



Ontogenetic and Caste Differentiation in the Expression of Water-soluble Proteins and Some Isozymes in *Reticulitermes lucifugus* (Rossi, 1792) (Isoptera: Rhinotermitidae)

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Abstract: Ontogenetic and caste differentiation in the expression of water-soluble proteins, non-specific esterases, malic enzymes, lactate dehydrogenases and superoxide dismutase in the species *Reticulitermes lucifugus*, spread out in Bulgaria has been investigated. A total of 41 loci have been analysed. Stage and caste specific protein and isoenzyme expressions have been established as follows: 11 of the protein loci analysed have been characterised by a permanent activity without varying of the product expression in all of the castes; for seven of the protein loci, a constant action has been found in ontogenesis but with a caste-specific variability in their expression; for four of the protein loci analysed differential gene regulation, expressed in gene activity in some of the castes and inactivation in the others has been established; eight of the detected protein loci have demonstrated a ontogenetic and caste-specific expression; four of the sixth esterase loci have been characterised by a permanent action in the course of the ontogenesis; one of the two malic enzyme loci has been expressed only in reproductive castes (neotenic and queen) and the other one has been expressed in all stages of individual development; both lactate dehydrogenase loci have been expressed specifically in ontogenesis, depending on caste differentiation; superoxide dismutase locus has been found in all of the castes and stages of ontogenesis in *R. lucifugus*.

Key words: *Reticulitermes lucifugus*, proteins, isoenzymes, stage and caste specificity

Introduction

Reticulitermes lucifugus (Rossi, 1792) is the only one of the eight known species of the genus *Reticulitermes* Holmgren, 1913 occurring in Europe (KRISHNA et al. 2013, GHESINI & MARINI 2015, CONSTANTINO 2019) recorded for Bulgaria (POPOV & CHOBANOV 2004). It dwells in the soil and builds

its nests in decaying wood. The number of families depends on the abundance of food. Swarming occurs once a year, usually between April and May. The species is characterised by significant tolerance to environmental conditions, high plasticity and viability. This makes it relatively easy to cultivate in laboratory conditions and use as a suitable object for various studies.

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Various protein systems are increasingly used as highly sensitive markers to unravel the complex mechanisms of differentiation during ontogenesis. Each step of the individual development corresponds to a set of proteins, varying from one stage to another and specific for the different species.

One of the most interesting objects for ontogenetic and population genetic studies among the members of the class Insecta are social insects – various species of termites (Isoptera), ants, bees and wasps (Hymenoptera). Different studies indicate that electrophoretic approaches are suitable for the analysis of various protein and enzyme systems. In recent decades, a number of species of Isoptera, characterised by social lifestyles, have been investigated by different molecular genetic approaches (BAGINE et al. 1989, LUYKX 1993, OGINO et al. 1993, YAMAMURA 1993, MARTINELLI & SBRENNNA 1994, DAVIS et al. 1995, KAMBHAMPATHI et al. 1996, THOMPSON & HEBERT 1998, AUSTIN et al. 2005, 2006, SZALANSKI et al. 2008, LO PINTO et al. 2017). In general, however, studies on stage and caste specificity in the expression and genetic control of water-soluble proteins and of various isoenzyme systems in termite representatives are incomplete, and such kind of studies in Bulgaria have hardly been conducted (VULCHEV et al. 1999, IVANOVA et al. 1999, MURLEVA et al. 1999).

Unlike the social species of Hymenoptera, termites develop through incomplete metamorphosis. Their castes are more diverse and more clearly differentiated than in other social insects. In the course of individual development, termites go through several stages, each involving one or more ages. Thus, each individual have the characteristics of the caste. The development discontinued in the soldiers and imago caste. Workers are a specialised caste, with several ages. In many species of termites they could become soldiers or secondary reproductives. Insects of all castes in different ages can be found in termite nests during the swarming period (ZHUZHNIKOV 1979).

