

## Article

# Influence of Temperature, Relative Humidity and Protein Content on the Growth and Development of Larvae of the Lesser Mealworm, *Alphitobius diaperinus* (Panzer)

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**Abstract:** The human population is rapidly growing, subsequently leading to an increase in food and protein demand. Therefore, alternative protein sources have to be evaluated as food and feed. Among the most promising alternative protein sources with significant advantages are insects. Nevertheless, insect rearing conditions have to be optimized prior to insect mass production. In the present study, using laboratory bioassays, we evaluated the effect of several biotic and abiotic factors on the development of the larvae of the lesser mealworm, *Alphitobius diaperinus* (Panzer). In the first series of bioassays, we investigated *A. diaperinus* larval growth at three temperatures (25, 30 and 32 °C) and two relative humidity (r.h.) levels (55 and 75%). Furthermore, in the second series of bioassays, the larval growth was assessed on wheat bran-based substrates with different percentages of yeast, i.e., 0%, 10%, 17.5%, 25%, 32.5% and 40%. According to our results, the temperature was shown to be highly important for larval development, with *A. diaperinus* larvae performing better at the higher temperatures tested, i.e., 30 and 32 °C. In contrast, relative humidity did not have a significant effect on *A. diaperinus* growth, at least for the relative humidity levels tested. Finally, the increase in the percentage of yeast in the diet increased larval growth, development and survival. Our study aims to highlight the significance of several biotic and abiotic factors for the rearing of *A. diaperinus* larvae, providing parameters that can be further utilized in mass rearing protocols of this species.

**Keywords:** alternative protein source; edible insects; insect protein; insects as food and feed; larval growth; lesser mealworm



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## 1. Introduction

Traditionally, insects have been an important component of the human diet in many tropical countries around the world [1]. In contrast, they were never really integrated into the Western dietary patterns and they were rarely given any consideration as part of the sustainability and food security agendas of international organizations [2]. However, in recent years, the rapidly increasing demand for nutrient sources due to the high global population growth, along with the imperative need for more environmentally friendly and sustainable food practices and habits, eventually led to the re-evaluation of insects as an alternative nutrient source for human food and animal feed [2–5]. As an outcome of the increasing willingness to adopt insect-based feeds, in 2017 the European Union (EU) enforced regulations for the utilization of insects as ingredients of aquafeeds [6]. More recently, in September 2021 the EU Regulation 2021/1372 came into force and authorized insect proteins in poultry and pig diets, widening the perspectives of the insect sector and further boosting the exploitation of insects in animal nutrition [7]. Apart from the use of insects as feed, recently the larvae of the yellow mealworm, *Tenebrio molitor* L. (Coleoptera: Tenebrionidae), have been approved by the European Food Safety Authority (EFSA) as food for human consumption [8].

Among the insect species that have been registered for aquafeeds, most of the research conducted so far refers to two insect species, *T. molitor* and the black soldier fly, *Hermetia illucens* (L.) (Diptera: Stratiomyidae) [5]. An insect species with great potential as a nutrient source for food and feed that has lately attracted considerable scientific and commercial interest is the lesser mealworm, *Alphitobius diaperinus* (Panzer) (Coleoptera: Tenebrionidae) [9]. Its larvae hold a high protein and lipid content, whereas their essential amino acids are comparable to the ones of soybean protein [9–12]. Recent research on *A. diaperinus* thoroughly evaluated different diets, emphasizing the exploitation of various organic side-streams as diet components [13–17]. For instance, recent studies evaluated the suitability of several byproducts of the cereal and legume seed cleaning process [17], as well as diets based on side-streams from the agri-food sector, e.g., rapeseed meal, rice bran, etc. [16] for the rearing of *A. diaperinus* larvae. However, there are still several knowledge gaps regarding the impact of key biotic and abiotic factors on *A. diaperinus* larval growth that have to be addressed. Hence, apart from the diet *per se*, there is still an inadequate amount of quantified information on the temperature and relative humidity levels that can be used with success in mass rearing protocols of this species. In fact, available data are focused on the evaluation of these parameters in individual development, since in these studies *A. diaperinus* was examined as a pest species, and not as an edible species that can be mass-reared ([17] and references therein). The same holds true for the effect of certain diet components, such as the protein content, where there are disproportionately few data for *A. diaperinus*, when compared with *T. molitor* or *H. illucens* [18–21].

Based on the above, the objective of the present study was to examine the effect of a series of biotic and abiotic factors on the growth and development of *A. diaperinus* larvae. Particularly, in two series of laboratory bioassays, we assessed the impact of temperature and relative humidity, as well as the effect of the diet protein content on the growth of *A. diaperinus* larvae.

