Contents

VOLUME 138 (2)

ISSN: 0777-6276

Belgian Journal of Zoology

2008 138 (2)

Belgian Journal of Zoology

AN INTERNATIONAL JOURNAL PUBLISHED BY
THE ROYAL BELGIAN SOCIETY FOR ZOOLOGY

Volume 138 (2) – July 2008

BELGIAN JOURNAL OF ZOOLOGY

THE BELGIAN JOURNAL OF ZOOLOGY IS COVERED IN CURRENT CONTENTS, SCIENCE CITATION INDEX AND IN OTHER LEADING REFERENCE PUBLICATIONS

Belgian Journal of Zoology

The *Royal Zoological Society of Belgium* and its *Belgian Journal of Zoology* continue in a long scientific tradition devoted to the promotion of zoology and to the publication of research in zoology. This tradition goes back to 1863, when the "Société Malacologique de Belgique" was founded in Brussels and began to publish the "Annales de la Société Malacologique de Belgique." Name changes took place in 1903 ("Annales de la Société Royale Malacologique et Zoologique de Belgique"), and 1923 ("Annales de la Société Royale Zoologique de Belgique"). With the internationalisation of the editorial board in 1989, the journal was opened to the international scientific community, and the name "Belgian Journal of Zoology" was chosen. The Belgian Journal of Zoology is now distributed to the about 400 Belgian and international members of the Society, to bookshops, and is accessible, through an active exchange policy, in 200 libraries of various institutions in more than 50 countries in the world.

The Belgian Journal of Zoology is an open source journal, where all published papers will be made available through the journal website (address see below).

The Belgian Journal of Zoology publishes, in English or French, original papers and notes in all fields of zoology, reviews or original points of view on topics of general or current interest. All manuscripts (including short notes) are peer reviewed by at least two referees.

The Belgian Journal of Zoology appears in two regular issues per volume (January and July) and occasional special issues.

All manuscripts should be sent electronically to BJZ@ua.ac.be.

SUBSCRIPTION INFORMATION TO THE BJZ

Membership of the Royal Belgian Zoological Society includes a free subscription to the Belgian Journal of Zoology. Reduced annual dues exist for regular members, PhD students (up to four years after graduation) and undergraduate students. Membership includes the free subscription to the Belgian Journal of Zoology, as well as a subscription to the zoologist's mailing list. Rates for annual dues can be found on the Society website (see below).
Payments are accepted only in these currencies by cheque via a Belgian Bank or via an international money order directed to "Royal Belgian Society of Zoology, B-1000 Brussels" on account number 000-0049113-31 (Fortis Bank). For international transfers: Fortis account number: 001-1870002-15, IBAN: BE83 0011 8700 0215, BIC: GEBABEBB, Fortis Bank NV, Warandeberg 3, B-1000 Brussels (Belgium). Payments through VISA or MASTERCARD are no longer accepted.

Back issues/volumes and a price list are available on request to the treasurer (erik.verheyen@naturalsciences.be). For large orders a substantial reduction can be offered.

Membership and subscription

Dr. Erik Verheyen (erik.verheyen@naturalsciences.be)
Treasurer
Royal Belgian Institute of Natural Sciences
Vautierstraat 29
B-1000 Brussels
Belgium

Requests for exchange, and other library matters

Dr. Laurent Meese (laurent.meese@natuurwetenschappen.be)
Library Administrator
Royal Belgian Institute of Natural Sciences
Vautierstraat 29
B-1000 Brussels
Belgium

The Belgian Journal of Zoology is published with the financial support of the "Direction générale de l'Enseignement supérieur de la Recherche scientifique" and of the "Universitaire Stichting van België/Fondation Universitaire de Belgique".

General information about the Society and the Belgian Journal of Zoology, including detailed instructions for authors, can be found on http://www.naturalsciences.be/institute/associations/rbzs_website/rbzs/

INSTRUCTIONS FOR AUTHORS

The Belgian Journal of Zoology publishes, in English or French, original papers, reviews and notes in all fields of zoology. Papers in French must include English translations of the title and of the abstract.

