum and, initial density increases over the experimental time according to an asymptotic function indicating that 111.27 mg live weight / g dry horse manure would be the maximum density the population can support.

4.  The effect of body mass on metabolic expenditure has been exposed in this work and a common exponent of 0.66 was obtained irrespective of the culture conditions (grouped or isolated), observing lower metabolic rates with increasing in size. The study also demonstrates that the presence of co-specific individuals produces a decrement in metabolism (elevation for group-cultured 2.63; elevation for isolated 3.19), explained by the earlier development of reproductive structures and by the intraspecific competition for food and space that take place in group-cultured organisms.

5.  Regarding the experiment with grass composted for different periods, survival of hatchlings decreased linearly with composting period of grass and two groups became evident regarding maximal size attained: 120 mg in grass composted for 1 to 3 weeks (OM % over 80) *vs.* 60 mg in grass composted for more than 7 weeks (OM % below 77.7). From our results, standing crop of *E. andrei* in grass litter appears determined by the proportion of readily available carbohydrates extracted from plant cells by the assemblage between earthworms and soil microbiota. Impoverishment of quality associated to aging appears linked to the decline of hydrated, readily extractable sugars within the fibre pool redounding in decreasing survival and growth.

6.  In substrates sampled from the three vegetation units (with two control substrates: HM and CC), substrate respiration ($\mu LO_2$ $h^{-1}$ $g^{-1}$), with the exception of HM, was negatively related to the proportion of fibre (% of organic matter), and the highest respiratory rate was recorded in HM (279.18), followed by EP (32.43), FQR (21.31), SC (9.66) and CC (~ 0). In this line, survival and growth of *E. andrei* individuals were positively related to substrate respiration in both developing stages.

    A decrease of the non-fibre organic content (% of dry matter) of substrates and an increase of the proportion of their fiber content (% of organic matter) would account for the lower digestibility of the organic matter for both soil microorganism and earthworms. In substrates with high microbial biomass, degradation of the most refractory pool of organic matter would be enhanced and also provides food in the form of microorganisms. So, a decrease of the proportion of easily metabolizable components in soil, with a concomitant reduction in soil microbial content, would reduce live expectancy and growth of *E. andrei*.

# Chapter 9

## 9. References

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# Annex 1

## A1. Resumen general

# Resumen general

La especie de lombriz sujeta al estudio presentado en esta tesis es *Eisenia andrei* (Bouché 1972), también conocida con el nombre de lombriz roja. Pertenece a la familia Lumbricidae, una de las familias más importantes de lombrices de tierra. Es una lombriz de pequeño tamaño, presentan un cuerpo cilíndrico con simetría bilateral, y se encuentra externamente dividida por segmentos a lo largo del cuerpo, coincidiendo con los septos que dividen al animal internamente (Edawards & Lofty 1977; Brusca & Brusca 2003). Son hermafroditas, generalmente con una reproducción sexual cruzada (Edwards & Lofty 1977; Domínguez *et al.* 2003). Al llegar la madurez sexual, se produce un ensanchamiento corporal denominado clitelo. Muestra altas tasas de crecimiento y son muy prolíficas (Domínguez *et al.* 2003; Toccalino *et al.* 2004; Domínguez & Gómez-Brandón 2010). Todas estas características hacen que sea una de las especies más utilizadas en la industria del vermicompostaje, en donde el efecto de la densidad poblacional se encuentra maximizado. Por otro lado, al igual que el resto de lombrices de tierra, tienen gran interés conservacionista, por su implicación en los ciclos de materia orgánica y su fuerte impacto positivo sobre la fertilidad del suelo. Luego el mantenimiento de poblaciones activas de lombrices en el medio edáfico, ayuda a preservar y/o restaurar la salud del suelo (Lavelle *et al.* 1989; Jouquet *et al.* 2014).

En este contexto, esta tesis está dividida en tres secciones principales. En el primer bloque se han desarrollado dos técnicas que permitirán la consecución de los demás objetivos. El segundo bloque, trata acerca de la influencia de la densidad poblacional sobre los parámetros fisiológicos de los individuos de *E. andrei*. En la última sección de la tesis se aborda la cuestión de los efectos de la dieta, en términos de calidad y cantidad de materia orgánica, sobre el ciclo de vida de estos individuos lo cual nos permitirá

conocer los requerimientos nutricionales de la especie y poder aplicar estos conocimientos en estrategias de restauración y conservación de suelo.