## 2. Materials and Methods

### 2.1. Insects

Individuals of *A. diaperinus* from the colonies maintained at the Laboratory of Agricultural Zoology in the University of Thessaly were used in the experiments. Larvae of *A. diaperinus* were originally purchased in September 2019 from a local retailer and had been reared since then with a diet comprised of wheat bran (90%) and dry instant yeast (10%) (Angel Yeast Co. Ltd., Yichang, China) supplemented with fresh potato slices twice a week. Insects were kept in plastic boxes (48 cm length × 28 cm width × 10 cm height) with a rectangular screen opening (19 cm × 27 cm) on the top cover to allow air circulation. To acquire eggs and newly hatched larvae, around 500 mixed sex adults (<1 month old) were transferred from the stock culture to a plastic container with 100 g of pre-sieved white flour, in order for the beetles to mate and oviposit. After 3 d, the flour was sieved with two different sieves: a 2 mm opening sieve and a 250 µm opening sieve. Adults were kept on the first sieve, whereas eggs were collected on the second sieve. Newly-emerged larvae (<2 d old) hatching from the collected eggs were used for experimentation. All rearing boxes with the stock *A. diaperinus* colonies were kept in incubators set at constant conditions, i.e.,  $26 \pm 1$  °C,  $55 \pm 5\%$  relative humidity (r.h.) and continuous darkness.

### 2.2. Bioassays Series I: Effect of Temperature and Relative Humidity

In this series of bioassays, the effect of temperature and r.h. on the growth and development of *A. diaperinus* larvae, as well as on their feed conversion efficacy, was evaluated under laboratory conditions. All the combinations of three temperatures (25, 30 and 32 °C) and two r.h. levels (55 and 75%) were tested, in incubators set at the desired conditions. Plastic cylindrical vials (7.5 cm in diameter, 8.8 cm in height) were filled with 1 g of wheat bran. Afterwards, a group of 50 newly-hatched larvae was transferred to each vial. Each group of larvae was weighed at the beginning of the experiment, and their initial weight was recorded. Larvae were provided with fresh carrot slices ( $0.6 \pm 0.1$  g)

three times per week, and old carrot pieces were removed. Larvae were allowed to feed undisturbed *ad libitum* for a 4-week period. Vials were monitored three times per week for food consumption to prevent the larvae from running out of food. If food was totally consumed, new food was weighed, added and recorded. After the 4-week interval, larval weight as a group and larval survival rate were monitored every two weeks until the emergence of the first pupa. The development time was calculated as the number of days between the start of the experiment and the day each vial was harvested. Food consumption and weight gain data were used to calculate food utilization parameters as previously described [22]. Specifically, the feed conversion ratio (FCR), i.e., the amount of feed needed (in kg) to obtain one kg of weight increase of the production animal, was calculated following the formula:

$$\text{FCR} = (\text{feed consumed})/(\text{weight gained}),$$

whereas the specific growth rate (SGR, % day<sup>−1</sup>) was calculated according to the following equation:

$$\text{SGR} = 100 \times (\ln\text{FBW} - \ln\text{IBW})/\text{days},$$

with FBW and IBW being the final and initial body weight, respectively. Both FCR and SGR were calculated on a fresh weight basis. For FCR and SGR calculations, we made the assumption that all provided feed was consumed, as previously described [23]. This assumption was verified by visual observations to ensure that the provided feed was converted to frass. However, a small amount of feed might have been left unconsumed in some cases. The weight of the provided carrots was excluded from the calculations. There were 6 vial replicates for each treatment, i.e., each temperature and r.h. combination.

### 2.3. Bioassays Series II: Effect of Protein Content in the Diet

In this series of bioassays, diets with different percentages of protein content were designed and evaluated. Specifically, the development of *A. diaperinus* larvae was investigated in mixtures of wheat bran with different percentages of dry instant yeast (0%, 10%, 17.5%, 25%, 32.5% and 40%). The nitrogen content of the wheat bran (2.67%) was determined on a dry matter basis by Kjeldahl analysis (behr Labor-Technik GmbH, Germany, K12-block standard digestion system, programmable infrared digestion device, S4 distillation unit) and was converted to protein using the Jones' default nitrogen-to-protein conversion factors (Kp) of 6.25 (16.7% protein). For yeast, the nutrient composition information provided by the manufacturer on the product label was used (50% protein), whereas the protein content of the diets tested was calculated by multiplying the inclusion level of each ingredient (wheat bran, yeast) with its protein content and summing these values per diet. Based on the above, the protein content of the diets tested was 16.7%, 20.0%, 22.5%, 25.0%, 27.5% and 30%, respectively. The experimental design described in Bioassays Series I was also followed in this series of bioassays. Briefly, there were 6 replicates per dietary treatment, whereas in all treatments larvae were provided with fresh carrot slices ( $0.6 \pm 0.1$  g) three times per week. All vials were kept at constant conditions, i.e., 26 °C, 55 r.h. and continuous darkness, arranged on a bench inside the incubator in a completely randomized design.