Manuscripts must contain significant new findings and must not have been published elsewhere nor be simultaneously under consideration for any other publication outlet. Single-species descriptions will not be accepted unless they have broader relevance such as implications for phylogeny or biogeography.

Authors benefit from a waiver of page charges for up to eight printed pages for members and six pages for non-members. Additional pages are charged to authors.

PREPARATION OF YOUR MANUSCRIPT

The page size of the Belgian Journal of Zoology is 17 x 24.6cm, in two columns of 8.15cm. Please keep this in mind, particularly with regard to the size of figures and tables.

Manuscripts must be submitted in electronic version only (double spaced, with numbered pages and margins of at least 2.5cm), as well as the original figures and tables. Figures and tables should be kept separate from the text; the legends should be at the end of the manuscript. Manuscripts have to be submitted to the editorial office at BJZ@ua.ac.be.

Text. Papers should be written in clear, concise language and consist of an abstract (summarizing the essential results and conclusions), introduction, material and methods, results and discussion. Sections and sub-sections should not be numbered, but authors may suggest a printing format to give structure to the article. The title must be sufficiently informative. Please provide a short running title and (additional) key words (separated by commas). The full first name (first name first) and the address of each author should be given, as well as the e-mail address of the first or the corresponding author. Keep following matter in mind: units should have the international format, and no **space** separates the value and the unit; do not use a space **before** interpunction, but do use a space **after** it.

Figures. Figures are referred to in the texts by "Fig**.** n" (capital F and full stop) or "Figs n-m" (no full stop). Drawings should be clearly readable after reduction to page width, column width or in between. Photographs should be sharp and with good contrast. Label size should be adequate and the same in all illustrations after reduction. Magnification or reduction should be given by a scale bar. Photocopies of photographs or figures are not acceptable. Indicate the preferred location of each figure in the manuscript. An electronic version of all illustrations must be provided (see below). Inclusion of colour prints is at additional charge. Legends to the figures come on a separate page.

Tables. The sizes of tables should take account of page width and length, or column width. Tables are referred to in the text by "Table n" (with capital T). The legend to a table is given on top of the table. Indicate the preferred location of each table in the manuscript.

Electronic version. An electronic version of the text should be provided, exclusively by e-mail as an attached file, preferably in MSWord or RTF. Files of figures and tables should remain separated from the text file. Figure files should have the JPG, PICT or TIFF format and those scanned should have a resolution of at least **300dpi**. Tables and graphs may be sent as an Excel file. Do not incorporate figures in a Word document because this may result in loss of quality. All files should have the name of the first author and should begin with the MS number once it has been allocated after submission (see below).

Species names. Species names and the names of genera should be in italics. Names of species (and names of higher taxa) should be followed, on the first occasion of use, by the name of the author who described the species or introduced the taxon name (in lower case) and the year. This rule does not apply for names of plants. If a table of all the species names used in the text is provided, the author names can be mentioned there. Do not use the genus name to indicate a species or individuals of a species.

References. Author names in text citations should be in small capitals and in the following format: NAVAS (1996), LAUDER & LIEM (1980) for two authors or WILSON et al. (1987) for more than two authors. Consecutive references must be separated by a semicolon (;). All authors quoted in the text should be found in the reference list.
Examples of literature citations:

* *Paper in a journal*: NAVAS CA (1996). Implications of microhabitat selection and pattern of activity on the thermal ecology of high elevation neotropical anurans. Oecologia, 108: 617-626. (Abbreviation of the journal according to the "World list of Scientific Periodicals".)
* *Paper in a book*: MALLEFET J, VANHOUTTE P & BAGUET F (1992). Study of *Amphipholis squamata* luminescence. In: ALERA-LIACI & CANICATTI TI (eds), Echinoderm Research, L. Balkema, Rotterdam: 125-130.
* BELLAIRS: BELLAIRS R (1991). Egg incubation: its effects on an embryonic development in birds and reptiles. Cambridge University Press, Cambridge.