## Bloque 1: Técnicas

### Método fotogramétrico

Se ha desarrollado un método de estimación de medidas de biomasa en organismos tubulares basado en técnicas fotográficas. El objetivo de este trabajo es estandarizar las condiciones que permitan una cuantificación fiable de medidas de biomasa, longitud, superficie, volumen y grado de hidratación en estudios a largo plazo. Las fotografías de los animales se pueden obtener con cámaras digitales de bajo coste y el número de fotografías por individuo que deben ser tomadas puede reducirse a dos (C.V 3.5 %) incluso en individuos recién nacidos (~10 mg). Para el análisis de imagen se ha utilizado el programa CobCal 2.0©. La relación entre la superficie fotografiada (PS) y el peso vivo (LW) es independiente de la posición del animal. Sin embargo, la pérdida de agua a consecuencia del ayuno implica que los animales se encuentren más aplanados, por lo que presentan una proporción mayor de área expuesta a la cámara (un factor de 1.136). No se han encontrado diferencias entre los dos métodos que se han desarrollado (laboratorio y campo) por lo que se ha establecido una función común que relaciona el área de la foto con el peso vivo ($PS = 3.27*LW^{0.681}$). Por debajo de 8 mg, el exponente del peso se mantiene pero el valor del coeficiente aumenta a 4.21. Como conclusión, se ha demostrado que este método no invasivo puede ser empleado en individuos entre 0.2 a 3000 mg de peso vivo, para determinar parámetros biométricos como la longitud, el volumen, la superficie o el peso vivo, que son factores clave para interpretar las respuestas fisiológicas subyacentes a los patrones de crecimiento.

**Método de marcaje**

Nuestro objetivo en este estudio ha sido la estandarización del uso de los elastómeros fluorescentes como método de marcaje en individuos de *E. andrei*. Para ello se marcaron 87 individuos juveniles y adultos con tallas comprendidas entre 27 y 1702.65 mg de peso vivo. Como control, 40 individuos recién nacidos sin marcar se mantuvieron individualizados durante 160 días. En cuanto al tiempo de permanencia del elastómero, después de un periodo de tres meses el 75 % de los individuos continuaban marcados. No se encontró efecto del número de pinchazos sobre la supervivencia. Sin embargo, la mortalidad asociada al método de marcaje es dependiente de la talla del individuo: de 110 a 270 mg la supervivencia aumenta de 45 a 80 %, alcanzando valores del 100 % en individuos mayores que 270 mg. Este método permite el seguimiento individualizado de animales dentro de una población pudiendo trabajar con todas las etapas del ciclo vital (desde 27 mg de peso vivo). Esta técnica, resulta interesante en estudios de densidad y dinámica poblacional, en experimentos de comportamiento o incluso en ensayos de ecotoxicidad.

## Bloque 2: Efecto del aislamiento y densidad poblacional

**Efecto de la densidad poblacional sobre el crecimiento**

Los muchos trabajos realizados con *E. andrei* acerca del efecto de la densidad poblacional sobre su crecimiento y reproducción utilizan criterios de valoración globales en el que los parámetros poblacionales actúan como descriptores. Dada la presumible existencia de variaciones interindividuales con respecto al crecimiento tanto somático como gonadal, en este trabajo se ha efectuado un seguimiento individualizado de cada individuo dentro de la población en un esquema de mesocosmos, utilizando la técnica de marcaje para el seguimiento de los individuos mantenidos en grupo. Para ello, 33 individuos marcados se repartieron en tres cajas de 1100 cm$^3$ (11 individuos por caja) obteniendo una densidad

inicial media de 56.84 mg de peso vivo por gramo de bosta de caballo seca. Por otro lado, para poder realizar un estudio comparativo, 18 animales fueron individualizados en cajas de 450 cm³. Los animales fueron mantenidos en oscuridad, a temperatura y humedad constante (20 °C; 80 % RH), durante un periodo de tres meses, tomando medidas de biomasa, mediante técnicas fotogramétricas, cada 3-4 días. Los resultados revelan que los individuos mantenidos en grupo mostraban principalmente un crecimiento asintótico (50%), mientras que los individualizados se regían principalmente por un patrón de crecimiento rectilíneo (~50%). El peso máximo alcanzado por los animales en mesocosmos contrasta con el de los individualizados: 552 mg *vs.* 1453 mg, respectivamente. El peso máximo en los individuos en grupo (AP) se relaciona con el peso en el momento de adquisición del clitelo ($AP = 63.759* e^{(0.005LWC)}$; $R^2$=0.897). La densidad inicial aumenta a lo largo del periodo experimental mediante una función asintótica ($Density = (111.27-55.102)*(1-e^{(-0.032Time)}) + 55.102$; $R^2$=0.845) que indica una capacidad de carga de 111.27 mg de peso vivo por gramo de bosta de caballo seca.