### 2.4. Statistical Analysis

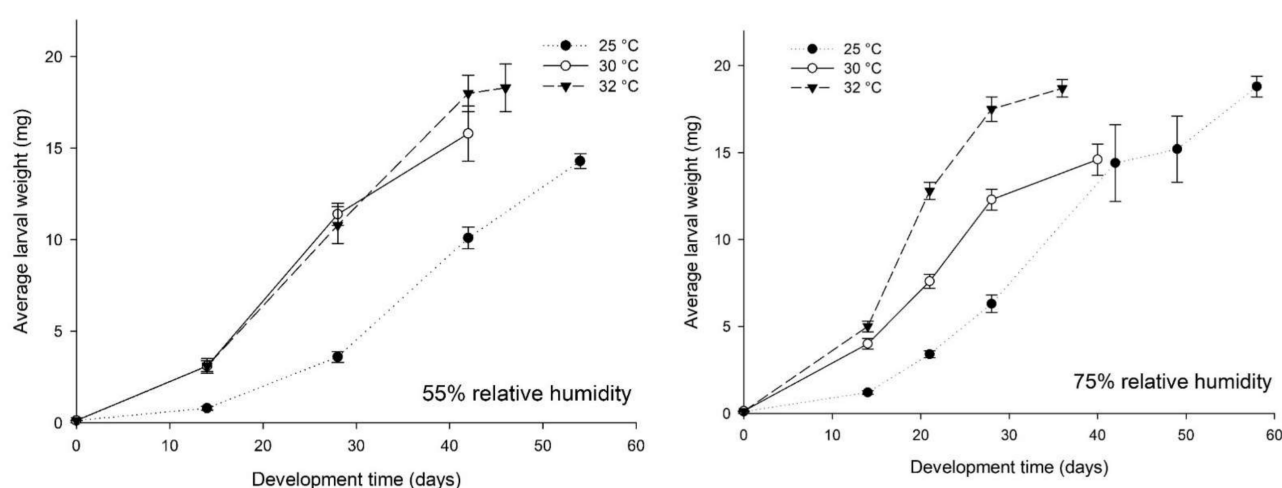
Prior to analysis, the data of Bioassays I and II (FCR, SGR, total produced larval biomass) were first checked for normality and homogeneity of variances using the Shapiro–Wilk and Levene's tests, respectively. Since the assumptions for parametric analysis were met for both bioassays, the data of Bioassay I were submitted to a two-way ANOVA, with FCR, SGR and total larval biomass production as the response variables and temperature with relative humidity as the main effects, whereas the data of Bioassay II were analyzed using an one-way ANOVA [24]. When significant differences were found among treatments (different temperature and relative humidity conditions in Bioassay I, different protein content in Bioassay II), the Tukey–Kramer HSD test was used to statistically compare

the values obtained for the different treatments. For both bioassays, the Kaplan–Meier method was used to analyze development time and a Mantel–Cox test was used to detect differences among treatments. The Pearson correlation test was used to determine the correlation between development time and SGR. All data were analyzed using SPSS 26.0 (IBM Corporation, Armonk, NY, USA).

### 3. Results

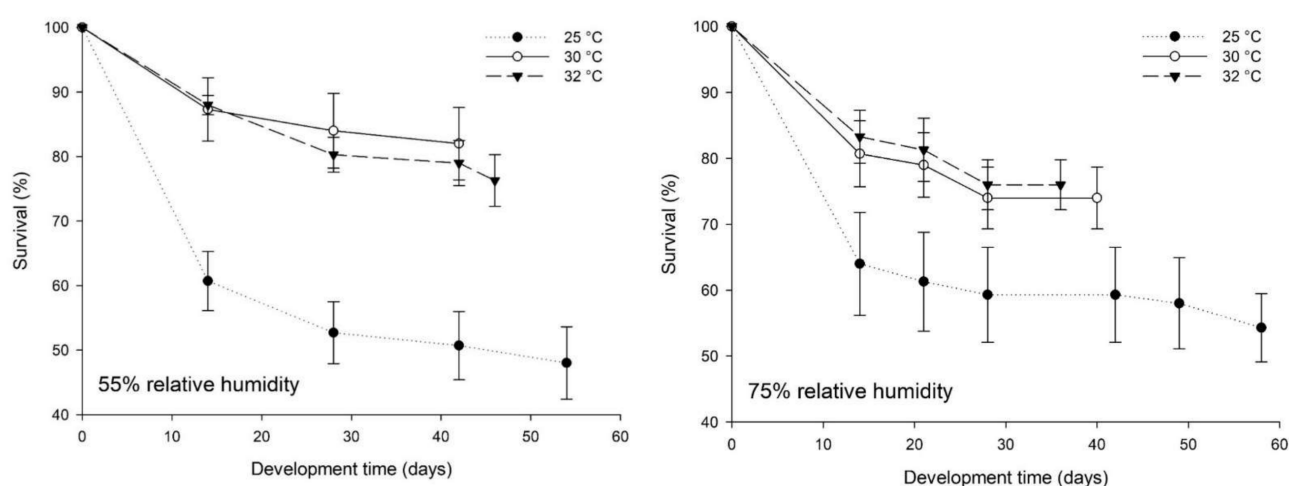
#### 3.1. Bioassays Series I: Effect of Temperature and Relative Humidity