Unpublished reports, master theses, etc… are not considered publications and should be referred to as "unpublished data" or "personal communication" in the text. A report can be given in a footnote. Ph.D. theses are accepted in the reference list. Author names that only appear in the text after a taxon, do not appear in the reference list.

Short notes. Short notes do not exceed two printed pages (incl. figures and tables –approx. 10 000 characters minus the figures and references). They are the appropriate form for new findings of temporary importance, comments on papers published in the journal, important new records of fauna, curiosa, etc. They should be written as a continuous text without the various divisions of regular papers (and without an abstract). References for notes must be indicated in the text by numbers. Short notes will be reviewed as normal papers.

PROCESSING

Manuscripts should be sent to the editor. On receipt of the manuscript, the corresponding author is notified by e-mail, and will receive the number under which the manuscript has been registered. Please mention this MS# first in the subject of e-mails and in the names of all files subsequently sent to the editor. The MS is then sent to at least two referees, and a reply may be expected six to eight weeks after receipt. If the MS is accepted, the author is requested to prepare the final version, taking into account the remarks of the referees. Only one galley proof is sent as a PDF file, to the corresponding author. The proof must be carefully corrected and sent back without delay.

Publication delay is between three and nine months, but is highly dependent of the willingness of the referees and the collaboration of the authors.

BJZ

Belgian Journal of Zoology

Published by the
"KONINKLIJKE BELGISCHE VERENIGING VOOR DIERKUNDE —
SOCIÉTÉ ROYALE ZOOLOGIQUE DE BELGIQUE"

Volume 138 (2)

(July, 2008)

Managing Editor of the Journal:

Ronny Blust
Department of Biology/EBT
University of Antwerp, Campus Groenenborger
B-2020 Antwerp (Belgium)

Printed in Belgium (July 2008) by
Drukkerij George Michiels, N.V., Tongeren

Belg. J. Zool., 138 (2) : 115-127 July 2008

Genus *Armascirus* (Acari: Prostigmata: Cunaxidae) from Pakistan

M. Hamid Bashir[1*], M. Afzal[2] & Bilal Saeed Khan[3]

[1] **Assistant Professor, Department of Agri. Entomology, University of Agriculture, Faisalabad, Pakistan. (hamid_uaf@yahoo.com). +92 321 6650959**

[2] **Associate Professor, Department of Agri. Entomology, University of Agriculture, Faisalabad, Pakistan. (chafzal64@yahoo.com). +92 300 7634963**

[3] **Lecturer, Department of Agri. Entomology, University of Agriculture, Faisalabad, Pakistan. (bsk_1703@yahoo.com). +92 321 6646852**

Corresponding author : * Prof. M. Hamid Bashir, e-mail: hamid_uaf@yahoo.com

ABSTRACT. Four new species of the genus *Armascirus,* namely *A. akhtari* n. sp., *A. jasmina* n. sp., *A. sabrii* n. sp. and *A. gojraensis* n. sp. are described and illustrated from Pakistan. Distribution records of four already known species (*A. satianaensis* Bashir & Afzal*, A. asghari* Bashir & Afzal*, A. mactator* Muhammad & Chaudhri and *A. pluri* Muhammad & Chaudhri) are given. A key to the species of the genus *Armascirus* from Pakistan is prepared and phenetic affinities of these species have been inferred.

KEY WORDS: Acari, Cunaxidae, *Armascirus*, Pakistan.