The eggs are elongated, whitish, released individually, and then collected by the workers in special nest chambers. Larva is a stage of ontogenesis without visible signs of caste differentiation, involving several ages.

The nymph is a stage of development that individuals, after passing through several ages, become a winged imago. In the absence of a “royal couple” in the nest, the nymphs can develop into secondary (neotenic) reproductive individuals. In *R. lucifugus*, the nymphs can develop also into workers (pseudergates) and subsequently in soldiers.

Presoldier is an intercaste and an intermediate stage in the transformation of larvae, pseudergates or nymphs into soldiers. In *R. lucifugus*, pseudergates play the role of workers.

The imago stage is actually a caste of wing individuals that develops from nymphs in last age. During the swarming, royal couples are formed, which organise separate nests. These individuals are primary reproductives. In the case of the royal couple death in the nest, secondary reproductive individuals (neotenic) are formed from the pseudergates or nymphs (through the intermediate stage – pseudonymph).

Soldiers are wingless individuals with a specifically differentiated head and mandibles. Their main function is the nest protection, in connection with which they have specific features – large head, elongated and powerful mandible, frontal gland. The soldiers develop from the presoldiers and represent one of the final stages of individual development (ZHUZHNIKOV 1979).

All the peculiarities in the individual development of termites in complex with their caste differentiation determine the complicated mechanisms of genetic control regarding the stage and caste specific expression of proteins and enzymes in the organism.

The purpose of the present study is to characterise the tendencies in the expression of water-soluble proteins and some isoenzymes in *R. lucifugus* from Bulgaria on the basis of polyacrylamide gel electrophoresis (PAGE) and to reveal, by comparative analysis, the specificity of their genetic control during ontogenesis and according to caste differentiation of individuals in the termite nest.

Materials and Methods

Materials

These are relatively small termites, living in moist soils and rotting, dead wood in which they organise diffuse nests. The number of families depends on the abundance of food. The swarming takes place once a year, usually in April-May. The species is characterised by significant tolerance to environmental conditions, high plasticity and viability. This makes it relatively easy to cultivate under laboratory conditions and to use it as a convenient object for various studies.

The individuals included in this study have been collected from *R. lucifugus* nests inhabiting abandoned vineyard areas south of Sozopol in Bulgaria. The collected specimens have been grown under laboratory conditions in large glass baths, at the bottom of which lies the soil from the natural

termite habitat. Termite storage and cultivation has been carried out at room temperature 25-27°C, soil humidity close to 20% and relative air humidity above 95% (ZHUKHNIKOV 1979). Under these conditions, termite colonies were maintained for months and individuals of different castes and ages, including reproductive individuals, have been used for experimental work.

Totally, 645 individuals of *R. lucifugus* belonging to different castes and stages of ontogenesis have been included in the PAGE analysis (Table 1).

Individual total extracts (0.8 M Tris-phosphate extraction buffer with pH 6.7) have been used. Centrifugation has been performed at 900 g for 20 minutes.

Polyacrylamide gel electrophoresis – PAGE

PAGE has been performed by the method of DAVIS (1964) and the first system of MAURER (1971) with some modifications (IVANOVA & POPOV 1997) in 6% and 7.5% polyacrylamide gel. Polymerization has been done in two buffer systems – Tris-chloride (pH 8.9) and Tris-phosphate (pH 6.7). The electrophoretic separation has been carried out in Tris-glycine buffer with pH 8.3 at temperature 4°C.

Protein and isoenzyme systems

Common water-soluble proteins and the isoenzyme systems of EST – esterases (EC 3.1.1), ME – malate enzymes, NADP-dependent malate enzyme (EC 1.1.1.40); LDH – lactate dehydrogenase (EC 1.1.1.27) and SOD – superoxide dismutase (EC 1.15.1.1), controlled by a total of 41 genes (Sp1 - Sp30, Est1, Est2, Est3, Est4, Est5, Est6, Ldh1, Ldh2, Me1, Me2 and Sod1) have been subjected to electrophoretic analysis. The staining of the protein and isoenzyme systems has been done according to IVANOVA & POPOV (1997), IVANOVA et al. (2000) and MEIXNER et al. (2013).

