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Tenebrio molitor for food or feed

Rearing conditions and the effect of pesticides on its performance

Nuno Tiago Garrucho Martins Ribeiro

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Abstract

Tenebrio molitor is one of the most reared species of insect at industrial scale. The entire life cycle of mealworms takes place in the same ecosystem and the duration of the different stages is highly dependent on environmental and physical conditions such as temperature, relative humidity, diet and population density. Most of the environment conditions as well as diet have already been studied and are documented.

Tenebrio molitor is deeply adaptable to extreme dry conditions, obtaining water from both food ingestion (even substances with low water content) and/or atmosphere, however larvae grows faster in moist conditions upper than 70%. At the industrial scale, water can be provided as pure water, through wet paper, fresh vegetables with high water content or even polymers capable of absorbing water (polyacrylamide). Chapter 1 is a review of the optimal conditions for *Tenebrio molitor* rearing. In Chapter 2, the effects of relative humidity and the effects of ingesting fresh vegetables contaminated with pesticides and polyacrylamide are evaluated. Relative humidity affected the development of mealworm larvae, with higher pupation and body mass attained at 65% and with faster growth rates attained at 40%. No effects of pesticides were detected. However, the chemical analysis of body mass will allow knowing if there was bioaccumulation.).

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1 Introduction

The world agriculture lived, between 1870 and 2000, an *Agriculture Treadmill,* a period characterized by a high food production rate at declining prices for an increasing number of humans. However, at the turn of the millennium this period came to an end (Von Witzke and Noleppa, 2007). The prices on the world market for agricultural goods have been rising since the early 2000s because the increase in global demand exceeds the global supply, and this trend will continue in the foreseeable future. The demand for food will continue to grow, mainly due to two reasons. On the one hand, due to the continuing growth of the world population, expected to reach 9.3 billion people by 2050. On the other hand, due to the raising of living standards in newly industrialized countries with the consequent increase of per capita food (FAO, 2009).

In addition to human consumption pressures on agriculture production, the demand for commonly used protein sources for feed, such as fishmeal and soybean, is also increasing. Soybean is used for food and feed but is also as feedstock for the energy industry (biodiesel). However, soybean cultures are highly dependent on weather and lead to environmental issues related with deforestation and pesticides utilization. The demand for fishmeal, essentially used for feed, has been increasing due to intensification of aquaculture in Asia. Thus, the search for alternative protein sources which are appropriate, environmentally sustainable and financially viable for use in feed industry will be the most critical factor in the development of intensive exploration in the future where, alongside algae and pulses, insects are foreseen as one of the best options.

Insects, the largest group of living organisms, are known as human food at several parts of the world (DeFoliart, 1997). Insect rearing as a novel source of protein both to human food and animal feed has been gaining followers and several production projects at an industrial scale already exist. Insects can grow and reproduce quickly and easily, have high feed conversion efficiency while contribute to food and feed security (van Huis et al., 2013).

Among several species that can be reared at an industrial scale, *Tenebrio molitor* (mealworm), a species of darkling beetle (order Coleoptera), is one of the most used due to the relative ease of culturing. However, its culture requires much manpower and there is a need to optimize and automatize the process of cultivation in order to allow competitive prices.

At artificial rearing, mealworms are commonly fed with wheat bran with protein supplementation. For drinking, vegetables are the best option, since *T. molitor* can intake water without the problems involved with fresh water supply, i.e., drowning or mold development in substrate. The drawback to the use of vegetables as a source of water is the possibility of containing pesticides residues and the potential bioaccumulation in larvae tissues which may have effects on humans or animals feeding on mealworms.

This work is composed of a scientific review of *Tenebrio molitor*'s environmental, physical and diet conditions during rearing (Chapter I), and an experimental research on the potential effects of humidity and pesticide-contaminated diet on the performance of mealworms (Chapter II).

2 Objectives

The main objectives of this work are:

- to review the *Tenebrio molitor* optimal rearing conditions and the influence of those conditions on the nutritional quality of the insect;
- to study the effects of relative humidity rearing conditions, polyacrylamide as a water source, and the presence of residual concentrations of the pesticides Deltamethrin, Manacozeb, and Metribuzin in vegetables on *T. molitor* development and survival.

3 Chapter I – Optimal conditions for *Tenebrio molitor* rearing: a review

3.1 Introduction

It is widely recognized and accepted that, maintaining the trend observed in the world population growth and resources consumption it will be imperative to pursue new sources of human food and feed to preserve the stock of natural resources and sustainable development. Since the publication of "Edible insects - Future prospects for food and feed security", by FAO (van Huis et al., 2013), the idea of rearing insects for food and feed is a growing tendency all over the world there are already several start-up companies that bet on this new source of protein as business opportunity.

From hundreds of thousands existing insect species, only a few have been reared to be used as potential protein sources: black soldier fly, common housefly, termites, silkworms, grasshoppers, locusts and yellow mealworm (*Tenebrio molitor*), under more industrialized or more home-made scales. *Tenebrio molitor* is easy to culture and many small farmers have been rearing it in Europe and elsewhere, either for pet feeding, fishing bait or human consumption.

Insect use in feed in food will only be a reality if production costs are competitive when compared to other protein sources, such as soybean and fishmeal for feed or other vegetable and animal protein for food. The decrease of insect protein and insect fat production costs requires automation of insect rearing and optimal growth conditions.

This review presents an overview of studies that identify minimum and optimal environmental, physical and diet conditions.

3.2 Biology and Life Cycle

Tenebrio molitor is a species of the Tenebrionidae Family, commonly known as Darkling Beetles, wherein life cycle is characterized by 4 stages of development, egg, larvae, pupae and adult. This cosmopolitan beetle feeds primarily on farinaceous materials, and is usually found in flour mills, barns, etc., being considered a pest.

The females lay up an average of 400-500 eggs (Cotton, 1927; Hardouin and Mahoux, 2003; Hill, 2002; Manojlovic, 1987; Spencer and Spencer, 2006) singly or in small clusters, that are attached to the substrate or the walls and floor of the containers where they are bred. After a period varying between 4 days at 26-30 ºC and 34 days at 15 ºC (Kim et al., 2015), tiny larvae emerge from the eggs and the larval stage begins. This period is reported to have a duration varying from 57 days in controlled conditions (Weaver and McFarlane, 1990) to 629 days in nature (Cotton, 1927) with an average time of 112 – 203.3 days (Martin, Rivers and Cowgill, 1976; Miryam, Bar and Oscherov, 2000). Shorter durations of the entire lifecycle of *T. molitor* have been reported by (Spencer and Spencer, 2006) and (Hardouin and Mahoux, 2003) with 75 and 90 days, respectively. During the larval stage the larvae undergo several molts, varying from a minimum of 9 (Cotton, 1927; Hill, 2002) to a maximum of 23 (Ludwig, 1956), with an average of 11-19 instars (Ludwig, 1956; Miryam et al., 2000).

After the larval stage, the larvae begin a short period of latency gaining a “C” shape before turning into a pupa, condition in which metamorphosis takes place. The pupa state takes 6 (Cotton, 1927; Ghaly et al., 2009) to 20 days (Hill, 2002). The young adults emerge as white, soft exoskeleton beetles, gradually darkening, and are able to start oviposition in about 3 days after emerging (Manojlovic, 1987). The adult stage of *Tenebrio molitor* is reported to last from 16 to 173 days (Miryam et al., 2000), with an average of 31.8 (Urs and Hopkins, 1973) to 62 days (Miryam et al., 2000).

The entire life cycle takes place in the same ecosystem and the duration of the different stages is highly dependent on environmental and physical conditions such as temperature, relative humidity, diet and population density that involve mealworm rearing, as can be concluded from the above mentioned data (Table 1).

Table 1 -Biological and lifecycle parameters of *Tenebrio molitor*

		Minimum	Average	Maximum
Number of eggs		**77** (Cotton, 1927) **250** (Ghaly et al., 2009)	**250** (Spencer and Spencer, 2006) **276** (Cotton, 1927) **280** (Hardouin and Mahoux, 2003) **414** (Manojlovic, 1987) **400-500** (Ghaly et al., 2009)	**500** (Hill, 2002) **576** (Cotton, 1927) **1000** (Ghaly et al., 2009)
Length (mm)	**Larvae**	**20** (Ghaly et al., 2009) **28** (Hardouin and Mahoux, 2003)	**16** (Barker et al., 1998) **28** (Hill, 2002)	**25** (Ghaly et al., 2009) **31.6** (Park et al., 2014) **32** (Hardouin and Mahoux, 2003)
	Adult	**12** (Hill, 2002) **15.5** (Kim et al., 2015)	-	**16** (Hill, 2002; Kim et al., 2015)
Body mass (mg)	**Larvae**	**75** (Martin et al., 1976) **130** (Makkar et al., 2014) **140** (Tschinkel and Willson, 1971)	**111** (Martin et al., 1976) **120** (Connat et al., 1991) **126** (Finke, 2002) **191 220** (Kim et al., 2016)	**134** (Ramos Elorduy et al., 2002) **145** (Martin et al., 1976) **160** (Makkar et al., 2014) **182.7** (Ghaly et al., 2009) **190** (Tschinkel and Willson, 1971) **220** (Kim et al., 2016)
	Adult	-	**136** (Finke, 2002)	-
Duration (days)	**Lifespan (complete cycle)**	**75** (Spencer and Spencer, 2006) **90** (Hardouin and Mahoux, 2003) **181** (Urs and Hopkins, 1973) **280** (Hill, 2002)	**80-83.7**(Park et al., 2012) **189** (Urs and Hopkins, 1973) **294** (Miryam et al., 2000)	**90** (Spencer and Spencer, 2006) **120** (Hardouin and Mahoux, 2003) **196** (Urs and Hopkins, 1973) **630** (Hill, 2002)
	Egg stage	**4** at 26-31 ºC (Cotton, 1927) **4-6** (Siemianowska et al., 2013) **5** at 35 ºC (Kim et al., 2015) **7** (Miryam et al., 2000) **10** (Hill, 2002)	**7** (L. Li et al., 2016)(Hardouin and Mahoux, 2003) **7.55** at 25 ºC (Manojlovic, 1987) **7-8** (Park et al., 2014) **9.2** (Miryam et al., 2000) **12.6** at 20 ºC (Manojlovic, 1987) **15** (Ghaly et al., 2009)	**10** (Spencer and Spencer, 2006) **12** (Hill, 2002) **15** (Miryam et al., 2000) **19** at 18-21 ºC (Cotton, 1927) **34** at 15 ºC (Kim et al., 2015)
	Larval stage	**57** (Weaver and McFarlane, 1990) **87.7** (Urs and Hopkins, 1973) **110.8** at 30 ºC (Koo et al., 2013)	**112** (Martin et al., 1976) **120** at 25 ºC (Ludwig, 1956) **151.15** (Urs and Hopkins, 1973)	**202.5** (Urs and Hopkins, 1973) **216** (Kim et al., 2015; Morales-Ramos et al., 2015a) **240** (Ghaly et al., 2009)

		Minimum	Average	Maximum
Duration (days)	**Larval stage**	**120** (Hill, 2002) **127** (Kim et al., 2015) **129** (Morales-Ramos et al., 2015a) **143** (Morales-Ramos et al., 2010) **180** (Ghaly et al., 2009) **280** in nature , (Cotton, 1927)	**173** at 30 ºC (Ludwig, 1956) **180** (Cotton, 1927) **203.3** (Miryam et al., 2000) **241.9** (Park et al., 2014)	**244** at 17 ºC (Koo et al., 2013) **540** (Hill, 2002) **629** in nature (Cotton, 1927)
	Pupal stage	**6** at 27 ºC (Cotton, 1927) **6** at 28 ºC (Ghaly e Alkoaik, 2009) **6.51** at 27.5 ºC (Kim et al., 2015) **7.7** (Urs and Hopkins, 1973)	**5-6** (Siemianowska et al., 2013) **7** (Hardouin and Mahoux, 2003) **7.3** (Morales-Ramos et al., 2010) **7.6** (Miryam et al., 2000) **8.9** (Urs and Hopkins, 1973) **9** (Hill, 2002)	**10.1** (Urs and Hopkins, 1973) **18** at 18 ºC (Cotton, 1927; Ghaly et al., 2009) **20** (Hill, 2002; Kim et al., 2015)
	Adult	**16** (Miryam et al., 2000) **25** (Urs and Hopkins, 1973) **30** (Hill, 2002) **37** (Ghaly et al., 2009) **38** (Cotton, 1927)	**31.8** (Urs and Hopkins, 1973) **61.5** (Cotton, 1927) **62.05** (Miryam et al., 2000)	**60** (Hill, 2002) **94** (Cotton, 1927) **96** (Ghaly et al., 2009) **105** (Urs and Hopkins, 1973) **173** (Miryam et al., 2000)
Number of larval instars		**9** (Cotton, 1927; Hill, 2002) **11** at 25 ºC (Ludwig, 1956) **12.2** (Urs and Hopkins, 1973) **14** (Morales-Ramos et al., 2015a) **15** at 30 ºC (Ludwig, 1956)	**11** (Miryam et al., 2000) **13** (Koo et al., 2013) **13.2** at 25 ºC (Ludwig, 1956) **14** (Morales-Ramos et al., 2010) **14.15** (Urs and Hopkins, 1973) **15** (Hardouin and Mahoux, 2003) **15-17** (Park et al., 2014) **17-19** (Cotton, 1927) **19.1** at 30 ºC (Ludwig, 1956)	**15** at 25 ºC (Ludwig, 1956) **15** (Morales-Ramos et al., 2010) **16.1** (Urs and Hopkins, 1973) **18** (Morales-Ramos et al., 2015a) **20** (Cotton, 1927; Hill, 2002; Park et al., 2014) **23** at 30 ºC (Ludwig, 1956)

3.3 Rearing diets and environmental conditions

3.3.1 Temperature

The most common temperatures to rear mealworms fall within the interval 25-28 ºC (Kim et al., 2015; Koo et al., 2013; Ludwig, 1956; Punzo, 1975; Punzo and Mutchmor, 1980; Spencer and Spencer, 2006). The minimum temperature for growth is reported to be 10 ºC (Punzo and Mutchmor, 1980) and the maximum temperature 35 ºC (Martin et al., 1976; Punzo and Mutchmor, 1980). However, these maximum and minimum are not healthy, and the extreme values for the normal development of *Tenebrio molitor* are 17 ºC (Koo et al., 2013) and 30 ºC (Koo et al., 2013; Ludwig, 1956). There are no significant differences in the temperature requirements of the different stages of development of this species. The maximum lethal and chill-coma temperatures are 40-44 ºC (Altman and Katz, 1973; Martin et al., 1976) and 7-8 ºC (Mutchmor and Richards, 1961), respectively, for exposure periods of 24 hours (Table 2).