The average larval weight and survival of *A. diaperinus* larvae grown at the different combinations of temperature and r.h. levels are presented in Figures 1 and 2, respectively. FCR and the total insect biomass were significantly affected by temperature, but not by relative humidity and their associated interaction, whereas SGR was significantly affected by both main effects, but not by their interaction (Table 1). At 55% r.h., high larval survival rates were recorded at 30 and 32 °C, as 80 and 76% of the individuals were alive at the end of the observation period, respectively. At 25 °C and 55% r.h., larval survival rates were lower and reached 60% after 4 weeks of development and 48% at the end of the observation period. A similar pattern was noted for survival rates at 75% r.h., where 54, 74 and 76% of the individuals were alive at the end of the observation period at 25, 30 and 32 °C, respectively. Regarding larval weight at 55% r.h., larvae were heavier at 32 °C (18.3 mg final average larval weight) compared to 25 °C (14.3 mg) and 30 °C (15.8 mg). At 75% r.h., the final larval weight was 18.8, 14.6 and 18.7 mg at 25, 30 and 32 °C, respectively. The larval development time was influenced by diet (Mantel–Cox  $\chi^2 = 31.1$ ,  $df = 5$ ,  $p < 0.001$ ) and varied between 36 and 57 d over treatments (Figure 3). The shortest development time was found for larvae grown at 32 °C and 75% r.h. (36 d), whereas development was slower at 25 °C at both relative humidity regimes tested (54 and 57 d at 55 and 75%, respectively). Furthermore, these shorter developmental times correlated with a higher SGR ( $r = -0.843$ ,  $p < 0.001$ ) that allowed a faster growth. FCR took its lowest value at 32 °C, both at 55% (2.0) and 75% r.h. (2.2), indicating a better feed utilization (Table 2), which subsequently resulted in the highest total larval biomass production (654 and 688 mg at 32 °C and 55 or 75% r.h., respectively).

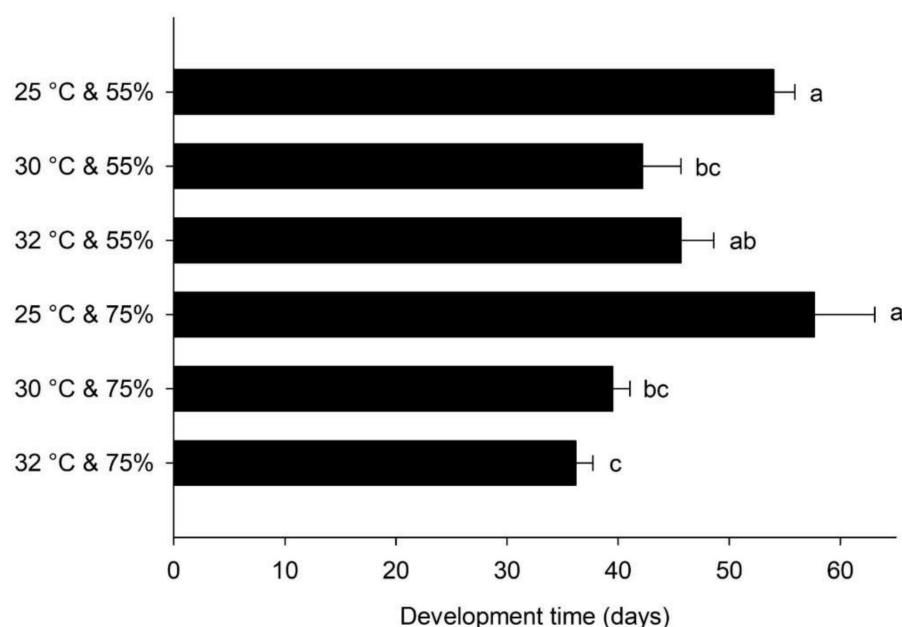


**Figure 1.** Average larval weight (mg  $\pm$  SE) of *Alphetobius diaperinus* larvae reared on wheat bran at three temperatures (25, 30 and 32 °C) and two relative humidity levels (55% (left) and 75% (right)) ( $n = 6$ ) (Bioassay I).





**Figure 2.** Survival (%  $\pm$  SE) of *Alphitobius diaperinus* larvae reared on wheat bran at three temperatures (25, 30 and 32 °C) and two relative humidity levels (55% (left) and 75% (right)) (n = 6) (Bioassay I).



**Figure 3.** Development time (days  $\pm$  SE) of *Alphitobius diaperinus* larvae reared on wheat bran at three temperatures (25, 30 and 32 °C) and two relative humidity levels (55% (left) and 75% (right)). Bars followed by the same lowercase letter are not significantly different. In all cases, n = 6; df = 5;  $p = 0.05$  (Bioassay I).