### Efecto del aislamiento sobre el metabolismo

La tasa metabólica es una medida del coste de la vida que está influenciada por factores tanto endógenos como exógenos. En este trabajo, nos hemos centrado en el peso del animal como factor endógeno principal, mientras que la densidad poblacional, entendida como los efectos añadidos de competencia por los recursos y el comportamiento reproductivo, se ha escogido como una mezcla de presiones endógenas y exógenas. En este marco, el propósito de este trabajo ha sido analizar si el metabolismo respiratorio (consumo de oxígeno, en $\mu L$ $O_2$ $h^{-1}$ $animal^{-1}$) en *E. andrei* es dependiente de las condiciones de cultivo (en grupo o individualizados) introduciendo el peso del animal como covariable. En nuestro diseño experimental, se han tomado medidas de consumo de oxígeno una vez al mes de 51 individuos: 18 individualizados con una densidad

inicial de 4 mg de peso vivo por gramo de bosta de caballo seca; y 33 en grupo cuya densidad inicial era de 56.84 mg de peso vivo por gramo de bosta de caballo seca. Los animales fueron mantenidos en oscuridad, a temperatura y humedad constante (20 °C; 80 % RH), durante un periodo de tres meses, tomando medidas de biomasa, mediante técnicas fotogramétricas, cada 3-4 días. Los resultados muestran un exponente del peso común en ambas condiciones de cultivo de 0.66 y un incremento del nivel metabólico por un factor de 1.21 en los animales individualizados. Esta disminución en el nivel metabólico de los animales creciendo en grupo se asocia a que presentan una maduración temprana, en términos de talla, de las estructuras reproductivas y a la competencia intraespecifica por los recursos alimenticios.

## Bloque 3: Efecto de la dieta

### Ciclo de vida de *Eisenia andrei* en hierba pre-compostada

Las lombrices epigeas juegan un papel fundamental en el mantenimiento de la fertilidad de suelo por su habilidad para degradar el aporte espontáneo de material vegetal y los desechos de las prácticas de siega en los ecosistemas agrarios. En este contexto, en un intento por entender las capacidades de colonización y reclutamiento de esta especie en este tipo de ecosistemas, hemos analizado la eclosion, supervivencia y crecimiento de individuos recién nacidos de la especie *Eisenia andrei* en hierba cortada y compostada por distintos periodos de tiempo. Para este propósito, se seleccionaron 5 substratos de hierba en distinto grado de descomposición (1, 3, 7, 11 y 13 semanas), utilizando bosta de caballo como substrato control. En este esquema, los capullos y recién nacidos fueron individualmente incubados en los distintos substratos, en oscuridad, a temperatura y humedad constante (20 °C and 80 % RH). Se realizó el análisis bioquímico de los substratos para poder tener una aproximación a la calidad del substrato: el incremento en la mineralización y decremento en el porcentaje de fibra

asociados al envejecimiento de la hierba reducía el éxito en la eclosión, la supervivencia y el crecimiento de los individuos de esta especie. La supervivencia presentaba una relación lineal positiva con el contenido de materia orgánica del substrato, mientras que los pesos máximos alcanzados descienden de ~120 mg (Bosta de caballo y hierba compostada por 1 y 3 semanas) a ~60 mg (hierba compostada por 7, 11 y 13 semanas). Esta reducción de la supervivencia y el crecimiento a pesar de que el contenido orgánico siga siendo alto (62.02 %-77.7 %), puede ser explicada por la disminución de la proporción de carbohidratos fácilmente asimilables dentro de la fibra, incrementándose así los costes de procesamiento del alimento y reduciéndose su eficiencia de absorción.

**Ciclo de vida de *Eisenia andrei* en suelo perteneciente a tres tipos de vegetación**

Dada la implicación de los organismos edáficos en la preservación de la salud del suelo, en este trabajo se ha analizado la idoneidad de suelo perteneciente a tres diferentes unidades de vegetación (plantacion de *Eucalyptus globulus* "EP", bosque dominado por *Quercus robur* "FQR" y matorral dominado por *Crataegus monogyna* "SC") para sostener poblaciones activas de la lombriz epigea *Eisenia andrei*, fijándonos en su valor nutricional. Se emplearon dos substratos control: bosta de caballo (HM) y corteza de coco (CC). Al igual que en el apartado anterior, se analizó la eficiencia en la eclosión, supervivencia y crecimiento de individuos recién nacidos. Así mismo, para tener una idea global del reclutamiento de individuos reproductores, se analizó en un segundo experimento el crecimiento de individuos juveniles de *E. andrei*. El análisis de las características iniciales de los substratos revelaba los mayores porcentajes orgánicos en CC (93 %) y HM (86 %), contrastando con porcentajes de 29, 22 y 20 en EP, FQR y SC, respectivamente; sin embargo, los valores menores para la respiración del suelo fueron encontrados en CC. En esta línea, con la excepción de HM, se estableció una relación negativa entre la respiración del substrato ($\mu LO_2$ $h^{-1}$ $g^{-1}$) y su proporción de