3.3.2 Relative Humidity

Compared to temperature, the mealworms show more flexibility to relative humidity, justifying the wider range of relative humidity values that are used in laboratory tests. In all studies that have focused on the influence of relative humidity on the development of *Tenebrio molitor*, an optimum interval was identified from 60% (Manojlovic, 1987) to 75% (Punzo, 1975; Punzo and Mutchmor, 1980). Some authors refer that the mealworm growth rate increases with relative humidity higher than 70% (Fraenkel, 1950) or even 90-100 % (Hardouin and Mahoux, 2003) but this values are unfeasible because they mold development on the substrate. Overall, depending on temperature, mealworms are quire resilient regarding relative humidity (Table 3).

Table 2 - Temperature conditions used on the rearing of mealworms (ºC)

	Minimum	Optimal	Maximum	Other laboratorial values*
Eggs	**10** (Punzo and Mutchmor, 1980) **15** (Kim et al., 2015) **17** (Koo et al., 2013)	**23-27** (Manojlovic, 1987) **25** (Murray, 1968, 1960; Punzo and Mutchmor, 1980) **25-27** (Siemianowska et al., 2013)	**30** (Manojlovic, 1987) **35** (Kim et al., 2015; Punzo and Mutchmor, 1980)	
Larvae	**10** (Punzo and Mutchmor, 1980) **17** (Koo et al., 2013) **20** (Martin et al., 1976)	**25** (Ludwig, 1956; Murray, 1968, 1960; Punzo, 1975; Punzo and Mutchmor, 1980) **27-28** (Kim et al., 2015; Koo et al., 2013; Spencer and Spencer, 2006)	**30** (Koo et al., 2013; Ludwig, 1956) **35** (Martin et al., 1976; Punzo and Mutchmor, 1980)	**18** (Lardies et al., 2014; Urrejola et al., 2011) **22.4** (Miryam et al., 2000) **25** (Allen et al., 2012; Connat et al., 1991; Fraenkel, 1950; Ghaly et al., 2009; Houbraken et al., 2016; Lv et al., 2014; Mellandby and French, 1958; Menezes et al., 2014; Oonincx et al., 2010; Park et al., 2014; Ravzanaadii et al., 2012; Vinokurov et al., 2006a) **26** (Baek et al., 2015; Tang et al., 2012; Tindwa and Jo, 2015; Urs and Hopkins, 1973) **27** (Davis, 1970a; Davis and Leclercq, 1969; Davis and Sosulski, 1973a; Hardouin and Mahoux, 2003; Morales-Ramos et al., 2013, 2012) **28** (L. L. Li et al., 2016; Ramos Elorduy et al., 2002; van Broekhoven et al., 2015) **30** (Fraenkel, 1950; Weaver and McFarlane, 1990) **35** (Mellandby and French, 1958)
Pupae	**10** (Punzo and Mutchmor, 1980) **18** (Cotton, 1927) **21** (Hardouin and Mahoux, 2003)	**25** (Murray, 1968, 1960; Punzo, 1975) **27** (Cotton, 1927) **27.5** (Kim et al., 2015) **28** (Ghaly et al., 2009) **27-33** (Hardouin and Mahoux, 2003)	**35** (Punzo and Mutchmor, 1980)	
Adult	**10** (Punzo and Mutchmor, 1980) **14-16** (DICK, 2008)	**25** (Murray, 1968, 1960; Punzo, 1975)	**35** (Punzo and Mutchmor, 1980)	

* Temperature on tests where this parameter was not the study object.

Table 3 - Relative humidity conditions on the rearing of mealworms (%)

	Minimum	Optimal	Maximum	Other laboratorial values*
Eggs	**12** (Punzo, 1975; Punzo and Mutchmor, 1980)	**60-75** (Manojlovic, 1987) **70** (Murray, 1968, 1960) **75**(Punzo and Mutchmor, 1980)	**98** (Punzo, 1975; Punzo and Mutchmor, 1980)	
Larvae	**12** (Punzo, 1975; Punzo and Mutchmor, 1980) **30** (Fraenkel, 1950)	**75** (Punzo, 1975; Punzo and Mutchmor, 1980) **60-70** (Spencer and Spencer, 2006) **70** (Fraenkel, 1950; Fraenkel and Blewett, 1944; Hardouin and Mahoux, 2003; Martin et al., 1976; Murray, 1968, 1960)	**98** (Punzo, 1975; Punzo and Mutchmor, 1980)	**43** (Allen et al., 2012) **50** (Morales-Ramos et al., 2010; Ravzanaadii et al., 2012; Urs and Hopkins, 1973) **55** (Tang et al., 2012; Weaver and McFarlane, 1990) **60** (Gerber and Sabourin, 1984; Tindwa and Jo, 2015) **60-70** (Ramos Elorduy et al., 2002; Rho and Lee, 2014; Zheng et al., 2013) **65** (Davis, 1970b; Davis and Leclercq, 1969; Davis and Sosulski, 1977; Morales-Ramos et al., 2013; van Broekhoven et al., 2015) **70** (Connat et al., 1991; Ghaly et al., 2009; Li et al., 2015; Ludwig, 1956; Menezes et al., 2014; Oonincx et al., 2015) **74.**2 (Miryam et al., 2000) **75** (Baek et al., 2015) **79.**2 (Oonincx et al., 2010) **80** (Alves et al., 2016) **85** (Lardies et al., 2014)
Pupae	**12** (Punzo, 1975; Punzo and Mutchmor, 1980)	**70** (Murray, 1968, 1960) **75** (Punzo, 1975; Punzo and Mutchmor, 1980)	**98** (Punzo, 1975; Punzo and Mutchmor, 1980)	
Adult	**12** (Punzo, 1975; Punzo and Mutchmor, 1980) **20** (DICK, 2008; Hardouin and Mahoux, 2003)	**70** (Murray, 1968, 1960) **75** (Punzo, 1975; Punzo and Mutchmor, 1980) **90-100** (Hardouin and Mahoux, 2003)	**98** (Punzo, 1975; Punzo and Mutchmor, 1980)	

* Relative humidity on tests where this parameter was not the study object.

3.3.3 Diet

Tenebrio molitor can be fed only with bran, that contains all necessary nutrients but not in optimum proportions. Adding a water source to the bran is the most common diet composition in mealworm industrial/laboratorial rearing facilities. Several studies tested the influence of diet on the growth rate and on the mass of the insect at the different development stages (Table 4). Studies to test the influence of environmental conditions on the performance of mealworms usually use diets of cereal bran or flour and fresh vegetables (e.g. carrot, apple, potato or cabbage) as a water source, or flour with water and a source of protein. Protein sources commonly used to complement the diet include beer yeast, casein and soy protein.

The influence of several diets was studied both for the adult and for the larvae phases (Leclercq, 1948; Urrejola et al., 2011). Mealworms have the ability to select foods to balance their diet ratio/intake according to their nutritional needs (Morales-Ramos et al., 2011; Rho and Lee, 2016, 2014; Urrejola et al., 2011).

Table 4 - Diets and protein sources used on the rearing of mealworm

Diet	Reference
Bran/Flour + water source (vegetable or water)	(Allen et al., 2012; Baek et al., 2015; Dick, 2008; Greenberg and Ar, 1996; Houbraken et al., 2016; Li et al., 2015; Ludwig, 1956; Lv et al., 2014; Miryam et al., 2000; Morales-Ramos et al., 2012; Punzo, 1975; Ravzanaadii et al., 2012; Siemianowska et al., 2013; Spang, 2013; Tschinkel and Willson, 1971; Urs and Hopkins, 1973; Vinokurov et al., 2006b; Wang Xuegui, Zheng Xiaowei, Li Xiaoyu, Yao Jianming, Jiang Surong, 2010)
Bran/Flour + water source (vegetable or water) + Protein Source	(Connat et al., 1991; Ghaly et al., 2009; Kim et al., 2015; Lardies et al., 2014; L. L. Li et al., 2016; Manojlovic, 1987; Murray, 1968; Oonincx et al., 2010; Ramos Elorduy et al., 1997; Tang et al., 2012; Weaver and McFarlane, 1990)
Varied artificial diet	(Alves et al., 2016; Davis, 1978, 1974, 1970a; Davis and Sosulski, 1973b; Fraenkel, 1950; Gerber, 1975; Hardouin and Mahoux, 2003; John et al., 1978; Leppla et al., 2013, Martin and Hare, 1942; Martin et al., 1976; Menezes et al., 2014; Morales-Ramos et al., 2010; Murray, 1960; Oonincx et al., 2015; Rho and Lee, 2015, 2014; Tindwa and Jo, 2015; Urrejola et al., 2011; van Broekhoven et al., 2016)
Protein Source	**Reference**
Beer yeast	(Gerber, 1975; Ghaly et al., 2009; John et al., 1978; Lardies et al., 2014; Oonincx et al., 2015, 2010; Ramos Elorduy et al., 1997; Tang et al., 2012; Tindwa and Jo, 2015; Urrejola et al., 2011; van Broekhoven et al., 2015; Weaver and McFarlane, 1990)
Casein	(Davis, 1978, 1970a; Davis and Leclercq, 1969; Fraenkel, 1950; Murray, 1960; Rho and Lee, 2014)
Dried yeast	(Connat et al., 1991; Murray, 1968, 1960)
Albumin	(Morales-Ramos et al., 2013, 2010; Murray, 1960; Rho and Lee, 2014)
Soy	(Davis and Sosulski, 1973a; Hardouin and Mahoux, 2003; Manojlovic, 1987; Morales-Ramos et al., 2013)
Dry potato	(Morales-Ramos et al., 2015b)
Lactalbumin	(Davis, 1970a; Davis and Leclercq, 1969)
Lactalbumin hydrolisate	(Davis, 1970a; Davis and Leclercq, 1969)
Aminoacid mixture	(Davis, 1974; John et al., 1978)
Bird feed	(Menezes et al., 2014)
Bocaiuva (*Acrocomia aculeata*)	(Alves et al., 2016)
Cookie remains	(Oonincx et al., 2015; van Broekhoven et al., 2015)
Beef (blood, muscle, liver)	(Martin and Hare, 1942)

3.3.4 Population Density, Photoperiod and Oxygen

The development and growth of *Tenebrio molitor* is directly dependent on the number and the duration of the larval molts, which are influenced by population density (Connat et al., 1991; Morales-Ramos et al., 2012; Morales-Ramos and Rojas, 2015; Tschinkel and Willson, 1971; Weaver and McFarlane, 1990), photoperiod (Cloudsley-Thompson, 1953; Tyshchenko and Ba, 1986) and oxygen levels (Greenberg and Ar, 1996).

3.3.4.1 Population Density

Early studies have noted pupation inhibition and cannibalism associated with crowding in tenebrionid beetles (Tschinkel and Willson, 1971) as well as the occurrence of incomplete transformations and lower growth rates due to reduced feeding opportunity induced by conspecific competition (Weaver and McFarlane, 1990). On the other hand, although high population density conducts to a reduction on food consumption, the lower growth rates are also a consequence of the reduction in efficiency of digested food conversion (ECD) and efficiency of ingested food conversion (ECI), in response to increased larval density (Morales-Ramos et al., 2015a). In addition to the effect of overcrowding on the consumption of food (ingestion and efficiencies), changes on females and reproductive process were also identified, with single couples being the most productive in terms of progeny produced per female (Morales-Ramos et al., 2012). A further aspect of overpopulation is the effect on pre-pupae stage with isolated larvae having accelerated larval-pupal apolyses and reduced number of larval instars (Connat et al., 1991). Moreover, it is not irrelevant the increase in temperature generated by the metabolism of larvae that can easily be lethal under overcrowded conditions (Table 5).

3.3.4.2 Photoperiod

Another environmental parameter influencing growth development is photoperiod (Tyshchenko and Ba, 1986). The mealworm is a negative phototropic or phototactic species (Balfour and Carmichael, 1928; Cloudsley-Thompson, 1953) with adults and larger larvae positioning near the top of substrate and coming to surface in darkness. The response to photoperiod tends to disappear under constant conditions and *T. molitor* becomes arrhythmic (Cloudsley-Thompson, 1953). Even when there is a response to photoperiod recent studies showed that larval development was optimal in long-day conditions, with lower development times achieved in photoperiodic conditions of 14L:10D (Kim et al., 2015). The eclosion rate is also dependent on photoperiod, with 45.5% at 14L:10D versus 24.2% at 10L:14D.

The pupation is directly influenced by photoperiod, being induced by a 12L:12D regime at 25 ºC and resulting in more protracted delays than shorter days. However, Tyshchenko and Ba (1986) reported a reversal of photoperiodic response at 30 ºC with a 12 hour day inhibiting and a 18 hour day stimulating pupation (Tyshchenko and Ba, 1986) (Table 6).