Results

Data concerning expression of the soluble proteins and isoenzymes studied in different castes and ontogenesis stages of *R. lucifugus* are presented in Table 2 and 3. Information concerning number of soluble protein and isoenzyme fractions and total number of fractions found in the different ontogenetic stages and castes of *R. lucifugus* is presented in Figure 1.

Table 1. Number and percentage (%) of individuals studied by caste and stage of ontogenesis: L – larvae (1, 2 and 3 age); PE – pseudergates; PS – presoldiers; S – soldiers; PN – pseudo nymphs; SR – secondary reproductives (neotenic queens); N – nymphs (1, 2, 3 age); I – imago (IM – male; IF – female); Q – queen.

Castes and Stages	L	PE	PS	S	PN	SR	N1	N2	N3	IM	IF	Q	Total
Number	24	349	5	84	19	3	15	20	65	32	28	1	645
%	3.72	54.2	0.8	13.0	2.95	0.5	2.33	3.1	10.1	4.95	4.35	0.16	100

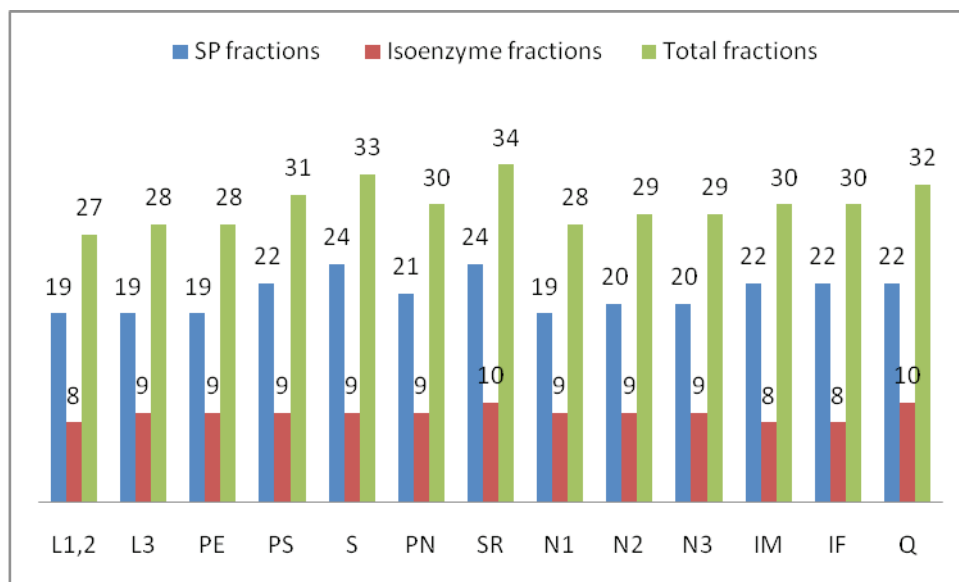


Fig. 1. Number of soluble protein and isoenzyme fractions and total number of fractions expressed in spectrums of *R. lucifugus*: L – larvae (1, 2, 3 age); PE – pseudergates; PS – presoldiers; S – soldiers; PN – pseudo nymphs; SR – secondary reproductives (neotenic queens); N – nymphs (1, 2, 3 age); I – imago (IM – male; IF – female); Q – queen.

Table 2. Soluble proteins expressed in different castes and ontogenesis stages: L – larvae; PE – pseudergates; PS – presoldiers; S – soldiers; PN – pseudo-nymphs; SR – secondary reproductives (neotenic queens); N – nymphs (1, 2, 3 age); I – imago (IM – male; IF – female).