INTRODUCTION

Mites belonging to the family Cunaxidae are well-known predators of harmful mites and small soft bodied insects (SMILEY, 1992). WALTER & KAPLAN (1991) found *Coleoscirus simplex* colonizes in greenhouse pot cultures infested with root knot nematodes (*Meloidogyne* spp.) in Florida, where it feeds on this nematode and other soil arthropods. They also studied the feeding behaviour of Cunaxidae. TAGORE & PUTATUNDA (2003) reported that cunaxid mites were important predators in ornamental plants in Haryana, India.

Armascirus, an important genus of the family Cunaxidae, was erected by DEN HEYER (1978) designating *A. huyssteeni* as its type species. MUHAMMAD & CHAUDHRI (1991) collected and described two new species, *A. mactator* and *A. pluri* from Pakistan. BASHIR & AFZAL (2005) reported two new species *A. satianaensis* and *A. asghari* from this region. During surveys of different climatic regions of Punjab- Pakistan four new species of the genus *Armascirus,* namely *A. akhtari* n. sp., *A. jasmina* n. sp., *A. sabrii* n. sp. and *A. gojraensis* n. sp were collected from leaf debris and are herein described and illustrated, making a total of eight species of this genus now known from Pakistan.

MATERIALS AND METHODS

Mites were extracted from samples of leaf debris processed through Berlese's funnel for at least 24 hours, collected in a beaker containing 50% alcohol, then preserved in small vials containing 70% alcohol and few drops of glycerine. The cunaxid mites were sorted out under a binocular microscope and permanent slides were prepared using Hoyer's medium. The mounted specimens were identified using a phase contrast microscope and sketches were prepared with the aid of an ocular grid. Identification of the species was done with the help of the existing keys and literature of SMILEY (1975; 1992), DEN HEYER (1978) and BASHIR & AFZAL (2005). The setal nomenclature of SMILEY (1992) has been followed. All the measurements (in µm) and ranges are given in the description. The following abbreviations are used in this manuscript.

asl	attenuated solenidion
bsl	blunt-ended solenidion
sts	simple tactile setae
T	trichobothrium

The phenetic relationships of all the known species of this genus from Pakistan have been worked out by multivariate cluster analysis using average linkage method and Euclidean distance by applying the computer software Minitab 13.1. Twenty four morphological characters were used (Table 1), and the result of cluster analysis is depicted in the dendrogram (Fig. 5).

RESULTS

Key to species of genus *Armascirus* known from Pakistan

1)Dorsal hysterosoma with lateral reticulated shields. . . 2
Dorsal hysterosoma without lateral shields 7

2)Venter with 6 pairs of simple setae between coxae II and genital region. 3
Venter with 7 pairs of simple setae between coxae II and genital region. 6

3)Palp genu with 3 spine-like setae and one simple seta. 4
Palp genu with only 3 spine-like setae . . . . . *satianaensis* Bashir & Afzal

4)Coxa IV with 2 setae; genu II with 8 setae . . . . . *akhtari* n. sp.
Coxa IV with 3 setae; genu II with 7 setae 5

5)Genu I with 9 setae; genital valve with longitudinal rows of dot-like lobes
. . . . . *mactator*-Muhammad & Chaudhri
Genu I with 8 setae; genital valve with random dot-like lobes . . . . . *pluri* Muhammad & Chaudhri
6)Basifemora I-IV with 4-5-4-2 setae; genu I with 8 setae
. . . . . .*asghari* Bashir & Afzal
Basifemora I-IV with 5-5-3-2 setae; genu I with 7 setae
. . . . . *jasmina* n. sp.
7)Palp genu with 2 spine-like setae; genu I with 6 setae .
. . . . . *sabrii* n. sp.
Palp genu with 3 spine-like setae; genu I with 8 setae
. . . . . *gojraensis* n. sp.