### 3.2. Bioassays Series II: Effect of Protein Content in the Diet

In general, the increase in the dietary inclusion of yeast in the diets of *A. diaperinus* larvae improved larval growth and development. The highest survival rates were recorded when larvae were fed diets with 32.5 and 40% of yeast, which correspond to diet protein contents of 27.5 and 30%, respectively (Figure 4). In these diets, survival was in the range of 80% at the end of the observation period, whereas for the rest of the diets, survival ranged between 62 and 68%. Similarly, the lowest (12.0 mg) and the highest final larval weight (17.9 mg) was recorded for the diets with the lowest (100% wheat bran) and highest (wheat bran 60% + yeast 40%) yeast content (Figure 5). Developmental time was not significantly affected by the treatments (Mantel–Cox  $\chi^2 = 6.2$ ; df = 5;  $p = 0.285$ ) and ranged between 45 and 53 days for the different yeast dietary inclusion percentages (Figure 6). When larvae were fed on diets with 32.5 and 40% of yeast, FCR took its lowest values and was 1.6 and 1.5, respectively, being significantly different from the wheat bran control (3.1) (Table 3).

Similarly, the increase in yeast percentage in the diet resulted in a higher SGR (18.5 and 18.3% in the diets with 32.5 and 40% yeast, respectively) and subsequently led to a higher total larval biomass production (660 and 683 mg, respectively), which was significantly different from the biomass produced in wheat bran alone (control).

**Table 1.** Two-way ANOVA parameters for main effects (temperature, relative humidity) and associated interaction for the feed conversion ratio (FCR), specific growth rate (SGR, %) and total produced larval biomass (mg) of *Alphitobius diaperinus* larvae reared on wheat bran at three temperatures (25, 30 and 32 °C) and two relative humidity levels (55 and 75%) (Bioassay I).

Source	df	FCR		SGR		Total Produced Larval Biomass	
		F	p	F	p	F	p
Corrected model	5	7.1	<0.001	7.3	<0.001	7.4	<0.001
Intercept	1	390.3	<0.001	1464.8	<0.001	711.3	<0.001
Temperature	2	16.2	<0.001	13.0	<0.001	14.9	<0.001
Relative humidity	1	0.6	0.440	5.3	0.028	1.0	0.317
Temperature × Relative humidity	2	1.3	0.298	2.4	0.106	3.0	0.066

**Table 2.** Feed conversion ratio (FCR), specific growth rate (SGR, %) and total produced larval biomass (mg) ( $\pm$  SE) of *Alphitobius diaperinus* larvae reared on wheat bran at three temperatures (25, 30 and 32 °C) and two relative humidity levels (55% and 75%) (Bioassay I).

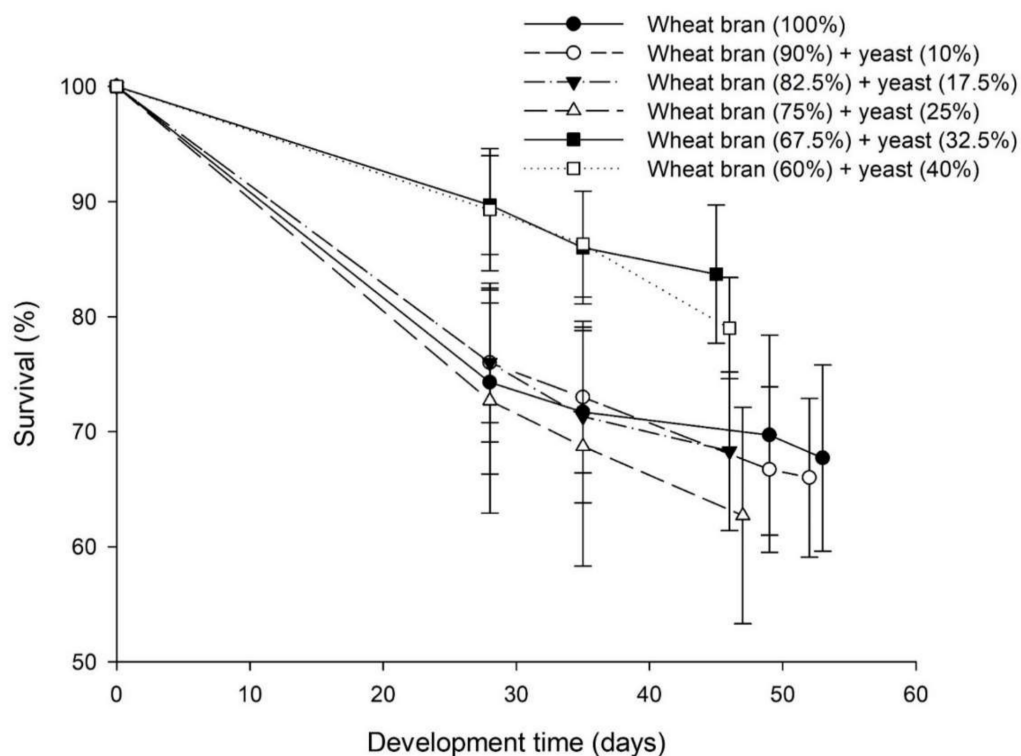
Temperature/ Relative Humidity	Feed Conversion Ratio (FCR)	Specific Growth Rate (SGR, %)	Total Produced Larval Biomass (mg)
25 °C–55%	4.3 $\pm$ 0.6 a	8.8 $\pm$ 0.3 c	317.8 $\pm$ 37.9 b
30 °C–55%	2.2 $\pm$ 0.2 b	11.6 $\pm$ 1.0 abc	589.3 $\pm$ 58.1 a
32 °C–55%	2.0 $\pm$ 0.2 b	11.0 $\pm$ 0.8 bc	654.4 $\pm$ 55.0 a
25 °C–75%	3.9 $\pm$ 0.5 a	9.4 $\pm$ 0.8 bc	483.7 $\pm$ 58.3 ab
30 °C–75%	3.1 $\pm$ 0.3 ab	11.9 $\pm$ 0.4 ab	513.0 $\pm$ 46.1 ab
32 °C–75%	2.2 $\pm$ 0.1 b	14.2 $\pm$ 0.7 a	688.8 $\pm$ 38.2 a