	L	PE	PS	S	PN	SR	N ₁	N ₂	N ₃	IM	IF
SP1	+	+	+	+	+	+	+	+	+	+	+
SP2	+	+	+	+	+	+	+	+	+	+	+
SP3	+	+	+	+	+	+	+	+	+	+	+
SP4	+	+	+	+	+	+	+	+	+	+	+
SP5	+	+	+	+	+	+	+	+	+	+	+
SP6				+							
SP7			+	+	+	+					
SP8	+	+	+	+	+	+	+	+	+	+	+
SP9				+							
SP10						+					
SP11						+					
SP12	+	+	+	+	+		+	+	+	+	+
SP13	+	+	+	+	+	+	+	+	+	+	+
SP14	+	+	+	+	+	+	+	+	+	+	+
SP15	+	+	+	+	+	+	+	+	+	+	+
SP16	+	+	+	+	+	+	+	+	+	+	+
SP17	+	+	+	+	+	+	+	+	+	+	+
SP18				+							
SP19	+	+	+	+	+	+	+	+	+	+	+
SP20										+	+
SP21		+	+	+	+	+		+	+	+	+
SP22	+	+	+	+	+	+	+	+	+	+	+
SP23				+		+			+	+	+
SP24	+	+	+	+	+	+	+	+	+	+	+
SP25						+					
SP26	+	+	+	+	+	+	+	+	+	+	+
SP27	+	+	+	+	+	+	+	+	+	+	+
SP28			+								
SP29	+	+	+		+		+	+	+	+	+
SP30	+	+	+	+	+	+	+	+	+	+	+

Table 3. Expression of EST, LDH, ME and SOD isoenzymes studied in different castes and ontogenesis stages of *R. lucifugus*: L – larvae; PE – pseudergates; S – soldiers; SR – secondary reproductives (neotenic queens); N – nymphs (1, 2, 3 age); I – imago (IM – male; IF – female); Q – queen.

	L _{1,2}	L ₃	PE	S	SR	N1	N2	N3	IM	IF	Q
EST1	+	+	+	+	+	+	+	+	+	+	+
EST2		+	+	+	+	+	+	+	+	+	+
EST3	+	+	+	+	+	+	+	+	+	+	+
EST4	+	+	+	+	+	+	+	+			+
EST5	+	+	+	+	+	+	+	+	+	+	+
EST6	+	+	+	+	+	+	+	+	+	+	+
LDH1	+	+							+	+	
LDH2			+	+	+	+	+	+			+
ME1					+						+
ME2	+	+	+	+	+	+	+	+	+	+	+
SOD1	+	+	+	+	+	+	+	+	+	+	+

Discussion

Soluble proteins

The data from the electrophoretic study of the water-soluble proteins in *R. lucifugus* showed the presence of a total of 30 fractions – 19 in larvae, pseudergates and nymphs, first age, 20 in second- and third-age nymphs, 21 in pseudo nymphs, 22 in presoldiers and imagoes of both sexes and 24 in soldiers and neotenic queens. These fractions could be grouped as follows (Table 2):

- Group 1 – fractions expressed in all castes (stages of individual development) of the species without variability in the intensity;
- Group 2 – fractions expressed in all castes (stages of individual development), but with variabilities in the intensity;
- Group 3 – fractions observed in two or more castes (stages of individual development) and missing in the others;
- Group 4 – fractions specific to the representatives of a particular caste (stage of individual development).

Group 1 includes 11 fractions – SP1, SP2, SP3, SP14, SP15, SP16, SP17, SP22, SP26, SP27 and SP30 expressed in all samples with equal intensity (Table 2). Their expression is the result of the action of nine monoallelic genes, active in all of the ontogenesis stages and in all of the *R. lucifugus* castes.