3.3.4.3 Oxygen needs

Larvae mortality is higher in lower oxygen conditions (Greenberg and Ar, 1996). Although development time in hyperoxia and normoxia are similar, hyperoxia conditions induce lower number of instars thus resulting in lower final larval biomass (Greenberg and Ar, 1996)

Table 5 - Population density used on the rearing of mealworms

Population density (larvae/cm^2)	Tray dimensions (cm)	Area (cm^2)	Volume (cm^3)	References
0.84				(Morales-Ramos et al., 2012)*
0.5-1.5	11×18×3	198	594	(Connat et al., 1991)*
0.8 -1.45	16×21.5×7	344	2408	(Park et al., 2012)
0.48	48×49.5×10.5	2376	24948	(Park et al., 2014)
0.02	30×15	450		(Spang, 2013)
0.3	17.5×9.3×6.3	163	1025	(Oonincx et al., 2015)
0.3	40×30×25	1200	30000	(Alves et al., 2016)
0.4-0.6	40×17×11	680	7480	(Rho and Lee, 2016)
0.3	10 (diameter) ×28	79	2199	(DICK, 2008)
0.03	60×45×15	2700	40500	(Ghaly et al., 2009)
3.75 – 7.5		600		(Martin et al., 1976)
0.01 – 0.2	10 (diameter)	79		(Tschinkel and Willson, 1971)

*The population density was the object of study

Table 6 - Photoperiod regimes used in the rearing of with *Tenebrio molitor*

Light:Dark (h)	References
12:12	(Allen et al., 2012; Gerber and Sabourin, 1984; Greenberg and Ar, 1996; Kim et al., 2015; Lardies et al., 2014; Lv et al., 2014; Martin et al., 1976; Menezes et al., 2014; Oonincx et al., 2015; Rho and Lee, 2014, 2016; Tang et al., 2012; Urrejola et al., 2011; van Broekhoven et al., 2015)
Darkness	(Morales-Ramos et al., 2013; Oonincx et al., 2010; Punzo, 1975; Punzo and Mutchmor, 1980)
14:10	(Kim et al., 2015; Koo et al., 2013; Morales-Ramos et al., 2010; Ravzanaadii et al., 2012)
10:14	(Alves et al., 2016; Kim et al., 2015; Rho and Lee, 2016; Weaver and McFarlane, 1990)
16:8	(Tindwa and Jo, 2015; Urs and Hopkins, 1973)

3.4 Effects of diet and environmental conditions on the performance of mealworms

3.4.1 Temperature and relative humidity

Temperature and relative humidity affect both the number and length of the instars with direct effect on the development of mealworms, and influence the water absorption capacity of the different stages. The pupal stage is the most resilient to extreme conditions of temperature and relative humidity whereas the egg and young larval stages are the most sensitive (Punzo and Mutchmor, 1980).

The oviposition doesn't occur at temperatures below 14 ºC in a relative humidity of 65% but even at optimal temperature of 27 ºC in low humidity (20%) oviposition is greatly reduced (Dick, 2008). It was also reported that female adult activity is most relevant at relative humidity values of 90-100% (Hardouin and Mahoux, 2003).

At cold temperatures of 10 ºC (Punzo, 1975; Punzo and Mutchmor, 1980) and 12.5 ºC (Kim et al., 2015), water absorption is reduced, and the embryological development is not completed. On the other hand, extremely dry conditions of about 12% relative humidity potentiate water losses from the eggs, culminating on death due to desiccation (Punzo, 1975).

The growth rate of *T. molitor* larvae is highly dependent on moisture, being fastest at 70% relative humidity, very slow at 30% and hardly proceeding at 13% (Fraenkel, 1950). The higher the relative humidity, the higher the growth rate but high relative humidifies favor the growth of mold, other microorganisms and mites (Fraenkel, 1950). Larval molts are shorter and the number of instars is higher at 30 ºC than at 25 ºC, resulting in a lengthier total larval period at 30 ºC than at 25 ºC (Ludwig, 1956). Temperatures below 10 ºC and above 35 ºC clearly constitute stress conditions for this species although the chill-coma temperature is ≤7 ºC and the lethal temperature is ≥44 ºC (Punzo and Mutchmor, 1980).

The effect of temperature is potentiated at extreme moisture conditions and the effect of moisture is also more critical at extreme temperatures (Punzo and Mutchmor, 1980). For example, at optimal temperatures of 25-27.5 ºC, *T. molitor* shows no-stress even at extreme humidity conditions and long exposure periods. Moreover, a decrease in humidity was found to be inconsequential to adults, larvae, or pupae at a temperature of 25 °C, but resulted in

increased mortality at 10°C (Punzo and Mutchmor, 1980). At extremely dry conditions mealworm larvae may cease completely the food ingestion and become inactive until relative humidity gets favorable again (Urs and Hopkins, 1973).

3.4.2 Water intake

Tenebrio molitor can live under extremely dry conditions for long periods of time, being able to obtain water from both the food ingested (even substances with low water content) and the atmosphere (Fraenkel and Blewett, 1944). However, larvae grow faster in moist conditions higher than 70%, which enhance the development of mites, fungi and other microorganisms (Fraenkel, 1950). It was observed that mealworms reared on dry substrates reach higher growth rates when there is a source of water (Mellandby and French, 1958; Oonincx et al., 2015; Urs and Hopkins, 1973). In fact, on low-moisture substrates, when metabolic water per unit of food is low, development may become impossible if there is no drinking water. When suffering from water deprivation, larvae of *T. molitor* ingest less food and the conversion rate of ingested food into body mass decreases (Murray, 1968).

The reported effects of water intake on biomass composition are contradictory. Urs and Hopkins (1973) observed that the availability of water increases the concentrations of total lipids, while Oonincx (2015) reported that the supplementation of the diets with a water source increases the water content but not the total fatty acids content. Both authors found no influence of water on mealworm fatty acid profile.

3.4.3 Diet

Although mealworms can survive feeding exclusively on wheat bran, a diet supplementation benefits the species. Nutrient intake plays a central role on *T. molitor* life cycle, such as development time (Davis, 1970b; Fraenkel, 1950; Martin and Hare, 1942; Morales-Ramos et al., 2013, 2010; Rho and Lee, 2016; Urrejola et al., 2011; van Broekhoven et al., 2015), fertility (Gerber and Sabourin, 1984; Manojlovic, 1987; Morales-Ramos et al., 2013; Urrejola et al., 2011), number of instars (Morales-Ramos et al., 2010) and survival rate (Morales-Ramos et al., 2010; van Broekhoven et al., 2015).

Mealworm lifecycle is highly conditioned by the dietary ratio of protein (P) to carbohydrate (C) (Martin and Hare, 1942; Rho and Lee, 2016; Urrejola et al., 2011). Rho and Lee (2016) reported an optimal P:C ratio of 1:1 for lifespan and lifetime reproductive success, while Martin and Hare (1941) observed maximum growth at a minimum of 50% of carbohydrate and 15% or more than 25% of protein in diet. Fats don't have a determinant role on development, becoming an inhibitory factor only at values exceeding 3% (Martin and Hare, 1942). The most beneficial supplement to diet is protein and the addition of yeast in several concentrations proved to maximize weight gain and food conversion rates and to decrease mortality and development times (Martin and Hare, 1942; van Broekhoven et al., 2015).

The importance of nutrient balance was observed at different developmental stages. Besides the effect on larval development time, the intensity and period of oviposition is highly dependent on the quality of the ingested food (Manojlovic, 1987; Morales-Ramos et al., 2013). Good food quality favors progeny, by increasing the number of eggs and decreasing adult mortality (Gerber and Sabourin, 1984).

The ingestion of food results in body protein contents two-fold higher and body fat contents five to six-fold higher than that of food with a significant reduction in the values of crude fiber and carbohydrates (Ramos Elorduy et al., 2002). Mealworms fed on low P:C feed have higher body lipid content (Rho and Lee, 2014).

3.4.3.1 Protein

Dietary concentration of protein and the amino acid composition greatly influence *T. molitor* lifecycle, with direct benefits on larval development time, survival and weight gain (Morales-Ramos et al., 2013; Oonincx et al., 2015; van Broekhoven et al., 2015). Reported optimal ranges of protein concentration are 2-32% (Davis and Leclercq, 1969). Growth rate is enhanced by protein; with a fresh weight gain of 2.3-2.9 mg on a protein-free diet to 45.5-55.6 mg on a diet supplemented with yeast (John *et al*., 1979).

Yeast is reported to be the best source of protein, even acting as a feeding stimulant. Other efficient protein sources that provide optimal effects are casein, and at a lower level, lactalbumin (Davis and Leclercq, 1969; Fraenkel, 1950; Leclercq, 1948). Zein, gliadin and

enzymatic hydrolizates from casein or lactalbumin have residual effects when compared to casein and lactalbumin (Fraenkel, 1950).

Regarding amino acids, alanine, arginine, aspartic acid, cystine, histidine, isoleucine, leucine, methionine, proline and valine should be fed at equivalent levels to those found in larval tissues whereas phenylalanine should be provided at concentrations half of the values found in the larval tissues. Two limiting amino acids, threonine and tryptophan, should be given at twice the concentration found in larvae body (John et al., 1979). The presence of carnitine is absolutely necessary for the appropriate development of *T. molitor* (Hardouin and Mahoux, 2003).

Mealworms seem to have a highly constrained body protein content, since diets varying 2–3-fold in crude protein result in similar chemical body composition with regard to protein (van Broekhoven et al., 2015).

3.4.3.2 Fat

Mealworm fat composition is rather constant being rich in oleic, linoleic and palmitic acids when fed in different diets (Oonincx et al., 2015). The possible synthesis of fatty acids *ad novo* by mealworms could explain that palmitic and oleic acids remain almost stable independently of diet. On the other hand, linoleic acid may have to be supplied (van Broekhoven et al., 2015). In addition, higher ingestion of polyunsaturated C18 fatty acids results in lower proportion of C18 monounsaturated fatty acids in larval tissues (van Broekhoven et al., 2015).

The addition of low concentrations of lipid to dietary regimes is beneficial; however, high quantities are unfavorable and potentially pernicious (Morales-Ramos et al., 2013). While cholesterol is a necessary diet ingredient, fat concentrations higher than 1% do not show benefits on any mealworm lifecycle parameter (Fraenkel, 1950), and become an inhibitor factor at concentrations above 3% (Martin and Hare, 1942). Moreover, high fat foods promote the potential agglomeration of the substrate resulting in lower aeration and movement of mealworms thus negatively interfering in breathing (Alves et al., 2016).

3.4.3.3 Carbohydrates

Tenebrio molitor growth is almost nil in the absence of carbohydrates. The optimal range is 80-85% of carbohydrates and the ingestion of food with only 20% carbohydrates results in very slow growth (Fraenkel, 1950).

Although Fraenkel (1950) reported no significant differences between the growth of mealworms with glucose and starch, Davis (1974) observed smaller growth with starch, sucrose or lactose than with glucose, with diets containing amino acid mixtures. Similarly, bacteriological dextrin as a carbohydrate source induced weight gain of almost twice the one achieved with glucose (Davis, 1974).

3.4.3.4 Vitamins

Tenebrio molitor shows no growth in the absence of certain vitamins. Vitamins of B complex (thiamin, riboflavin, nicotinic acid, pyridoxin, or pantothenic acid) are essential and the lack of these vitamins results in no growth, while the absence of biotin or pteroylglutamic acid still allows a slow growth (Leclercq, 1948; Martin and Hare, 1942). On the contrary, the addition of vitamins A, C, D, E, K show no beneficial effect (Fraenkel, 1950; Martin and Hare, 1942). After mealworms reach half or fully-grown size no addition of vitamins is required to complete the larvae cycle and pupate (Leclercq, 1948).

3.5 Summary

The entire life cycle of mealworms takes place in the same ecosystem and the duration of the different stages is highly dependent on environmental and physical conditions such as temperature, relative humidity, diet and population density that involve mealworm rearing;

The most commonly used temperatures to rear mealworms fall within the interval 25-28 ºC (Kim et al., 2015; Koo et al., 2013; Ludwig, 1956; Punzo, 1975; Punzo and Mutchmor, 1980; Spencer and Spencer, 2006);

Tenebrio molitor has an optimum range of relative humidity between 60% (Manojlovic, 1987) and 75 % (Punzo, 1975; Punzo and Mutchmor, 1980);

Overpopulation can induce inhibition of pupation and cannibalistic behavior in mealworms (Tschinkel and Willson, 1971);

T. molitor is highly adaptable to extreme dry conditions, being able to obtain water from both the food (even substances with low water content) and the atmosphere. However larvae grow faster in moist conditions with relative humidity higher than 70% (Fraenkel and Blewett, 1944);

Even though mealworms can live feeding exclusively on what bran, supplementation with a source of protein, such as yeast or casein, benefits the development of the species;

Mealworm lifecycle is highly conditioned by dietary ratio of protein to carbohydrate (Martin and Hare, 1942; Rho and Lee, 2016; Urrejola et al., 2011);

T. molitor can regulate the ingestion of food in order to balance its nutritional needs (Morales-Ramos et al., 2011);

Optimal growth is achieved when the diets contain 5% to 10% of yeast (Martin and Hare, 1942);

The addition of fat to food at concentrations higher than 1% do not show any beneficial consequence on any mealworm lifecycle parameter (Fraenkel, 1950);

Mealworms show high growth rates when the range of carbohydrates is 80-85% of diet content, while an ingestion of food with only 20% carbohydrates induces a very slow growth (Fraenkel, 1950);

Vitamins of the B complex are essential to the development of mealworms (Leclercq, 1948; Martin and Hare, 1942) while there is no beneficial effect with the addition of vitamins A, C, D, E, K (Fraenkel, 1950; Martin and Hare, 1942).