Within each column (FCR, SGR, total produced larval biomass), means followed by the same lowercase letter are not significantly different according to the Tukey HSD test. In all cases, n = 6; df = 5; p = 0.05.

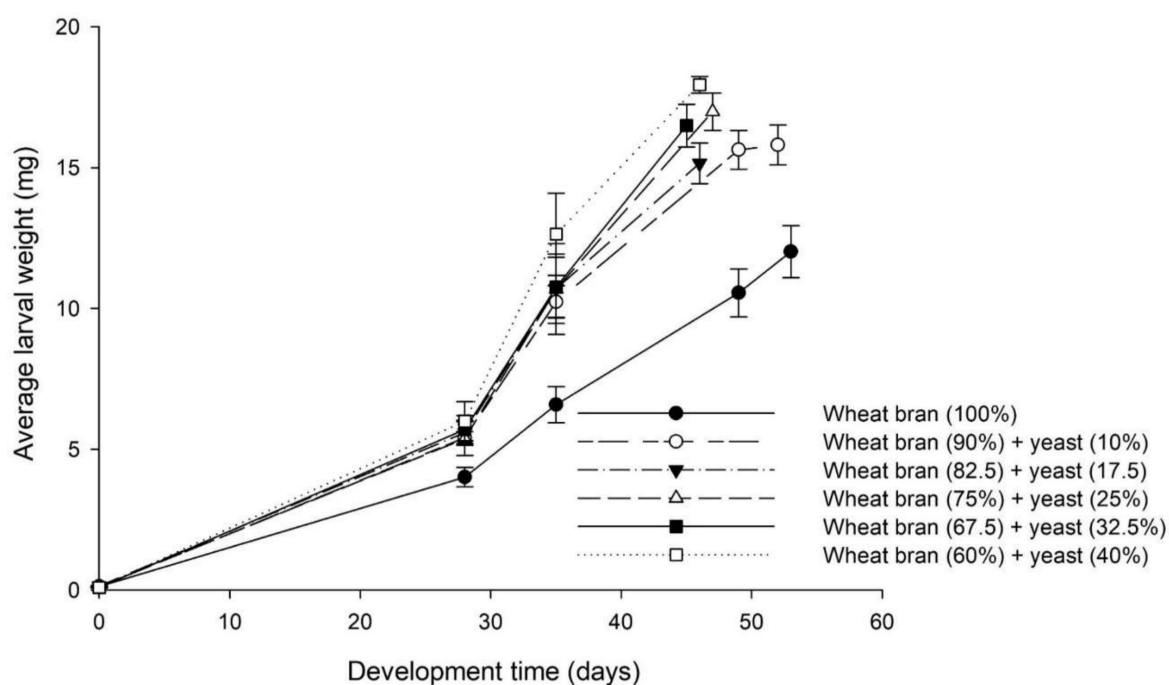
**Table 3.** Feed conversion ratio (FCR), specific growth rate (SGR, %) and total produced larval biomass (mg) ( $\pm$  SE) of *Alphitobius diaperinus* larvae reared on mixtures of wheat bran with different percentages of yeast (0%, 10%, 17.5%, 25%, 32.5% and 40%) (Bioassay II).