Group 2 includes seven fractions – SP4, SP5, SP8, SP13, SP19, SP21 and SP24, expressed at all castes and stages of ontogenesis, but with variable intensity as follows: SP4, SP5 and SP8 – strongly expressed in the soldier caste; SP13 – with reduced intensity in the same caste; SP19 – with the highest intensity in larvae, pseudergates, nymphs and imagoes of both sexes and with a lower intensity in pseudonymphs and presoldier; SP21 – with the highest intensity in soldiers and neotenic queens and SP24 – with a characteristic decrease in the intensity of expression in the castes of soldiers, neotenic queens and imago.

The variation in the intensity of the described fractions is a result of the differential gene regulation (gene activation and repression), related to the caste differentiation during the individual development.

Group 3 includes four fractions – SP7, SP12, SP23 and SP29. Fraction SP7 is expressed in soldiers and neotenic queens, as well as in part of the analysed samples of presoldiers and pseudonymphs, and is absent in the spectra of the other caste and stage groups (pseudergates, larvae, nymphs and imago). This protein is under the

control of a gene expressed only in the terminal castes of the sterile developmental pathway and its activation occurs immediately before the changing of the previous intercaste form. Fraction SP12 is expressed in samples from all castes except neotenic queens. In soldiers, the fraction shows a clear decrease in the intensity. This, as well as its absence in neotenic queens, is evidence for changing in gene expression in the soldier caste and a repression of the gene in the secondary reproductive individuals. Fraction SP23 is only expressed in the soldiers, third nymph age, neotenic queens and imagoes; this fact demonstrates that the gene responsible for this protein is active only in the final stages of ontogenesis. The absence of the fraction in some of the individuals studied is possibly associated with the presence of a null allele. A protein gene with null allele has been described also for *Apis mellifera* L. (Hymenoptera: Apidae) (IVANOVA 2000). Generally, the null alleles refer to the labile part of the genome and are characterised by adaptive value in the natural populations of different insects. Fraction SP29 has no expression in the spectrum of soldiers and neotenic queens, which also indicates caste-specific gene repression.

Group 4 includes eight protein fractions expressed only in individual castes of the species – SP6, SP9, SP10, SP11, SP18, SP20, SP25 and SP28. Three of the fractions listed, are specific for the soldier spectrum – SP6, SP9 and SP18, which demonstrates caste-specific gene expression. SP10, SP11 and SP25 are specific for the spectrum of neotenic queens. Their appearance is determined by the activity of three monoallelic genes characterised by distinct caste specificity. Fraction SP20 is expressed only in imagoes of *R. lucifugus*, demonstrating the presence of stage-specific expression. SP28 is only expressed in the presoldier spectrum, which demonstrates specific gene expression most likely associated with complex morphophysiological changes in the body, in this case, in the transition from pseudergate to soldier.

Isoenzymes

A general overview of isoenzyme fractions found in the spectrum of the *R. lucifugus* individuals by castes and stages of ontogenesis indicates the presence of the highest number of enzyme products (10) in the primary (queen) and secondary reproductive individuals (neotenic), and the smallest number – in the first and second larvae ages and the imagoes (8). For all other castes and stages, with some differences, totally nine fractions have been identified (Figure 1).

EST

The expression of esterase isoenzymes in *R. lucifugus* is associated with the activity of a total of six Est genes, four of which are permanently active during ontogenesis and in different castes – Est1, Est3, Est5 and Est6 (Table 3). The Est2 gene is not expressed at earlier larval ages and the Est4 gene is not found in male and female imago individuals. Although esterase isoenzymes have been used for population genetic and cross-species studies in representatives of *Reticulitermes* in Europe (CLÉMENT 1981, 1984), as well as to characterise their expression and activity in the gut of *Reticulitermes flavipes* (Kollar) (WHEELER et al. 2010), the esterase loci of *R. lucifugus* have not been studied previously in terms of their ontogenetic and caste differentiation. That is the reason comparisons with other investigations on this species not to be possible. But, the results obtained in the present study are similar to these reported by IVANOVA & POPOV (1996-1997) concerning the finding of stage specificity in the expression and genetic control of nonspecific esterases in another species of social insect – *A. mellifera*. For this species the authors have found differential activity of six genes two of which have been polymorphic.