1. *Armascirus satianaensis* Bashir & Afzal

Armascirus satianaensis Bashir & Afzal, 2005: 119

Known Distribution: Pakistan: Faisalabad

Known Source: Plant debris

2. *Armascirus asghari* Bashir & Afzal

Armascirus akhtari Bashir & Afzal, 2005: 117

Known Distribution: Pakistan: Faisalabad

Known Source: Plant debris

3. *Armascirus mactator* Muhammad & Chaudhri

Armascirus mactator Muhammad & Chaudhri, 1991: 50

Known Distribution: Pakistan: Faisalabad

Known Source: Cauliflower

4. *Armascirus pluri* Muhammad & Chaudhri

Armascirus pluri Muhammad & Chaudhri, 1991: 50

Known Distribution: Pakistan: Faisalabad, Toba Take Singh

Known Source : Plant debris and soil

5. *Armascirus akhtari* Bashir, Afzal & Khan, new species

(Figs 1 A – F)

Female

Gnathosoma

Gnathosoma 320 long and 110 wide. Hypostome covered with papillae, sub-rectangular in shape and cone-shaped distally, with 4 pairs simple hypognathal setae (hg_1-hg_4) and two pairs adoral setae (Fig. 1E).

Palp 5 segmented, all segments papillated, measuring 270. Chaetotaxy of palp as follows: trochanter none; basifemur with one simple seta; telofemur with one apophysis and one spine-like seta; genu with one long triangular apophysis, one simple seta and 3 spine-like setae; tibiotarsus terminating in a small claw, with 4 (1 long + 3 small) simple setae, and one small spine-like seta (Fig. 1C).

Chelicerae 160 long, terminating in a claw, dorsal and ventral sides with lobes, with one dorsolateral simple seta (Fig. 1D).

Dorsum

Body 470 long (without gnathosoma) and 330 wide. Propodosoma with reticulate sub-rectangular shield, originating behind the base of gnathosoma and extending to the anterior region of hysterosoma. Propodosomal shield with anterior and posterior sensillae PS_1, PS_2 measuring 250, 300 respectively and propodosomal setae P_1, P_2 both simple 8.75 and 10.0 long, respectively.

Hysterosoma separated from propodosoma by papillate striae. Hysterosoma with two reticulated lateral and one triangular reticulated median shield, latter with setae D_2 measuring 12.5. Setae L_1, D_1, D_3, D_4, D5 present on dorsal hysterosomal membrane. Setae L_1 11.25, D_1 11.25, D_3 15, D_4 22.5 and D_5 25 long, all simple. Hysterosoma with one pair of pores lateral in position, between setae D_3 and D_4 (Fig. 1A).