4 Chapter II – Effects of relative humidity, three pesticides and polyacrylamide on the performance of *Tenebrio molitor*

4.1 Introduction

From all species of insects with potential of rearing at industrial scale, the mealworm beetle, *Tenebrio molitor*, is one of the easiest and less sensitive to rearing conditions. Although optimal growth rates need optimal conditions, this species is resilient enough to withstand large temperature (Kim et al., 2015; Koo et al., 2013; Ludwig, 1956; Punzo, 1975; Punzo and Mutchmor, 1980; Spencer and Spencer, 2006), humidity (Punzo, 1975; Punzo and Mutchmor, 1980), light regimes (Cloudsley-Thompson, 1953; Tyshchenko and Ba, 1986) and diet amplitudes (Broekhoven, van *et al.*, 2015; Davis, 1970a,b; Fraenkel, 1950; Martin and Hare, 1942; Morales-Ramos *et al.*, 2010, 2013; Rho e Lee, 2016; Urrejola, Nespolo e Lardies, 2011).

Mealworms are commonly reared in plastic trays or boxes with about 65×50×15 cm, and fed exclusively with wheat bran supplemented with protein source such as yeast or casein or even with artificial mixtures. Though *T. molitor* can efficiently absorb atmospheric water at high relative humidity, at artificial rearing the water intake is usually promoted by the addition of fresh vegetables to the diet (Cortes Ortiz et al., 2016). As insects can be used directly by humans as food – in several countries, entomophagy has been gaining followers in recent years – but also indirectly by consumption of animals fed insect-based diets, the use of vegetables and the wheat based diet, if contaminated with pesticides, could increase the risk of contaminant intake by humans and result in food safety issues. In fact, lipophilic properties along with the large percentage of body fat give the mealworms a high potential for accumulating contaminants (Houbraken et al., 2016).

Besides the use of fresh vegetables to supply water to mealworms, the use of polymers such as polyacrylamide (PAM), a polymer that absorbs water and forms a gel where insects are able to "drink", have been gaining prevalence. This method is already used by the exotic animal breeders and has been adapted to the production of mealworm.

4.2 Objective

The objective of this work was to determine the influence of relative humidity, of the ingestion of carrots contaminated by three pesticides and of polyacrylamide on the performance of mealworm larvae.

4.3 Pesticides and Polyacrylamide

The mealworm larvae and beetle are commonly reared on wheat bran or flour with or without protein supplementation and a wide range of vegetables such as potato, carrot or chayote as source of moisture. As a substitute for vegetables there are several producers using a polymer, polyacrylamide, which has the ability to absorb water and become a gel ingested by the insects.

The pesticides used in the experimental trial were chosen to cover all three main types of pesticides, i.e., an herbicide, an insecticide and a fungicide according to their utilization in Portugal.

4.3.1 The insecticide deltamethrin

Deltamethrin ($C_{22}H_{19}Br_2NO_3$; Figure 1) is a semi-volatile pyrethroid ester insecticide with low aqueous solubility and low potential to leach to groundwater. It is not persistent in soil and is non mobile. The formulations of deltamethrin include emulsifiable concentrates, wettable powders and flowable formulations and granules (AERU, 2016a).

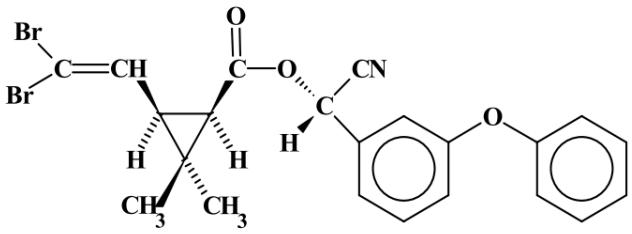

Figure 1- Chemical structure of the insecticide deltamethrin ($C_{22}H_{19}Br_2NO_3$)

Deltamethrin is commonly used in in olives, pome fruits, tree nuts, strawberries, vegetables, mushrooms, arable crops, forestry, flowers, ornamentals, as well as non-crop uses (European Commission, 2002) acting by contact and ingestion to sucking and chewing insects. Its high lipophilicity provides a high affinity with the insect cuticle" (Bayer, 2017). Although the mode of action of deltamethrin is thought to be mainly central in action, or at least originate in higher nerve centers of the brain, causing irreversible damage to the nervous system of insects when poisoning lasts more than a few hours (Rehman et al., 2014), the available data do not indicate that deltamethrin is a developmental neurotoxic agent (EFSA, 2009).

4.3.2 The herbicide metribuzin

Metribuzin ($C_8H_{14}N_4OS$; Figure 2) is a selective triazinone herbicide which inhibits photosynthesis. It is used to control weeds in cereals and a range of other crops as a pre- and post-emergence herbicide (AERU, 2016b). It is moderately adsorbed on soils with high clay or organic content, and the adsorption decreases with increasing soil pH. Although little leaching occurs in soils with a high organic content, metribuzin is readily leached in sandy soils. The soil half-life ranges from 14-60 days. (USEPA, 2003). Metribuzin is moderately to slightly toxic to birds and slightly toxic to fishes, non-toxic to honey bees, and has a moderate toxicity potential to earthworms.

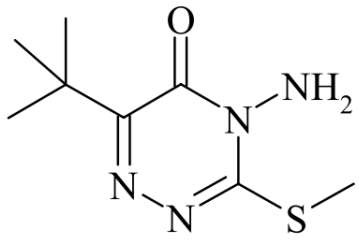

Figure 2 – Chemical structure of the herbicide metribuzin ($C_8H_{14}N_4OS$)

4.3.3 The fungicide mancozeb

Mancozeb ($C_8H_{12}MnN_4S_8Zn$; Figure 3) is a dithiocarbamate non-systemic fungicide used for the control of a wide range of pathogens including blights and scabs on crops. Mancozeb reacts with and inactivates sulfhydryl (SH) groups of amino acids and enzymes of fungal cells,

resulting in disruption of lipid metabolism, respiration and production of ATP. It is commonly applied as foliar applications by aerial or ground equipment (FAO, 1980).

Mancozeb is quite volatile, has a low aqueous solubility and it thus not expected to leach to groundwater. It is not persistent in soil systems but may be persistent in water under certain conditions. Mancozeb has a low mammalian toxicity but it has been associated with adverse reproduction/development effects. It is highly toxic to fish and aquatic invertebrates, moderately toxic to birds and earthworms and it show low toxicity to honeybees is low (AERU, 2016c).

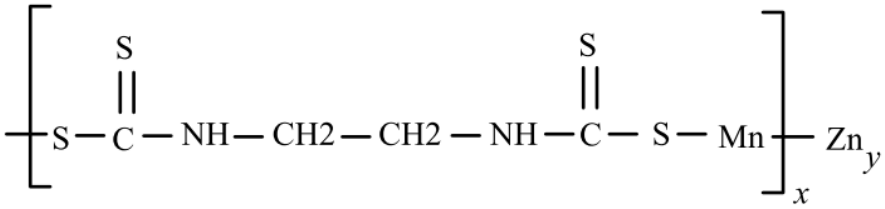

Figure 3 – Chemical structure of a monomer part of the polymeric complex of the fungicide mancozeb which contains 20% manganese and 2.5% zinc ($C_8H_{12}MnN_4S_8Zn$)

4.3.4 Polyacrylamide

Polyacrylamide gels are obtained by free radical crosslinking copolymerization of acrylamide and N,N-methylenebis(acry1amide) (Bis) monomers (Naghash and Okay, 1996). Although polyacrylamide is commonly accepted as being non-toxic, the acrylamide monomers cause peripheral neuropathy (Caulfield et al., 2003). Following ingestion, acrylamide absorbed from the gastrointestinal tract is metabolized, resulting in the production of glycidamide, the most likely cause of gene mutations and tumors in animals (EFSA, 2015). The level of acrylamide monomers in commercial polymers has been an important issue particularly for applications where human contact is involved. Moreover, there is a possibility of degradation of commercial polyacrylamide formulations to acrylamide (Caulfield et al., 2003).

4.4 Methods

4.4.1 Experimental design and methods

The experimental design consisted of two relative humidities (65% and 40%), six treatments (four pesticide concentrations + PAM + control) and five replicates per treatment, each with 16 to 20 larvae, in a total of 1119 larvae.

The experiment was carried out in plastic trays (20×10×5 cm) placed inside thermo-climatic chambers at 27±1 ºC under two different relative humidity (RH) atmospheres, 65±5% to simulate normal rearing conditions and 40±5% to simulate dry conditions that could stimulate water intake and, consequently, the ingestion of carrot or PAM.

The effect of the pesticides on the performance of *T. molitor* was assessed in a cocktail offered, via carrots, at four concentrations with five replicates and 16 to 20 larvae per replicate. Fresh carrots with no pesticides were used as the control.

Larvae with about one month were obtained from a local producer. On 16-June-2016, groups of 20 larvae were weighed (0.52 ± 0.79 SD; Table 9, Annex II) and acclimatized for one week at 27 ºC and 65% or 40% relative humidity. During the acclimation period the larvae were fed *ad libitum* with wheat bran (circa 20 g) and fresh carrots.

The experiment was started on 24-July-2016 and carried out for 21 days. At the beginning of the experiment, some of the larvae had already died and the initial number of larvae per replicate varied between 16 and 20 (Table 9, Annex II). The larvae were fed *ad libitum* with wheat bran (circa 20 g) and, at 2-3 days interval, sliced carrots containing the pesticide (treatments), PAM or fresh carrots (control).

4.4.2 Pesticides

The fresh vegetable selected to provide water to the mealworms was carrot, due to the easiness of manipulation and to the yearly availability in the market. Furthermore, carrots have a higher durability without rotting and becoming moldy, when compared with other fresh vegetables.

To select a way to inoculate the carrots with the pesticides, two similar pieces of carrot with about 2cm×2cm were weighed; one piece was lyophilized and stored in the fridge for 24 hours. The pieces were then submerged in water and weighed periodically until weight stabilization, which occurred after a submersion time of 120 minutes. Since lyophilization provided higher water absorption, the method selected to inoculate the carrots with the pesticides was to submerge lyophilized pieces in the pesticide solution for 120 minutes.

The pesticides selected are the most popular and widely used on crops in Portugal: the insecticide Deltamethrin (Sencor Liquid 600 $g.L^{-1}$, Bayer®), the herbicide Metribuzin (Decis 25 $g.L^{-1}$, Bayer®) and the fungicide Mancozeb (80%, Sapec). The concentrations were chosen based on the upper legal levels of concentration for pesticides in or on food or feed established by the European Commission (Maximum Residue Levels; Regulation (EC) Nº 396/2005).

Since the most commonly used vegetables as a source of water for mealworm larva are potatoes, carrot, and chayote (courgette), and the main feeding source is wheat, the Maximum Residue Level (MRL) for each pesticide was estimated as the average of the four (Table 7). A correction factor of +33% was established due to the carrot water content and pesticide diffusion (Table 7).

Table 7 - Legal MRL of the pesticides and values chosen for the experiment

Pesticide	MRL Potato	MRL Carrot	MRL Courgette	MRL Wheat	Mean MRL	Used MRL	2 MRL	½ MRL	¼ MRL
Deltamethrin	0.20	0.05	0.20	2.00	0.15	**0.20**	0.40	0.10	0,05
Metribuzin	0.10	0.10	0.10	0.10	0.10	**0.13**	0.27	0,07	0,03
Mancozeb	0.30	0.20	2.00	1.00	1.50	**2.00**	3.99	1.00	0,50

The pesticides were prepared in 2MRL concentration stock solutions using demineralized water, and this solution was diluted to provide 1MRL, ½MRL and ¼MRL solutions of each pesticide. Polyacrylamide was prepared according to the instructions using 5g of crystal in demineralized water, obtaining about 800ml of consumable hydrogel. Before the beginning of the experimental work, a sufficient amount of carrot for all the experimental work was sliced in similar pieces (circa 2cm×2cm×0.5 cm), lyophilized and stored at -20 ºC until needed.

At each supply, from each stock concentration (2MRL, MRL, ½MRL, ¼ LMR) 100 ml were allocated to a glass, 11 carrot pieces were submerged per concentration on a total of 44. This carrot pieces remained submerged for 120 minutes, and then were removed and dried on absorbent paper to remove excess water. Ten of these carrot pieces were used on the experiment and the other piece was collected and packaged in a freezer at -80 °C for later laboratorial analysis. A small amount of PAM of 2g was placed on a paper, over the substrate of the replicates.

4.4.3 Data analysis

Survival and pupation along time were calculated on basis of the larvae alive at the beginning of the experiment. Mortality was calculated as the percentage of larvae alive at the end of the experiment. Because the number of individuals per replicate was not uniform, mortality rate was corrected for the control with the Sun-Shepard's formula (Puntener, 1992):

$$Corrected\ (\%) = \left(\frac{Mortality\ in\ treated\ plot\ (\%) \pm\ Change\ in\ control\ plot\ population\ (\%)}{100 \pm\ Change\ in\ control\ plot\ population\ (\%)}\right) * 100$$

Where,

$$Change\ in\ control\ plot\ population\ (\%) = \left(\frac{Population\ in\ control\ plot\ after\ treatment - Population\ in\ control\ plot\ before\ treatment}{Population\ in\ control\ plot\ before\ treatment}\right) * 100$$

Because the larvae in each replicate were weighed in groups and mortality occurred during the experiment, a mean weight per individual was calculated at the beginning and at the end of the experiment.

Growth rate was calculated as,

$$Growth\ Rate = \frac{(Final\ weight - Initial\ weight)}{initial\ weight}$$

The effect of the four concentrations of the pesticide cocktail on the performance of the mealworm larvae was assessed by 2-way ANOVA using relative humidity and pesticide

concentration as factors. Data was tested for homeostadicity and normality and the arcsine transformation was used prior to statistical analysis whenever necessary. Tukey HSD was used as a post-hoc test whenever a significant effect of treatment was found. The statistical analyses were carried out with the software Statistica 13 with P set at 0.05.