Substrate	Feed Conversion Ratio (FCR)	Specific Growth Rate (SGR, %)	Total Produced Larval Biomass (mg)
Wheat bran (100%)	3.1 $\pm$ 0.7 a	14.7 $\pm$ 0.5 c	384.4 $\pm$ 58.6 b
Wheat bran (90%) + yeast (10%)	2.1 $\pm$ 0.2 ab	15.5 $\pm$ 0.7 bc	491.5 $\pm$ 49.4 ab
Wheat bran (82.5%) + yeast (17.5%)	2.2 $\pm$ 0.3 ab	16.8 $\pm$ 0.7 abc	493.8 $\pm$ 54.5 ab
Wheat bran (75%) + yeast (25%)	2.3 $\pm$ 0.4 ab	17.5 $\pm$ 0.5 ab	503.3 $\pm$ 75.9 ab
Wheat bran (67.5%) + yeast (32.5%)	1.6 $\pm$ 0.1 b	18.5 $\pm$ 0.4 a	660.1 $\pm$ 43.7 a
Wheat bran (60%) + yeast (40%)	1.5 $\pm$ 0.1 b	18.3 $\pm$ 0.5 a	683.1 $\pm$ 38.3 a

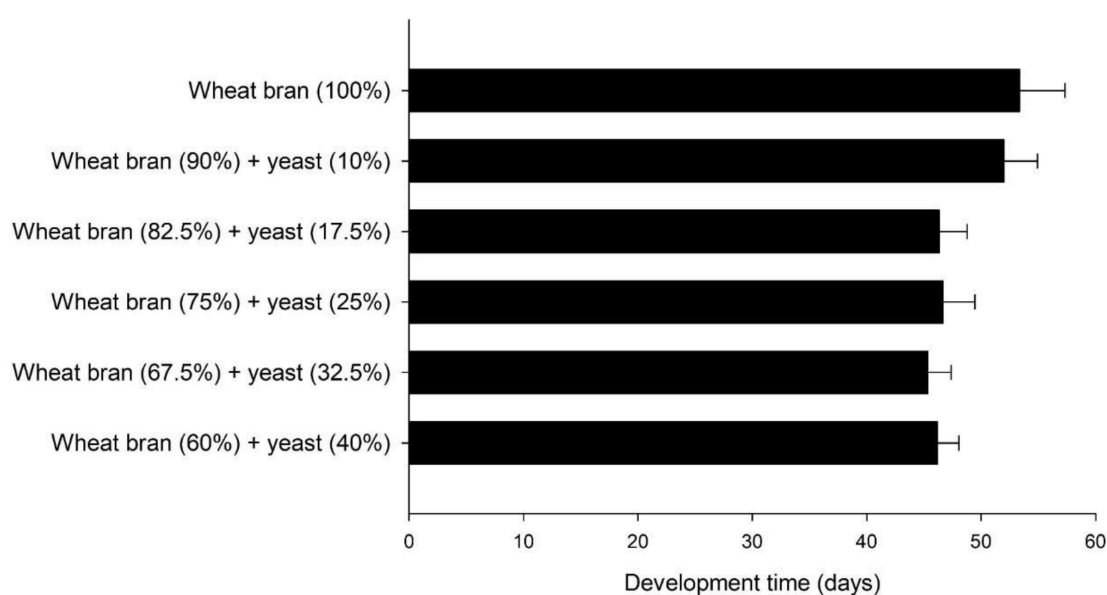
Within each column (FCR, SGR, total produced larval biomass), means followed by the same lowercase letter are not significantly different according to the Tukey HSD test. In all cases, n = 6; df = 5; p = 0.05.



**Figure 4.** Survival ( $\% \pm \text{SE}$ ) of *Alphetobius diaperinus* larvae reared on mixtures of wheat bran with different percentages of yeast (0%, 10%, 17.5%, 25%, 32.5% and 40%) ( $n = 6$ ) (Bioassay II).



**Figure 5.** Average larval weight ( $\text{mg} \pm \text{SE}$ ) of *Alphetobius diaperinus* larvae reared on mixtures of wheat bran with different percentages of yeast (0%, 10%, 17.5%, 25%, 32.5% and 40%) ( $n = 6$ ) (Bioassay II).



**Figure 6.** Development time (days  $\pm$  SE) of *Alphitobius diaperinus* larvae reared on mixtures of wheat bran with different percentages of yeast (0%, 10%, 17.5%, 25%, 32.5% and 40%) ( $n = 6$ ). No significant difference was noted among treatments. In all cases,  $n = 6$ ;  $df = 5$ ;  $p = 0.05$  (Bioassay II).