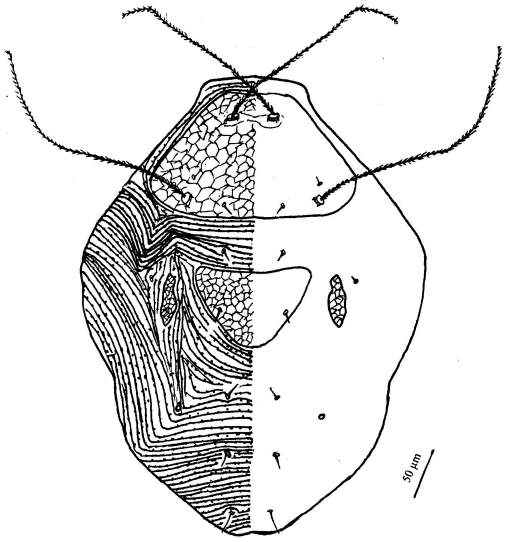


Fig. 1A. – *Armascirus akhtari* Bashir, Afzal & Khan, new species, Dorsal Side

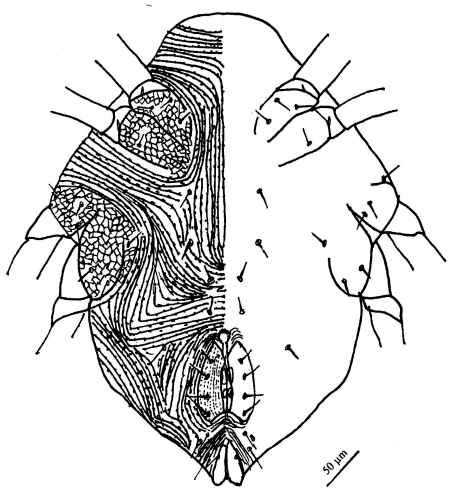


Fig. 1B. – *Armascirus akhtari* Bashir, Afzal & Khan, new species, Ventral Side

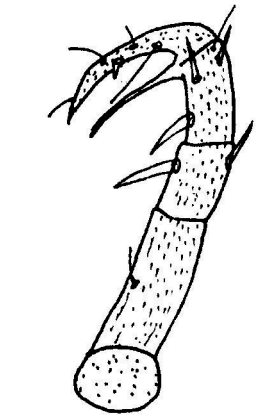


Fig. 1 C

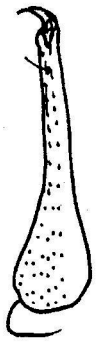


Fig. 1 D

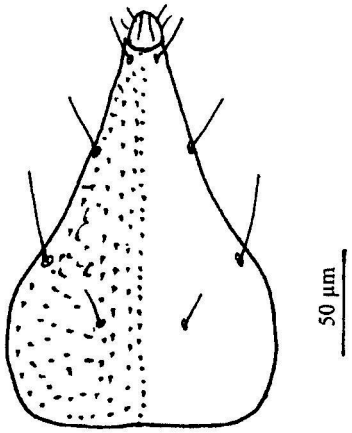


Fig. 1 E

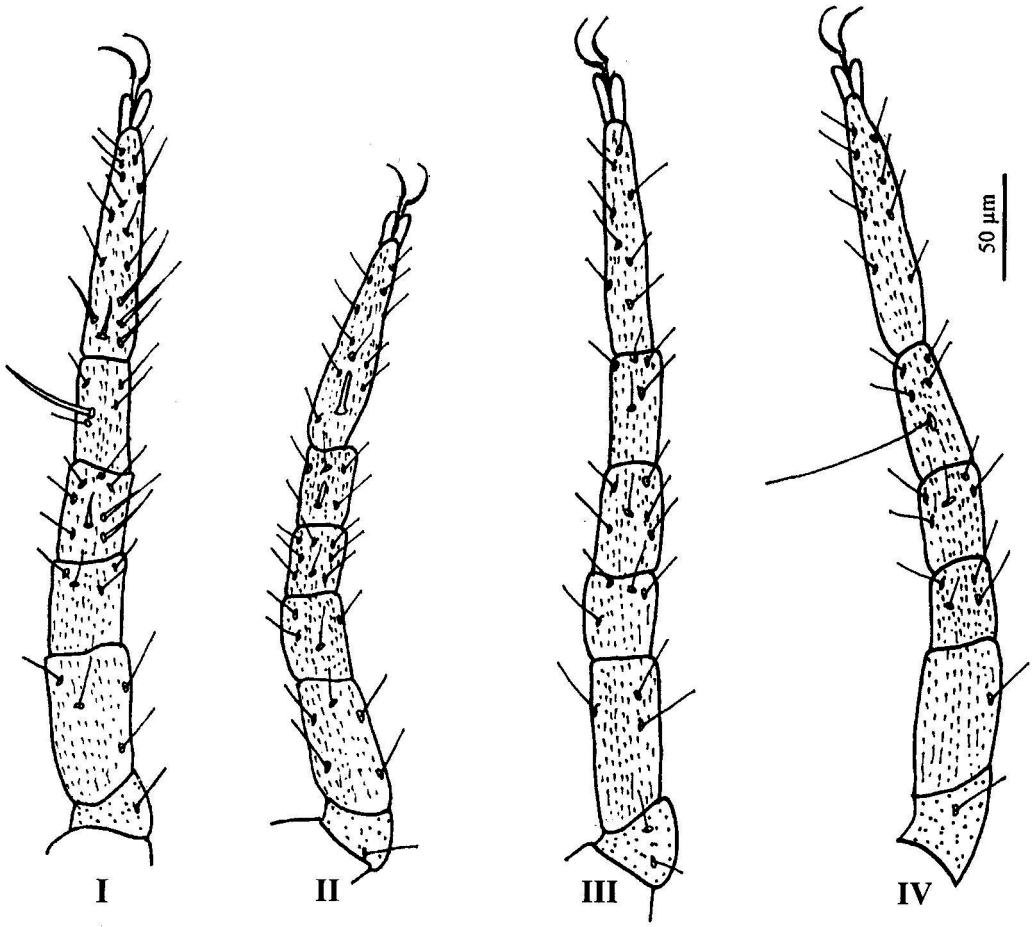


Fig. 1F

Fig. 1C. – *Armascirus akhtari* Bashir, Afzal & Khan, new species, Palp
Fig. 1D. – *Armascirus akhtari* Bashir, Afzal & Khan, new species, Chelicera
Fig. 1E. – *Armascirus akhtari* Bashir, Afzal & Khan, new species, E-Hypostome
Fig. 1F. – *Armascirus akhtari* Bashir, Afzal & Khan, new species, legs I-IV

Venter

Venter with papillated striations. Coxae I-II and coxae III-IV contiguous and reticulated. Hysterosoma with 6 pairs of simple setae between coxae II and genital region. Genital valves covered with papillae arranged in rows, each valve with 4 simple genital setae (g_1-g_4) longitudinally aligned and 2 genital suckers. Anal setae (a) 1 pair, paranal setae (pa) 2 pairs. One pair minute pores near anal shield (Fig. 1B).

Legs

Legs I-IV measuring (from trochanter base to the tip of tarsus) 333, 310, 370 and 380 respectively. All legs papillate, tarsi I-IV long, slender and attenuated, terminating in conspicuous lateral bilobed flanges. Chaetotaxy of legs I-IV as follows: Coxae 3-1-3-2; trochanters 1-1-2-1; basifemora 4-5-3-1; telofemora 4-4-4-4; genua 8 (3 asl + 5 sts)-8-6-5; tibiae 5(1 asl + 4 sts)- 6(1 bsl + 5 sts)-5-5(1 T + 4 sts) and tarsi 15(5 asl + 10 sts)- 12(1 bsl + 11 sts)-8-9 (Fig. 1F).