4.5 Results

During the one week acclimatization period larvae deaths were observed, probably due to handling in sample preparation. For this reason, the analysis of the results, when in percentage, will be in a percentage to the number of larvae that actually started the test.

4.5.1 Survival

Survival was higher at 65% than at 40% relative humidity (Figure 1). At 65% RH (Figure 1, top), the highest survival was observed in treatments 1/2MRL, MRL and PAM while the lowest survival occurred in treatments 1/4 MRL, 2MRL and control. At 40% RH, the highest survival was observed in treatment 2MRL and the lowest in treatment 1/4MRL (Figure 1, bottom).

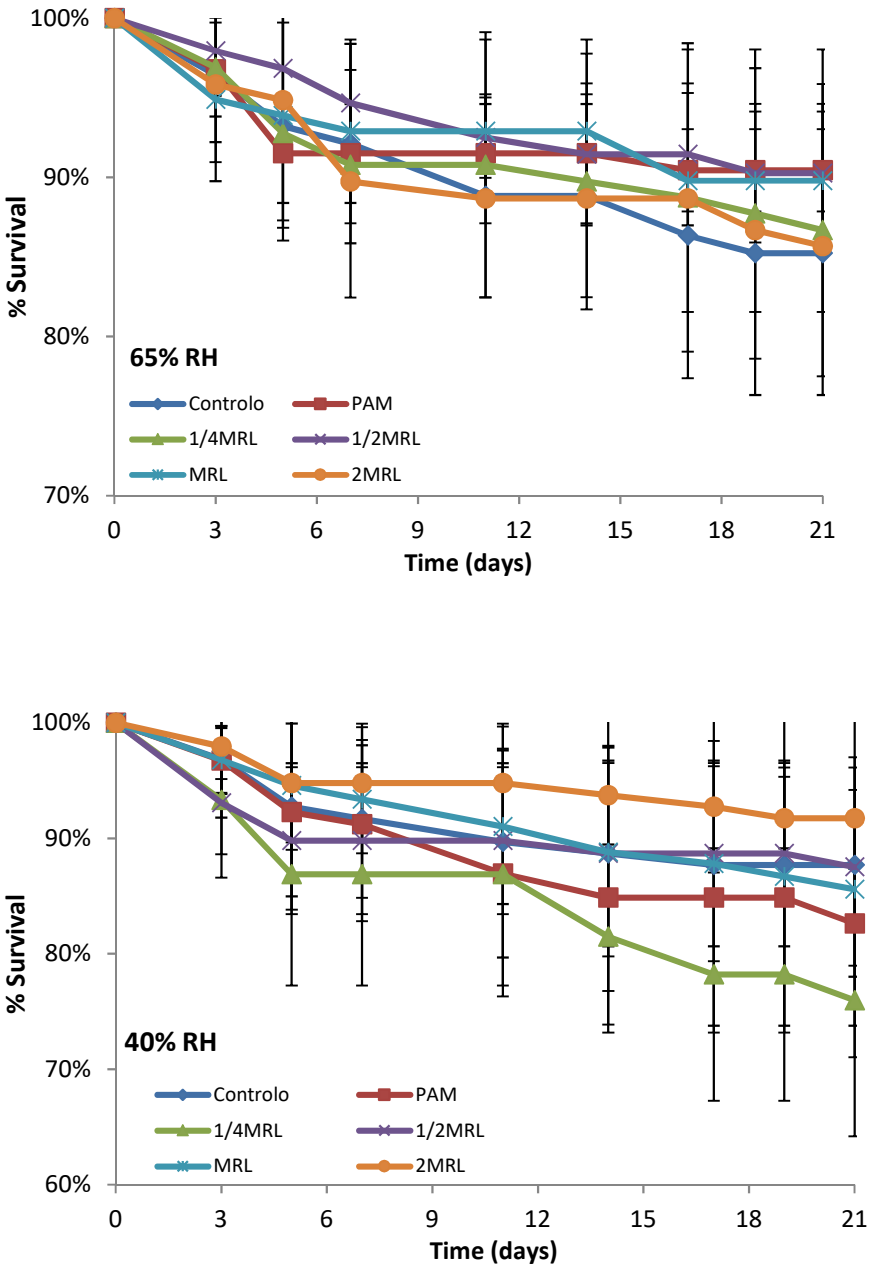

Figure 4 – Percentage survival of the mealworm larvae in the five treatments and the control at 65% (top) and 40% (bottom) relative humidity (average ± 1 standard deviation)

Mortality was highest in treatment 1/4MRL at 65% RH and lowest in the control for both relative humidities (Figure 2). However, there was a great data variability and there were no significant effect of treatment (F=0.939; P=0.464) or relative humidity (F=1,466; P=0.232) on mortality of the larvae.

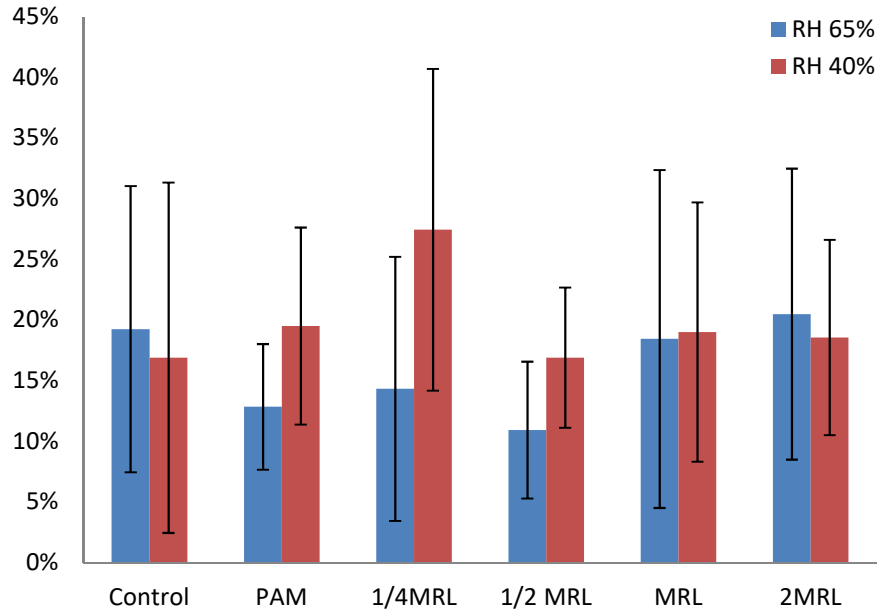

Figure 5 - Percentage mortality of the mealworm larvae in the five treatments and the control at 65% and 40% relative humidity (average ± 1 standard deviation)

When mortality was corrected with the Sun-Shepard's formula, negative values were observed for all treatments except for 2MRL at 65% RH. This means that the only treatment where mortality was higher than control was 2MRL. At 40% RH, all treatments had higher values of mortality than the control. The highest mortality achieved was observed for 1/4MRL at 40% RH and the lowest was 1/4MRL at 65% RH (Figure 3).

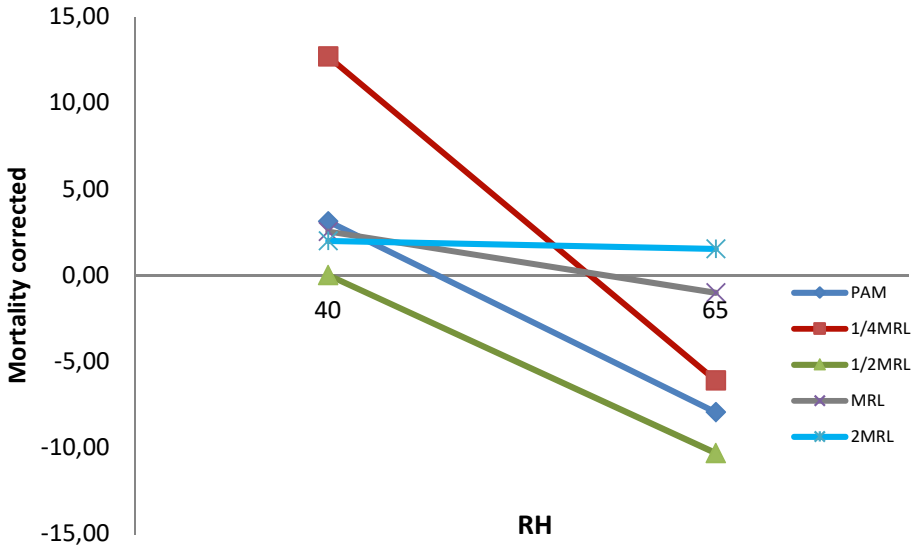

Figure 6 - Corrected mortality (Sun-Shepard's formula) of the mealworm larvae in the five treatments

4.5.2 Pupae

Pupation was significantly higher at 65% than at 40% relative humidity (F=24.09; P=0.000011; Figure 4). The treatment with the highest pupation was 1/2MRL with a pupation of 26% of initial larvae at the final of the trial, but there was not a significant effect of treatment in pupation (Figure 4; F=0.808; P=0.549).

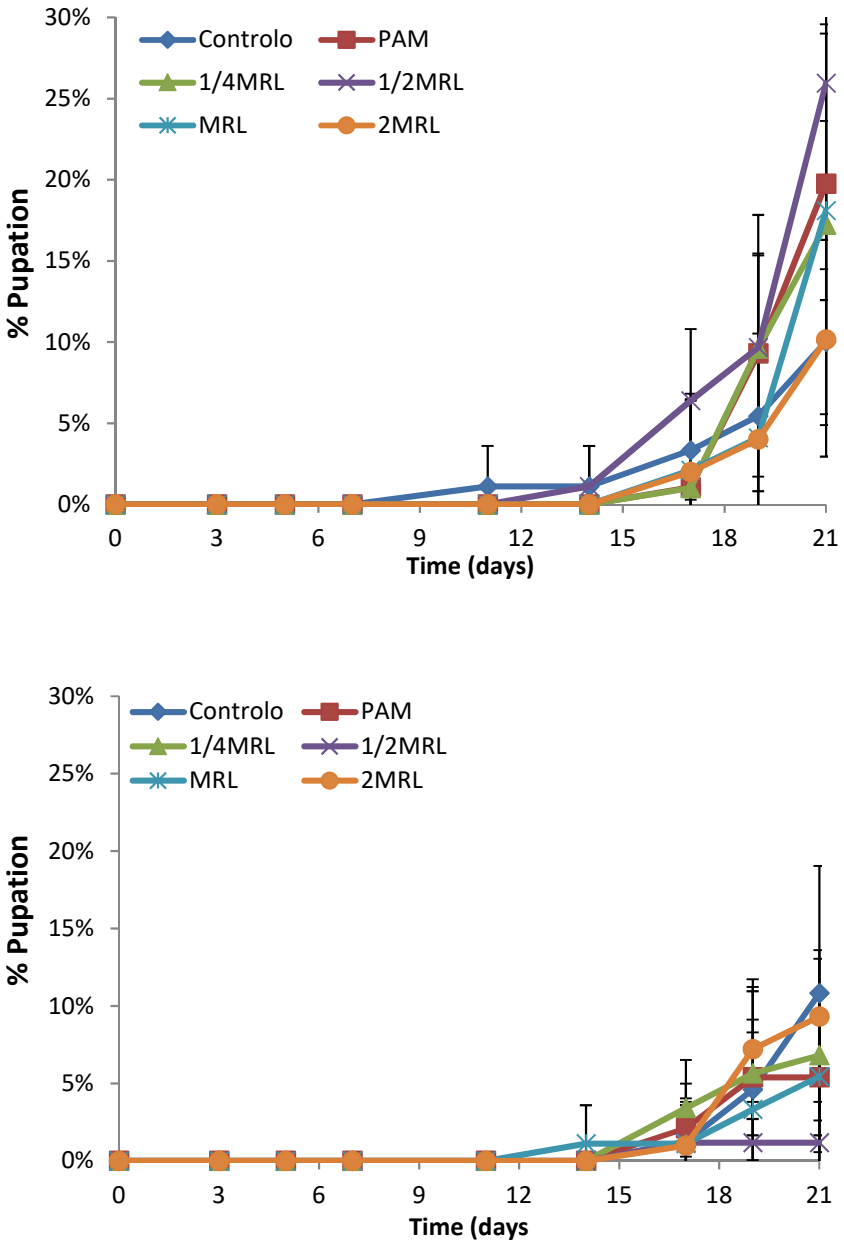

Figure 7 - Percentage pupation of the mealworm larvae in the five treatments and the control at 65% (top) and 40% (bottom) relative humidity (average ± 1 standard deviation)

4.5.3 Weight and Growth

Except for the control and treatment PAM, larva attained significantly higher body mass at 65% than at 40% relative humidity (Figure 5; F=13.69; P= 0.000554). The maximum average weight per larva was verified at 65% RH in treatments 1/4MRL, 1/2MRL and MRL but there was no significant effect of treatment on final body weight (F=1.558; P=0.1899).