#### 4. Discussion

The results of this study show the significance of a range of biotic and abiotic factors for the growth and development of *A. diaperinus* larvae. Temperature is one of the main abiotic variables that determine the metabolism and growth of animals, and particularly insects [25,26]. The effect of temperature on the growth of *A. diaperinus* has been shown earlier [27,28]. Although from another perspective, namely *A. diaperinus* as a pest in poultry facilities and as a reservoir and vector for various avian pathogens, Rueda and Axtell [28] studied the developmental rates, growth and survival of the immature stages of *A. diaperinus* under several constant temperatures, i.e., 20, 25, 30, 35 and 38 °C. Based on their results, development time was shown to be highly dependent on temperature, as the temperature increase from 20 to 35 °C considerably shortened insect development time from egg to adult, whereas at 38 °C, insect development was delayed. More recently, Bjørge et al. [29] investigated the temperature effect on the larval mass of *A. diaperinus* for temperatures ranging from 15.2 to 38.0 °C, trying to identify the optimum temperature for its rearing and reported that the highest survival rates, fastest development and best feed conversion efficacy were at 31 °C. However, in both of the aforementioned studies, air humidity was not controlled. In the study of Rueda and Axtell [28], relative humidity ranged between 50 and 60%, whereas in the study carried out by Bjørge et al. [29], air humidity was not regulated. In the present work, we explored the development of *A. diaperinus* larvae at three temperatures and two relative humidity levels. Our results are in agreement with the findings of the aforementioned studies suggesting that *A. diaperinus* larvae perform better at elevated temperatures in the range from 30 to 32 °C. However, no significant differences were detected in terms of development time, FCR, SGR and total larval biomass produced at 30 and 32 °C. Regarding relative humidity, larval growth was slightly enhanced at 75% r.h. compared to 55%, however, in most cases, differences were not significant, illustrating that *A. diaperinus* can be easily adopted at this temperature/relative humidity range. Our findings indicate that relative humidity does not have a considerable effect on *A. diaperinus* growth, at least for the relative humidity levels tested, supporting the hypothesis that temperature is much more pivotal than moisture in determining rates of development and survival [30].

Our results clearly demonstrated that protein content in the diet greatly affected larval growth and development, as increasing the percentage of yeast and subsequently

the protein content in the diet resulted in higher survival and growth rates, as well as in shorter development times. Although a lot of research has been directed to the nutrition of *T. molitor* and its nutrient requirements [18,19,31,32], the nutrient requirements of *A. diaperinus* larvae have not been precisely defined. When *A. diaperinus* larvae were fed on diets composed of side stream materials, i.e., spent grains and beer yeast, bread and cookie remains, potato steam peelings and maize distillers' dried grains with solubles, larvae performed better in the high protein diets, where protein content ranged between 24.1% and 32.5% [13]. These diets outperformed the commercial mealworm producers' diets that were used as a control, and which had a protein content that ranged between 15.5% and 18.8% [13]. These protein contents resemble the ones of the high-protein diets tested in our study, with a protein content between 25% and 30%, which gave, in most cases, the best results in terms of survival, FCR, SGR and larval weight gain. In the same context, when the suitability of seed cleaning process byproducts for *A. diaperinus* larvae was evaluated, the best results were obtained with lupin, which had the highest protein content (33%) among the substrates tested [17]. Taking into consideration all the above-mentioned results, diet protein content greatly affects the growth and development of *A. diaperinus* larvae, with its increase usually resulting in higher larval survival and growth rates. However, as was shown by the large differences in growth performance reported in a recent study in which *T. molitor* larvae were fed isonitrogenous diets [33], apart from protein content, other parameters such as diet digestibility and amino acid profile, its vitamin and mineral content, as well as the presence of antinutrient factors, have to be taken into consideration when designing diets for *A. diaperinus* [34].

The reason for why we used yeast as a protein source in our study is because its dietary inclusion has been associated with increased growth rates of *T. molitor* [23,32,35]. In the case of *A. diaperinus*, the high protein diets tested by Van Broekhoven et al. [13] that appeared favourable for larval rearing also contained yeast, particularly beer yeast. It becomes evident that, as is the case for *T. molitor*, yeast acts as a growth promoter for *A. diaperinus* larvae, as it contains, apart from high-quality protein, vitamins (in particular vitamin B), minerals and other valuable nutrients [36,37]; hence, the addition of yeast in the diet can be clearly considered as a "more than just protein" enhancement. Recently, it was shown that when added as a functional feed additive and probiotic, yeast can greatly enhance the growth performance of larvae of *T. molitor* and *H. illucens* [38,39]. Therefore, the utilization of yeast in *A. diaperinus* diets appears to be promising and merits further investigation in order to adjust the dietary inclusion ratio, achieving optimal growth.

To conclude, the results of the present study highlight the importance of temperature and diet protein content for the growth and development of *A. diaperinus* larvae. Larval growth was clearly favored by elevated temperatures (30–32 °C) and high protein content (25–30%). Such information is crucial for the optimization of *A. diaperinus* production and aims to promote and maximize the productivity of *A. diaperinus* rearing.

**Author Contributions:** Conceptualization, C.I.R. and C.G.A.; methodology, C.I.R. and C.G.A.; formal analysis, C.I.R.; investigation, C.I.R., K.K. and M.G.; resources, C.G.A.; writing—original draft preparation, C.I.R. and G.V.B.; writing—review and editing, C.I.R. and C.G.A.; visualization, C.I.R.; supervision, C.I.R. and C.G.A.; project administration, C.G.A.; funding acquisition, C.I.R. and C.G.A. All authors have read and agreed to the published version of the manuscript.

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**Data Availability Statement:** Not applicable.

**Conflicts of Interest:** The authors declare no conflict of interest.



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