fibra (% con respecto a la materia orgánica). En cuanto a la eficiencia en la eclosión, se encontraron diferencias significativas en cuanto al peso inicial de los recién nacidos y a la biomasa total por capullo, hallándose en CC y SC los valores más altos, lo cual se explica por el aumento en el tiempo de residencia de los no-natos dentro del capullo en ambientes poco favorables. El porcentaje de supervivencia variaba entre los distintos substratos obteniéndose valores de 100 %, 90 %, 80 %, 67 % y 60 % en HM, EP, FQR, SC y CC, respectivamente. De igual modo, los patrones de crecimiento también variaban entre los substratos, encontrando tallas máximas de 247, 117, 56, 19 y 10 mg en HM, EP, FQR, SC y CC, respectivamente para los recién nacidos y 1473, 881, 479, 282 y 100 en HM, EP, FQR, SC y CC, respectivamente para los juveniles. La supervivencia y el crecimiento se relacionan positivamente con el porcentaje de materia orgánica, con la excepción de CC, y con la respiración del substrato ($\mu LO_2$ $h^{-1}$ $g^{-1}$). En términos generales, el descenso de los componentes no fibrosos (% de peso seco) y el aumento de la proporción de fibra con respecto a la materia orgánica, hace que disminuya la digestibilidad de los componentes orgánicos tanto para los microorganismos del suelo como para las lombrices, produciendo una disminución de la esperanza de vida y del crecimiento en *E. andrei*.

# Annex 2

## A2. Contributions of this thesis

# Contributions of this thesis

## Scientific contributions

- *Biomass assessment in annelids: A photogrammetric method suitable for hatchlings and adults developed in Eisenia andrei.*(Submitted)

- *Life cycle traits of Eisenia andrei dwelling in aging grass: An approach to food quality of litter for epigeic earthworms.* (Submitted)

- MSc Thesis: *"Crecimiento individual de Eisenia andrei Bouché (1972) en mesocosmos a bajas densidades: marcaje con elastómeros y análisis de imagen"*. 2011-2012.University of the Basque Country. Financed by the Basque Government.

## Presence at scientific conferences

- Poster: "Crecimiento individual de *Eisenia andrei* (Bouché, 1972) en mesocosmos". IV Congreso de Biodiversidad. Bilbao. 2013. Authors: Esther Iparraguirre-Bolaños, Carlos Brea San Nicolás & M. Mercedes Ortega Hidalgo.

- Oral presentation: "Evaluación del crecimiento individual de *Eisenia andrei* en sustrato natural a baja densidad". IV Jornadas de Investigación de la Facultad de Ciencia y Tecnología. Leioa. 2014. Authors: Esther Iparraguirre-Bolaños & M. Mercedes Ortega Hidalgo.

- Poster: "Analysis of individual growth patterns in juveniles of *Eisenia andrei* grown on horse manure". First Global Soil Biodiversity Conference. Dijon, France. 2014. Authors: Esther Iparraguirre-Bolaños & M. Mercedes Ortega Hidalgo.

- Oral presentation: "Zizare Earthworm Lab. Educating soil ecology at preschool". First Global Soil Biodiversity Conference. Dijon, France. 2014. Authors: Oreina Orrantia & Esther Iparraguirre-Bolaños. http://www.globalsoilbiodiversity.org/?q=SoilBiodiversityEducation.

- Oral presentation: "Photogrammetric techniques applied to morphometric studies in soft-bodied invertebrates". XVth Spanish Biometric Conference and V[th] Ibero-American Biometric Meeting. Bilbao. 2015. Authors: M. Mercedes Ortega Hidalgo, Marta Barrenetxea & Esther Iparraguirre-Bolaños.

- Oral presentation: "Analysis of food quality of litter for epigeic earthworms: *Eisenia andrei* as a case study". V Jornadas de Investigación de la Facultad de Ciencia y Tecnología. Leioa. 2016. Authors: Esther Iparraguirre-Bolaños, Ander Villanueva Alonso y M. Mercedes Ortega Hidalgo.

- Oral presentation: "An approach to biological quality of pasture lands: viability of hatchlings of *Eisenia andrei* in aging grass". Jornadas doctorales de la UPV. Bilbao. 2016. Authors: Esther Iparraguirre-Bolaños & M. Mercedes Ortega Hidalgo.

- Poster: "An approach to biological quality of pasture lands: viability of hatchlings of *Eisenia andrei* in aging grass". 5th Interrnational EcoSummit Ecological Sustainability Engineering change. Montpellier, France. 2016. Authors: Esther Iparraguirre-Bolaños & M. Mercedes Ortega Hidalgo.