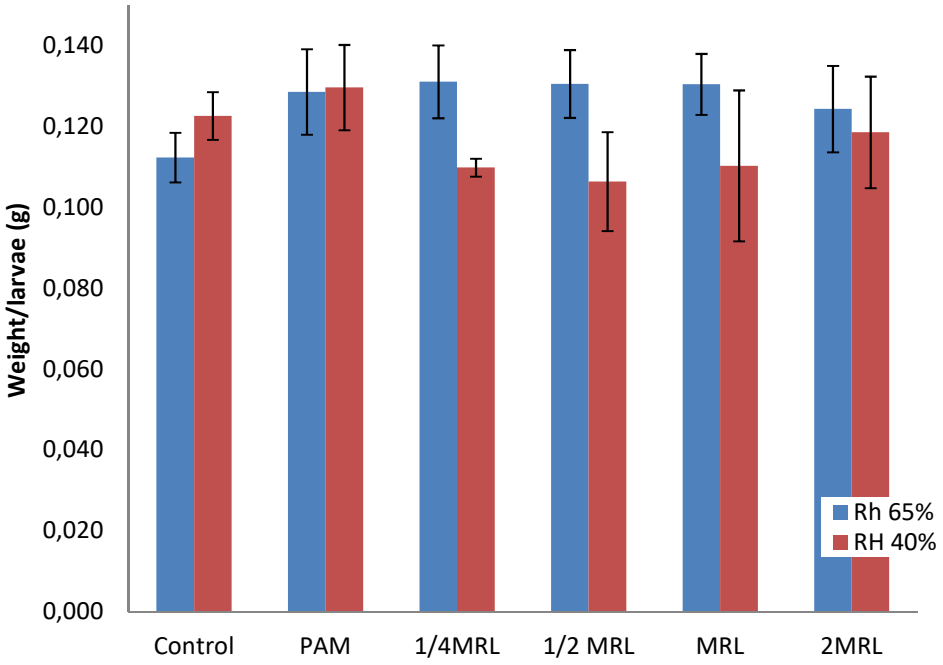

Figure 8 – Individual final weight of the mealworm larvae in the five treatments and the control at 65% and 40% relative humidity (average ± 1 standard deviation)

The highest growth rates were achieved at 40% relative humidity for all concentrations (Figure 6; F=7.421; P=0.0089). The highest growth rate occurred in the control where larvae grew, on average, approximately five times the initial weight. However, there was no significant effect of treatment on growth rates (F=0.667; P=0.650).

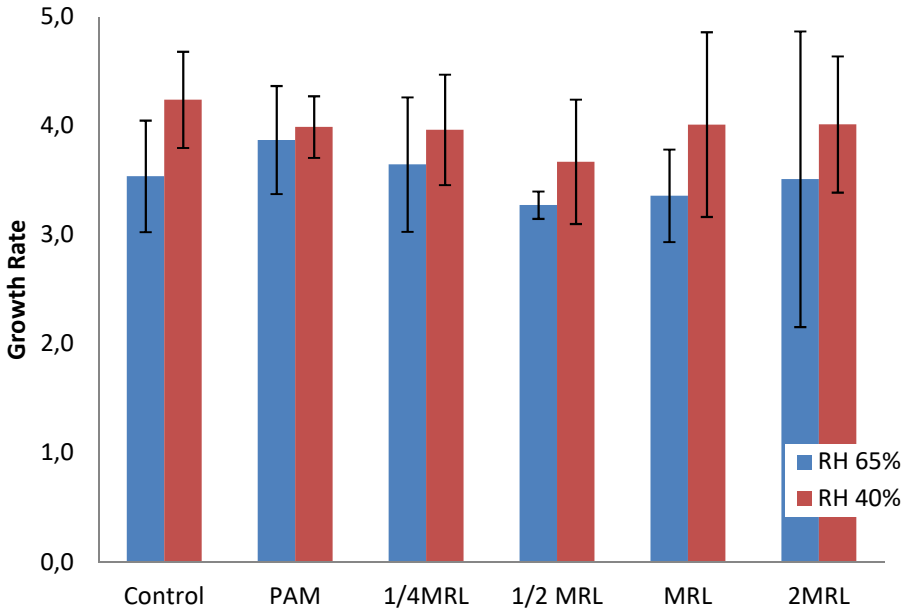

Figure 9 – Growth rate of the mealworm larvae in the five treatments and the control at 65% and 40% relative humidity (average ± 1 standard deviation)

As opposed to the values of weight per larva, the highest growth rates were achieved at 40% relative humidity for all concentrations. The highest growth rate occurred in "Control" samples where larvae grew, on average, approximately five times the initial weight. Statistically, only relative humidity had significant effects in mealworm growth rate (F=7.421; P=0.0089) with 40% promoting faster growth rates than 65% relative humidity, whereas treatments had none significance (F=0.667; P=0.650).

4.6 Discussion

No significant effect of treatment was found on any of the performance variables studied, even when the pesticide cocktail was provided at 2MRL. It could be that the pesticides commonly used in agriculture do not affect the performance of the mealworm larva. However, there are experimental evidences showing the pernicious effects of delthametrin in mealworm respiratory rhythm (Zafeiridou and Theophilidis, 2006) and pupae autonomic functions (Sláma and Miller, 1987). Even if the mealworm larvae are not affected by the pesticides, they may bio-accumulate them, thus constituting a contaminated food for humans or feed for animals. To elucidate this matter, the larvae used in the experiment were kept for chemical analyses.

Independently of the potential of the mealworm larvae to bio-accumulate the pesticides, the results obtained in the present study were conditioned by several experimental issues. The larvae deaths during the acclimatization period may, at least partially, have been caused by mishandling of the larvae due to the use of unsuitable tweezers. Because of that the experiment started with different numbers of larvae per replicate. Moreover, the size of the larvae was selected so that the end of the 21 days of the experiment coincided with the end of the larval stage or the beginning of pupation. However, the initial weight (and age) varied greatly among replicates (0.52 ± 0.79 SD), partially explaining the results obtained. At animals with such short lifecycles, the difference of a few days of age can have a clear influence in final weight, growth rate and pupation, thus masking the potential effects of the treatments and/or the relative humidity of rearing.

Contrarily to expected, at the end of trial there were still small larvae in some samples. It is not possible to know if it is a normal biological event, occurring only with younger larvae or naturally small individuals or whether there was any external factor of inhibition. Only in a complete lifecycle trial this could be verified.

Mortality during the experiment was probably also due to cannibalism since pieces of eaten larvae were found in samples. This led to the disappearance of larvae which were counted in mortality. Cannibalism is a relatively normal event at certain conditions such as overpopulation or low humidity (Hill, 2002; Weaver and McFarlane, 1990). In fact, mortality was higher at 40% than at 65% relative humidity.

Relative humidity of rearing had an effect on pupation, final body weight and growth rate. Growth rate was higher at 40% than at 65% relative humidity, in contradiction to previous studies that show the positive influence of moisture in the development of *Tenebrio molitor* (Fraenkel, 1950; Hardouin and Mahoux, 2003; Manojlovic, 1987; Punzo, 1975; Punzo and Mutchmor, 1980). The highest growth rate observed at 40% relative humidity was most probably the result of the smaller size of the larvae. In fact, growth in mealworms, like other animals, is represented by a Sigmoid curve (Urrejola et al., 2011), meaning that growth rate is faster in younger larvae. Thus, the smaller larvae of the 40% RH replicates grew faster than the bigger larvae of the 65% RH replicates. Similarly, the highest final weight and pupation obtained at 65% relative humidity were also most probably due to the bigger initial size of the larvae when compared to the larvae used at 40% RH.

In conclusion, several experimental issues prevented obtaining clear results in respect to the influences of relative humidity, the presence of pesticides and PAM on the performance of *Tenebrio molitor*.

5 Conclusions

In the continuous way for the development of new forms of food and feed and new sources of protein, the use of insects as a novel source is one of the leading trends. If on one hand, the industrial scale insect rearing is an appellative potential market, it is it is also true that only succeed if it can be profitable, ensuring a certain amount of factors. Issues such as the automation of production processes, food safety and environment sustainability are pressing needs that must be the subject of constant research and improvements.

Artificial insect rearing, both small and large scale has been object of intense research and will continue to be so in the coming times. In the list of most studied insects aimed to alternative protein productions are black soldier fly, locusts, grasshoppers, cockroaches and mealworms.

Tenebrio molitor is one of the easiest insects to rear at artificial conditions, due to the relatively wide range of physical and environmental conditions which can be successfully raised. Besides referred conditions it can also be fed on simple diets supplemented with water source provision such as vegetables.

The research carried out and reported in this report aimed to simulate some possible industrial scale conditions of water providing and assess potential pernicious effects in Tenebrio that could threaten food and feed safety. This assessment could not be completed since the biochemical results are not available yet, but expected to still be in useful time.

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Annexes

Annex I - Studies on *Tenebrio molitor* and variables studied

Mean Purpose	Conditions					
	Temperature range (°C)	**Relative humidity range (%)**	**Photoperiod**	**Diet**	**Physical conditions**	**Reference**
Effects of acclimation and rates of temperature change on critical thermal limits in *T. molitor* and *Cyrtobagous salviniae* (Curculionidae)	25 ± 0.5 ºC	43 ± 5%	12L:12D	Bran and carrots	-	(Allen, Clusella-Trullas and Chown, 2012)
Food value of mealworm grown on *Acrocomia aculeata* pulp flour	25 ºC	80%	10L:14D	**A (control diet): 50% wheat flour+ 50% soybean flour B: 50% diet A + 50% bocaiuva pulp flour C: 50% diet A + 50% ground bocaiuva kernel D: 50% bocaiuva pulp flour + 50% ground bocaiuva kernel**	400 insects per box (40×30×25cm)	(Alves *et al.*, 2016)
Response of *T. molitor* adults to potato	26 ± 2.5 ºC	75 ± 5.2%	-	oats	-	(Baek *et al.*, 2015)
The light reactions of the mealworm	-	-	-	-	-	(Balfour and Carmichael, 1928)
Two rearing substrates on *T. molitor* meal composition	25 ºC	70%	-	**Diets differing in fatty acids composition: linoleic (C18:2 n6) and α-linolenic (C:18:3 n3) acids content in diet A about 1/2 the one in diet B (29.5% vs 58.3% and 2.2% vs 4.1% respectively**	-	(Belforti M. *et al.*, 2014)
Growth performance and feed conversion efficiency of three edible mealworm species (Tenebrionidae) on organic by-products diets	28 ºC	65%	12L:12D	**Spent grains, beer yeast, bread remains, cookie remains, potato steam peelings and maize distillers' dried grains**	-	(Broekhoven, van *et al.*, 2015)
Photoperiodism and geotaxis in *T. molitor*	13.5-24 ºC	40-65%	**Varied**	Dry bran with rat cake ground	-	(Cloudsley-Thompson, 1953)
Metamorphosis in *T. molitor* larvae under grouped, isolated and starved conditions.	25 ± 1 °C	70%	-	Flour + 1% dried yeast	100-300 larvae per box Control larvae in plastic boxes (11×18×3 cm) + 1 cm height food; groups of ~20 animals	(Connat *et al.*, 1991)
Protein nutrition of *T. molitor*: nutritional values of various lactalbumins and lactalbumin hydrolysates	27 ºC	65%	-	**Varied artificial diets**	-	(Davis and Leclercq, 1969)

Mean Purpose	Conditions					
	Temperature range (°C)	**Relative humidity range (%)**	**Photoperiod**	**Diet**	**Physical conditions**	**Reference**
Protein nutrition of *T. molitor*: dietary protein concentration	27 ºC	65%	-	**Varied artificial diets**	-	(Davis and Sosulski, 1973)
Protein nutrition of *T. molitor*: Dietary Factors of Casein, Lactalbumin, and Lactalbumin Hydrolysate	27 ºC	65%	-	**Varied artificial diets**	-	(Davis, 1970a)
Protein nutrition of *T. molitor*: Improved Amino Acid Mixture and Dietary Carbohydrate	27 ºC	65%	-	**Varied artificial diets**	-	(Davis, 1974)
Growth response of larvae of *T. molitor* to concentrations of dietary amino acids	27 ºC	65%	-	**Varied artificial diets**	-	(Davis, 1978)
Oviposition in Coleoptera	27 ºC	-	-	Wheat flour	-	(Dick, 2008)
Nutrition of *T. molitor*	25 º C 30ºC (later tests)	70%	-	**Synthetic diet (casein, glucose, cholesterol, slat mixture, water) + vitamins**	-	(Fraenkel, 1950)
Oviposition Site Selection In *T. molitor*	18 ± 1 ºC	60%	12L:12D	**Control: fresh whole wheat flour + brewer's dried yeast (20:1)** **Treatments: 20:1 mixture of the material being tested + brewer's dried yeast (20:1):** • **Fresh whole wheat flour in all 14 layers** • **Conditioned whole wheat flour in top 7 layers and fresh whole wheat flour in bottom 7 layers** • **Fresh whole wheat flour in top 7 layers and conditioned whole wheat flour in bottom 7 layers** • **Conditioned whole wheat flour in all 14 layers** • **Wheat bran in all 14 layers**	Transparent plastic rings (2 cm deep × 10 cm diameter) taped together with transparent tape to form cylinders The cages covered on top by filter paper and a screened lid Filter paper wetted daily to provide water Bottom 13 rings filled with the tested foodstuff Top ring contained foodstuff in the bottom half and wheat bran in the top half to prevent foodstuff from becoming wet and sticking to the filter paper and beetles	(Gerber and Sabourin, 1984)
T. molitor as a Novel Source of Protein	25 ºC	70%-	8L:16D	Wheat flour + brewer's yeast (95:5 weight) + potato slices	100 larvae on plastic container (60 cm long × 45 cm wide × 15 cm deep)	(Ghaly and Alkoaik, 2009)
Effects of chronic hypoxia, normoxia and hyperoxia on larval development in *T. molitor*	25 ºC	-	12L: 12D.	Bran + occasional addition of lettuce and apples	30×70×20 cm	(Greenberg and Ar, 1996)