LDH

The results of the present study indicate that the expressed LDH isoenzymes are under the control of two lactate dehydrogenase loci – Ldh1 and Ldh2 with differential regulation in the termite castes and ontogenesis stages of *R. lucifugus*, as follows: Ldh1 – active in larvae and in male and female imagoes; Ldh2 – active in workers (pseudergates), soldiers, nymphs, neotenics and queen (Table 3).

In the literature, we have not found data concerning the genetic control of LDH in termites of different species, including *R. lucifugus*, which makes comparison with results of other authors impossible, but with respect to this isoenzyme group, IVANOVA & POPOV (1997) have found in *A. mellifera* more than 2 genes that are differentially active during the individual development. Authors report the presence of LDH product specific for fertile individuals (bee queen and drones) in the honey bee colony.

ME

The slower fraction (ME2) is characterised by a different intensity of expression – weaker in the spectrum of larvae and nymphs and stronger in imago, soldiers, workers and neotenics, and the strongest in the queen sample. Only in the spectrum of the queen and neotenics was the manifestation of the

faster fraction ME1, which was more pronounced in queen (Table 3).

The results obtained in the study suggest that the described ME products are under the control of two genes – Me1 and Me2, one of which – Me2 is active at all castes during all ontogenetic stages of *R. lucifugus*, but with variable expressiveness – strongest in the queen, intermediate – in workers (pseudergates), soldiers, neotenics and imago, and much weaker in larvae and nymphs. The second malic enzyme locus – Me1 is active only in reproductive castes of neotenics and queen, which demonstrates distinct caste specificity gene activity. This gene is probably inactivated in other *R. lucifugus* castes and functions with variable expression only in the final stages of the fertile developmental pathway in this termite species.

Data on the genetic control of this enzyme group are not available for *R. lucifugus* but STRONG & GRACE (1993) consider the presence of two ME loci for the species *Coptotermes formosanus* Shiraki (Isoptera: Rhinotermitidae). The control of malic enzymes in *A. mellifera* is also carried out by two loci, for which, however, no dependence on gene action has been established, neither on the stage of development and sex, nor on the fertility of individuals in the bee family (IVANOVA 1998).

SOD

Only one SOD product has been found in the spectrum of individuals of all *R. lucifugus* castes and stages of individual development which was under the control of one superoxide dismutase gene – Sod1. This gene has different intensity of expression – minimal in larvae stage and maximum in queen (Table 3), which is in agreement with TAsAKI et al. (2018) who have found high Cu/Zn-Superoxide dismutase activity in long-lived termite queens.

Although the genetic control of this enzyme group has been poorly studied in invertebrates and there is no enough data regarding their expression in termites, IVANOVA et al. (1997) reported the presence of two Sod genes active at all stages of the *A. mellifera* individual development.

It should be noted that the results obtained in the present investigation suggest that some of the esterase loci analysed as well as one of the lactate dehydrogenase genes are polymorphic with two or three allelic variants (including the presence of null alleles in esterase group) – a circumstance which could define the isozymes studied as potential genetic markers also suitable for population genetic studies of *R. lucifugus*.

Conclusions

Permanent gene expression in ontogenesis of *R. lucifugus* has been found for 24 of the studied water soluble protein and isoenzyme loci. Ontogenetic and caste specificity in the expression of the other 17 protein and enzyme genes studied has been established. The comparative results obtained in this study could be used in future ontogenetic, population genetic and phylogenetic studies of *R. lucifugus* and other termite species.

Acknowledgements: This study was supported by the Research Fund of the Plovdiv University "Paisii Hilendarski" through the Contract № SP 19 BF 011.

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