Mean Purpose	Conditions					
	Temperature range (°C)	**Relative humidity range (%)**	**Photoperiod**	**Diet**	**Physical conditions**	**Reference**
Breeding and use of insects in benefit of man and some animals	27 ºC	70%		**A: 400 g wheat bran** **B: 300 g wheat bran + 100 g soy flour** **C: 300 g wheat bran +100 g skim milk powder** **D: 200 g wheat bran + 100 g skim milk powder**	-	(Hardouin and Mahoux, 2003)
Pesticide contamination of T. *molitor* for human consumption	25 ± 1 ºC	40 ± 5%		Wheat bran + flour (50/50 wet weight) + slices of apple	-	(Houbraken *et al.*, 2016)
Protein nutrition of *T. molitor.* Growth response to dietary protein and of an amino acid mixture	27 ºC	65%	-	**Varied artificial diets**	-	(John, Davis and Sosulski, 1978)
Protein nutrition of *T. molitor.* Growth response of larvae to graded levels of amino acids	27 ± 0,25 ºC	65 ± 5%	-	**Varied artificial diets**	-	(John, Davis and Sosulski, 1979)
Growth characteristics of *T. molitor*	**12.5-35 ºC**	50-70%	**10L:14D** **12L:12D** **14L:10D**	-	48×49×33 cm	(Kim *et al.*, 2015)
Effects of Brewer's spent grain (BSG) on larval growth of *T molitor*	25 ºC	50-60%	14L:10D	**Wheat bran + fresh cabbage leaves or carrots as water source + different contents of Brewer's spent grain**	Plastic box (27×36×8)	(Kim *et al.*, 2016)
Temperature-dependent Development Model of Larvae of *T. molitor*	**15ºC to 30ºC**	60-70%	14L:10D	-	60×40×20cm	(Koo *et al.*, 2013)
Heritability of hsp70 expression in *T. molitor*	18 ± 1 ºC	85%	12L:12D	60% wheat flour + 20% oats + 10% wheat bran + 10% brewer's yeast	-	(Lardies *et al.*, 2014)
Nutritional requirements of *T. molitor*	26-27 ºC	90%	-	• **Wheat flour + 10% dry yeast** • **Wheat flour** • **Diet A= casein + glucose + cholesterol + McCallum's Salt mixture** • **Diet A + thiamine + riboflavin + nicotinic acid + pantothenic acid** • **Diet B = diet A + thiamine + riboflavin + nicotinic acid + pantothenic acid + pyridoxine** • **-Diet B + folic acid** • **-Diet B + folic acid + rice starch**	-	(Leclercq, 1948)

Mean Purpose	Conditions					
	Temperature range (°C)	**Relative humidity range (%)**	**Photoperiod**	**Diet**	**Physical conditions**	**Reference**
Feasibility of feeding *T. molitor* in bioregenerative life support systems as a source of animal protein for humans	28 ºC	70%	-	**Experimental group: wheat bran+ fermented straw +old cabbage leaves** **Control group: wheat bran +cabbage leaves**	2.55 larvae/cm^2	(Li, Zhao and Liu, 2012)
Effects of temperature and parental age on the life cycle of *T. molitor*	**25ºC and 30ºC**	70%	-	White flour	-	(Ludwig, 1956)
Stereoselectivity in bioaccumulation and excretion of epoxiconazole by *T. molitor* larvae.	25± 1 ºC	-	12L:12D	Bran soybean + corn + wheat (1:1:1)	50×30×30 cm	(Lv *et al.*, 2014)
Influence of the feeding of imagos and of climatic factors on the dynamics of oviposition and on the embryonal development of *T. molitor*	**Several**	**Several**	-	**Wheat flour** **Soybean flour**	-	(Manojlovic, 1987)
Nutritive Requirements of *T. molitor*	-	-	-	• **White wheat flour** • **Vitamin free diet: 20% casein + 74.5% corn starch + 4% salt mixture + 1% cholesterol + 0.5% crisco** • **Whole wheat flour** • **White wheat flour+ 5% beef blood** • **White wheat flour+ 10% beef muscle** • **White wheat flour+ 10% beef liver** • **Wheat flour+ 10% brewer's yeast** • **Vitamin wheat flour+10% brewer's yeast**	Immature larvae of same age and weight divided into groups of 25 Each group fed 15 grams of diet 2; each diet fed to three groups to obtain a triple check on results	(Martin and Hare, 1942)
Culturing *T. molitor* as food for animals in captivity.	25 ºC	70%	12L:12D	Wholemeal flour 685 g 47% (vol.) Bran 250 g 47% (vol) Yeastanin 35 g 3% (vol) Vionate 30 g 3% (vol)	Containers; internal surface area ~600 cm^2; substrate depth ~4 cm	(Martin, Rivers and Cowgill, 1976)
Importance of drinking water to larval insects	25 ºC, 35 ºC	30-35%	-	-	-	(Mellandby and French, 1958)
Effect of *Tenebrio* diet on predator *Podisus nigrispinus* development	25 ±-2 ºC	70 ± 10%	12L:12D	**T1: wheat bran (control)** **T2: pelleted feed for laying hens brand Extraovo 16 Guabi®** **T3: middlings for laying birds brand Posturave Guabi®** **T4: corn meal of brand Sinha**	-	(Menezes *et al.*, 2014)

Mean Purpose	Conditions					
	Temperature range (°C)	**Relative humidity range (%)**	**Photoperiod**	**Diet**	**Physical conditions**	**Reference**
Lifecycle of *T. molitor*	22,4 °C ± 5 °C	74,2% ± 20%	-	Pieces of fruit , vegetables and corn grains	114 and 96 mealworms; 250 cm^3 container	(Miryam, Bar and Oscherov, 2000)
Developmental plasticity in *T. molitor*	27 ± 1 °C	50 ± 5% RH	14L:10D	**10 g daily** **Wheat bran + 20% supplements:** • **dry potato flakes** • **dry egg white + dry potato (1:9)** • **dry egg white + dry potato (1:4)**	Plastic boxes 110×110×35 mm	(Morales-Ramos *et al.*, 2010)
Impact of Adult Weight, Density, and Age on Reproduction of *T. molitor*	28 ± 1 °C	75 ± 5%	-	Wheat bran and of reverse osmosis (RO) water twice a week.	Plastic tray 29×55×14.5 cm Plastic boxes 22×15×5.2 cm Glass tray 41×62.5×15 cm	(Morales-Ramos *et al.*, 2012)
Nutrient Self-Selection as a Diet Refining Tool in *T. molitor*	27 ± 1 °C	65 ± 5% RH	Darkness	**Different supplement %:** • **100% dry potato;** • **80% dry potato + 20% dry egg white;** • **80% dry potato + 20% soy protein;** • **80% dry potato + 5% dry egg white + 5% soy protein + 10% peanut oil** • **80% dry potato + 5% dry egg white + 5% soy protein + 10% canola oil** • **80% dry potato + 5% dry egg white + 5% soy protein + 10% salmon oil**	-	(Morales-Ramos *et al.*, 2013)
Effect of larval density on food utilization efficiency of *T. molitor*	26 ºC	70%	Darkness	**Wheat bran (>90%) + 5–10% dry potato squares once a week** **Adults misted with water twice a week with spray bottle**	-	(Morales-Ramos *et al.*, 2015)
Stimulus to feeding in larvae of *T. molitor*	25 ºC	70%	-	**Whole meal flour and middlings with ~ 5% dried yeast + endosperm, coarse bran flakes, cellulose powder, casein and egg albumin, soluble sugars, fats, fishmeal and dried yeast.**	large glass jar (7 lb capacity) containing	(Murray, 1960)
Importance of water in the normal growth of larvae of *T. molitor*	25 ºC	70%	-	Whole meal flour and middlings with ~5% dried yeast	large glass jar (7 lb capacity) containing	(Murray, 1968)
Low temperature tolerance of insects in relation to the influence of temperature on muscle apyrase activity	**Several**	**Several**	-	-	-	(Mutchmor and Richards, 1961)

Mean Purpose	Conditions					
	Temperature range (°C)	**Relative humidity range (%)**	**Photoperiod**	**Diet**	**Physical conditions**	**Reference**
Greenhouse gas and ammonia production by insect species suitable for animal or human consumption	25 ± 0 °C	79.8 ± 0.2% RH	Darkness	300 g mixed grain substrate (wheat, wheat bran, oats, soy, rye, corn + beer yeast) with on top pieces of carrot (6152 cm) weighing a total average of 637 g per repetition.	Plastic containers (50x30x8.7 cm).	(Oonincx *et al.*, 2010)
Feed Conversion, Survival and Development, and Composition of Four Insect Species on Diets Composed of Food By-Products	28 ºC	70%	12L:12D	**HPHF: 60% spent grains + 20% beer yeast + 20% cookie remains, HPLF: 50% beer yeast + 30% potato steam peelings + 20% beet molasses LPHF: 50% cookie remains + 50% bread LPLF: 30% potato steam peelings + 20% beet molasses + 50% bread**	Fifty larvae were placed in a plastic container (17.5 x 9.3 x 6.3 cm) with aeration slits in the sides	(Oonincx *et al.*, 2015)
Developmental characteristics of *T. molitor* larvae in different instars	25 ºC	-	-	-	T. molitor adults (about 1,000) were raised in acrylic boxes (width: 48 cm, length: 49.5 cm, height: 10.5 cm)	(Park *et al.*, 2014)
Effects of Temperature , Relative Humidity and Period of Exposure on the Survival Capacity of *T. molitor*	**25 °C (conclusions)**	**75% (conclusions)**	Darkness	-	-	(Punzo and Mutchmor, 1980)
Effects of temperature , moisture and thermal acclimation on the biology of *T. molitor*	**25 °C (conclusions)**	**75% (conclusions)**	Darkness	70% wheat bran + 30% corn meal	-	(Punzo, 1975)
Use of *T. molitor* to recycle organic wastes and as feed for broiler chickens.	28 ± 2 ºC	60-70% RH	-	**90 % bran + 10% yeast by weight; different combinations of organic wastes, yeast and excreta of *T. molitor***	30x20x15 cm, containing 500 g of diet for 90 d	(Ramos-Elorduy *et al.*, 2002)
Nutritional of *T. molitor* as Food Source	25±1 ºC	50% ±10%	14 L: 10D	Main food: wheat bran + fresh vegetables (cabbage, reddish, carrot) as water source twice a week	-	(Ravzanaadii *et al.*, 2012)

Mean Purpose	Conditions					
	Temperature range (°C)	**Relative humidity range (%)**	**Photoperiod**	**Diet**	**Physical conditions**	**Reference**
Nutrient balancing in T. *molitor*	25 ºC	-	12L:12D	**Nine foods differing in protein (P) to digestible carbohydrate (C) ratios (% dry mass):** • **0:42** • **5.6:28** • **7:35** • **14:28** • **21:21** • **28:5.6** • **28:14** • **35:75** • **42:0** **P= 3 casein:1 peptone:1 albumen; C= sucrose** **Other constituents: 2.4% Wesson's salt; 0.5% cholesterol; 0.5% linoleic acid, 0.3% ascorbic acid; 0.2% vitamin mix** **Remainder food: indigestible cellulose**	300–400 individuals per clear plasticbox (40x17x11cm) where water-soaked cotton was provided as a water source thrice a week.	(Rho and Lee, 2014)
Balanced intake of protein and carbohydrate and lifetime reproductive success in *T. molitor*	25 ºC	-	12L:12D	**Three synthetic foods; same amount of calories; different ratios (% dry mass) of protein (P) to digestible carbohydrate (C):** • **7:35** • **21:21** • **35:7** **P= 3 casein:1 peptone:1 albumen; C= sucrose** **Other constituents: 2.4% Wesson's salt, 0.5% cholesterol, 0.5% linoleic acid, 0.3% ascorbic acid, 0.2% vitamin mix** **Remainder food (ca. 54%): indigestible cellulose powder**	300–400 individuals per clear plastic box (40×17×11cm) water source provided 2× per week by water-soaked cotton	(Rho and Lee, 2016)
Larvae of *T. molitor* as European novel food	25 ˚C	-		Oat flakes + vegetables as water source	40 × 30 × 25 cm	(Siemianowska *et al.*, 2013)
Insects as food: Assessing the food conversion efficiency of *T. molitor*	20 °C – 23 °C	25-30%	-	Rolled oats + carrots	10 larvae per plastic storage container measuring 3015 cm	(Spang, 2013)

Mean Purpose	Conditions					
	Temperature range (°C)	**Relative humidity range (%)**	**Photoperiod**	**Diet**	**Physical conditions**	**Reference**
Regulatory effects of *T. molitor* on immunological function in mice	26±1 °C	55± 5%	12L:12D	Wheat bran + whole wheat flour + brewer's yeast (50:45:5 w/w)	-	(Tang, Dai e Zhou, 2012)
Molecular cloning and characterization of autophagy-related gene TmATG8 in Listeria-invaded hemocytes of *T. molitor.*	26 ± 1 °C	60 ± 5%	16L:8D	Autoclaved artificial diet: 200 g wheat bran + 20 g bean powder + 10 g brewer's yeast + 0.15 g chloramphenicol + 1.1 g sorbic acid + 1.1 ml propionic acid in 440 mL distilled water	-	(Tindwa and Jo, 2015)
Inhibition of pupation due to crowding in tenebrionid beetles.	25 ºC	-	-	Wheat bran + water	Petri dishes 10 cm diameter	(Tschinkel and Willson, 1971)
Diet-induced developmental plasticity in life histories and energy metabolism in a beetle	18 ± 1 ºC	-	12L:12D	**A) Low Protein: 10.25%** **B) Low Protein Control: 10.25%** **C) High Protein: 25.25%** **D) High Protein Control: 25.25%**	Eggs separated; after hatching, larvae placed individually on 6-well plates	(Urrejola, Nespolo and Lardies, 2011)
Effect of moisture on growth rate and development of two strains of *T. molitor*	26.7 ºC	50%	16L:8D	Screened wheat short + distilled water provided on cotton pads	Plastic dishes (3,8 cm high and 150 cm dia) with	(Urs and Hopkins, 1973)
Diversity of digestive proteinases in *T. molitor* arvae.	25 °C	-	-	Milled oat flakes + wheat bran (1:1)	-	(Vinokurov *et al.*, 2006)
Effect of larval density on growth and development of *T. molitor*	30 ± 1 ºC	55 ± 5%	14L:10D	Wheat bran + whole wheat flour + brewer's yeast (50:45:5 wet wt)	**4.55 L rearing jars with several densities**	(Weaver and McFarlane, 1990)
Potential of grease from *T. molitor* as a novel biodiesel feedstock	25–30 ºC	60–75%	-	Decayed vegetables provided as needed	-	(Zheng *et al.*, 2013)

Annex II – Registry of initial population characteristics

Group	Initial weight (g)	Weight/Larva average
Control	0,45	0,023
	0,47	0,024
	0,47	0,024
	0,57	0,029
	0,54	0,027
PAL	0,51	0,026
	0,51	0,026
	0,51	0,026
	0,59	0,030
	0,53	0,027
1/2 LMR	0,63	0,032
	0,65	0,033
	0,65	0,033
	0,57	0,029
	0,56	0,028
1/4 LMR	0,61	0,031
	0,53	0,027
	0,61	0,031
	0,44	0,022
	0,68	0,034
LMR	0,65	0,033
	0,62	0,031
	0,53	0,027
	0,59	0,030
	0,62	0,031
2LMR	0,58	0,029
	0,40	0,020
	0,62	0,031
	0,68	0,034
	0,60	0,030
MEAN 65% (RH)		0,0283
Control	0,45	0,023
	0,47	0,024
	0,47	0,024
	0,45	0,023
	0,51	0,026
PAL	0,51	0,026
	0,53	0,027
	0,50	0,025
	0,56	0,028
	0,50	0,025
1/2 LMR	0,48	0,024
	0,38	0,019
	0,45	0,023
	0,43	0,022
	0,56	0,028
1/4 LMR	0,39	0,020
	0,44	0,022
	0,45	0,023
	0,49	0,025
	0,46	0,023
LMR	0,45	0,023
	0,49	0,025
	0,51	0,026
	0,35	0,018
	0,42	0,021
2LMR	0,41	0,021
	0,52	0,026
	0,51	0,026
	0,45	0,023
	0,49	0,025
MEAN 40%		0,0235
GLOBAL MEAN		0,0259

Annex III – Trial monitoring registry

		Week	1						2						3						4			
		T (days)	Adaptation period				0		3		5		7		11		14		17		19		21	
	Group	Sample #	OBSERVATIONS (D-deaths; P – pupae)																					
			D	P	D	P	D	P	D	P	D	P	D	P	D	P	D	P	D	P	D	P	D	P
		1					1		1		2				1							2		
		2	1		1						1									1				
	Control	3			3		1		2										2					1
		4			2										2	1					1			1
		5	1		1								1							1				2
		6			1		1		1		1											1		1
		7							1		1											1		2
	PAL	8			1		1		1		1													3
		9					1				1								1	1		3		
		10									1											3		4
		11			2						1						1			1		1		2
		12							1						1					2		1		2
	1/2 MRL	13	2										1							1				6
		14			2		1								1						1	1		1
RH		15			1				1				1				1			1				4
65%		16			3																	2		2
		17			1				2		1											2		2
	1/4 MRL	18									1									1	1	1		
		19							1		1		2						1					
		20	1								1						1					3	1	3
		21					1		2										2	2				
		22							1													2		2
	MRL	23							2		1													5
		24																						4
		25											1						1					3
		26							1											2		1		
		27	1						3				2											
	2MRL	28			1										1									3
		29									1											1	1	2
		30											3								2			1
		31							1		2				2		1		1					3
		32	1		1				1															1
	Control	33					1						1									2		2
		34					1		1		2													
		35	2		1		1													1		1		
		36	1																	1				
		37			1		2				1											1	1	
	PAL	38			1						1				2		1					1		
		39			1				1				1		1		1			1		1	1	
		40					2		2		2				1									
		41	1				1		1															
		42	1								1													
	1/2 MRL	43	2						1		2						1							
		44	3						3														1	
RH		45			3				1											1				
40%		46	1						2		2						1		2	1		1	1	
		47					1		1		3						1							
	1/4 MRL	48			1		2		1								1			1			1	1
		49	1		2				2		1								1	1		1		
		50	1														2							
		51							1										1					1
		52	1				1															1	1	1
	MRL	53			2				1		1						2	1				1		
		54	1		2								1		2									
		55					2		1		1										1			
		56							1		1								1	1		1		
		57					1										1							1
	2MRL	58			1		1				1											1		
		59			1				1		1											2		1
		60																			1	2		
		Σ	22	0	36	0	23	0	43	0	37	0	14	0	14	1	14	2	13	21	7	41	8	67

n	T=0	1142
	Deaths	173
	T=21	969
	%	15%

Annex IV– Results of Statistical Analysis

Table IV.1 - Statistical results for 65% of Relative Humidity

Group	Sample #	Initial weight (gr)	Mean	n	Weight/larva Average	n T=0	T (days)	Massa final	Média	n	n+P	n+D+P	Weight/larva Average	Mean	Growth Rate	Death Rate	Mean
	1	0,45			0,023	19		1,454		11	13	18	0,112		5,0	0,32	
	2	0,47			0,024	18		1,763		15	16	19	0,110		4,7	0,11	
Control	3	0,47	0,50		0,024	16		1,251	1,6212	10	11	19	0,114	0,112	4,8	0,31	0,19
	4	0,57			0,029	18		1,571		13	15	20	0,105		3,7	0,17	
	5	0,54			0,027	18		2,067		14	17	20	0,122		4,5	0,06	
	6	0,51			0,026	18		2,055		13	15	19	0,137		5,4	0,17	
	7	0,51			0,026	20		2,08		15	18	20	0,116		4,5	0,10	
PAL	8	0,51	0,53		0,026	18		2,084	2,1212	12	15	19	0,139	0,129	5,4	0,17	0,13
	9	0,59			0,030	19		2,119		12	16	19	0,132		4,5	0,16	
	10	0,53			0,027	20		2,268		12	19	20	0,119		4,5	0,05	
	11	0,63			0,032	18		2,336		12	17	20	0,137		4,4	0,06	
	12	0,65			0,033	20		2,424		13	18	20	0,135		4,1	0,10	
1/2 MRL	13	0,65	0,61		0,033	18		2,339	2,1514	10	17	20	0,138	0,131	4,2	0,06	0,11
	14	0,57			0,029	17		1,668		12	14	19	0,119		4,2	0,18	
	15	0,56		20	0,028	19	21	1,99		11	16	20	0,124		4,4	0,16	
	16	0,61			0,031	17		2,332		13	17	20	0,137		4,5	0,00	
	17	0,53			0,027	19		2,226		12	16	20	0,139		5,3	0,16	
1/4 MRL	18	0,61	0,57		0,031	20		2,384	2,1326	16	18	20	0,132	0,131	4,3	0,10	0,14
	19	0,44			0,022	20		1,627		14	14	19	0,116		5,3	0,30	
	20	0,68			0,034	19		2,094		10	16	20	0,131		3,8	0,16	
	21	0,65			0,033	19		1,375		9	11	16	0,125		3,8	0,42	
	22	0,62			0,031	20		2,702		15	19	20	0,142		4,6	0,05	
MRL	23	0,53	0,60		0,027	20		2,231	2,126	12	17	20	0,131	0,131	5,0	0,15	0,18
	24	0,59			0,030	20		2,087		13	17	17	0,123		4,2	0,15	
	25	0,62			0,031	20		2,235		14	17	19	0,131		4,2	0,15	
	26	0,58			0,029	20		2,182		15	18	19	0,121		4,2	0,10	
	27	0,40			0,020	19		1,789		13	13	19	0,138		6,9	0,32	
2MRL	28	0,62	0,58		0,031	19		2,087	1,941	13	16	18	0,130	0,124	4,2	0,16	0,20
	29	0,68			0,034	20		2,23		15	18	20	0,124		3,6	0,10	
	30	0,60			0,030	20		1,417		12	13	18	0,109		3,6	0,35	
MEAN 65%			0,57		0,0283			2,016		12,7	15,9	19,2	0,126		4,5		

Table IV.2 - Statistical results for 40% of Relative Humidity

Group	Sample #	Initial weight (gr)	Mean	n	Weight/larva Average	n T=0	T (days)	Massa final	Média	n	n+P	n+D+P	Weight/larva Average	Mean	Growth Rate	Death Rate	Mean
	31	0,45			0,023	20		1,503		9	12	19	0,125		5,6	0,4	
	32	0,47			0,024	18		2,137		16	17	20	0,126		5,3	0,1	
Control	33	0,47	0,47		0,024	19		2,042	1,8598	14	18	20	0,113	0,123	4,8	0,1	0,17
	34	0,45			0,023	19		1,929		15	15	19	0,129		5,7	0,2	
	35	0,51			0,026	16		1,688		12	14	18	0,121		4,7	0,1	
	36	0,51			0,026	19		2,212		18	19	20	0,116		4,6	0,0	
	37	0,53			0,027	17		2,074		14	15	20	0,138		5,2	0,1	
PAL	38	0,50	0,52		0,025	19		1,838	1,908	13	14	19	0,131	0,130	5,3	0,3	0,19
	39	0,56			0,028	19		1,834		11	13	19	0,141		5,0	0,3	
	40	0,50			0,025	18		1,582		13	13	20	0,122		4,9	0,3	
	41	0,48			0,024	18		1,801		16	16	19	0,113		4,7	0,1	
	42	0,38			0,019	19		1,574		16	16	18	0,098		5,2	0,2	
1/2 MRL	43	0,45	0,46		0,023	18		1,249	1,5758	14	14	20	0,089	0,106	4,0	0,2	0,17
	44	0,43			0,022	17		1,473		13	13	20	0,113		5,3	0,2	
	45	0,56		20	0,028	17	21	1,782		14	15	19	0,119		4,2	0,1	
	46	0,39			0,020	19		1,125		8	10	19	0,113		5,8	0,5	
	47	0,44			0,022	19		1,546		14	14	20	0,110		5,0	0,3	
1/4 MRL	48	0,45	0,45		0,023	17		1,444	1,4472	11	13	19	0,111	0,110	4,9	0,2	0,27
	49	0,49			0,025	17		1,308		10	12	19	0,109		4,4	0,3	
	50	0,46			0,023	19		1,813		17	17	20	0,107		4,6	0,1	
	51	0,45			0,023	20		2,009		17	18	20	0,112		5,0	0,1	
	52	0,49			0,025	18		2,099		15	17	20	0,123		5,0	0,1	
MRL	53	0,51	0,44		0,026	18		1,296	1,6544	11	13	19	0,100	0,110	3,9	0,3	0,19
	54	0,35			0,018	17		1,02		12	12	18	0,085		4,9	0,3	
	55	0,42			0,021	18		1,848		14	14	19	0,132		6,3	0,2	
	56	0,41			0,021	20		1,57		12	14	17	0,112		5,5	0,3	
	57	0,52			0,026	19		2,347		16	17	19	0,138		5,3	0,1	
2MRL	58	0,51	0,48		0,026	18		1,608	1,857	15	16	19	0,101	0,119	3,9	0,1	0,19
	59	0,45			0,023	19		1,806		12	15	18	0,120		5,4	0,2	
	60	0,49			0,025	20		1,954		14	16	17	0,122		5,0	0,2	
MEAN 40%			0,47		0,0235			1,717	1,717	13,53	14,73	19,10	0,116		5,0		

Table IV.3 – Two-way Factorial ANOVA

Effect	Degr. of Freedom	%SURV SS	%SURV MS	%SURV F	%SURV p	GROWTH SS	GROWTH MS	GROWTH F	GROWTH p	%PUP SS	%PUP MS
Intercept	1	*44,99759*	*44,99759*	*5466,474*	*0,000000*	*846,2716*	*846,2716*	*2081,811*	*0,000000*	*0,208193*	*0,208193*
TREAT	5	0,03866	0,00773	0,939	0,464288	1,3564	0,2713	0,667	0,650044	0,015752	0,003150
RH	1	0,01206	0,01206	1,466	0,231979	*3,0166*	*3,0166*	*7,421*	*0,008966*	*0,093886*	*0,093886*
TREAT*RH	5	0,04891	0,00978	1,188	0,328976	0,5932	0,1186	0,292	0,915168	*0,050088*	*0,010018*
Error	48	0,39511	0,00823			19,5124	0,4065			0,187058	0,003897
Total	59	0,49474				24,4785				0,346784	

Effect	Univariate Results for Each DV (TENEBRIOII) Sigma-restricted parameterization Effective hypothesis decomposition								
	%PUP F	MORT SS	Weight SS	Weight MS	Weight F	Weight p	INITIAL weight SS	INITIAL weight MS	INITIAL weight F
Intercept	*53,42346*	*1,915021*	*0,882935*	*0,882935*	*8058,697*	*0,000000*	*0,040171*	*0,040171*	*4762,176*
TREAT	0,80842	0,031156	0,000854	0,000171	1,558	0,189925	0,000040	0,000008	0,956
RH	*24,09153*	0,020221	*0,001500*	*0,001500*	*13,692*	*0,000554*	*0,000348*	*0,000348*	*41,255*
TREAT*RH	*2,57059*	0,045206	*0,002460*	*0,000492*	*4,490*	*0,001949*	*0,000124*	*0,000025*	*2,930*
Error		0,570620	0,005259	0,000110			0,000405	0,000008	
		203	0,010072				0,000917		

Effect	Univariate Results for Each DV (TENEBRIOII)
	INITIAL weight p
Intercept	*0,000000*
TREAT	0,453824
RH	*0,000000*
TREAT*RH	*0,021831*
Error	
Total	