Male

Unknown.

Type

Holotype female, collected from University of Agriculture (Faisalabad) from leaf debris on 01-08-2004 (Hamid) and deposited in Acarology Research Laboratory, Department of Agri. Entomology, University of Agriculture Faisalabad – Pakistan.

Etymology

This species is named in honour of Prof. Dr. Akhtar.

Remarks

This new species can be separated from *Armascirus mactator* Muhammad & Chaudhri by the following characters.

1. Palp telofemur with 2 spine-like setae in *A. mactator* as against only one in this new species.

2. Chaetotaxy of legs I-IV in *A. mactator* is: coxae 3-2-3-3; basifemora 5-5-4-2; genua 9-7-6-7; tibiae 7-6-6-5 and tarsi 29-24-22-21 whereas in the new species chaetotaxy of legs I-IV is, coxae 3-1-3-2; basifemora 4-5-3-1; genua 8-8-6-5; tibiae 5-6-5-5 and tarsi 15-12-8-9.

This new species can also be separated from *A. pluri* Muhammad & Chaudhri by the following characters.

1. Palp telofemur with one spine-like seta and two apophyses in *A. pluri,* while palp telofemur has one spine-like seta and one apophysis in this new species.

2. In *A. pluri* genital shield with scattered papillae but in this new species genital shield with papillae arranged in rows.

3. In *A. pluri* leg segments I-IV coxae; basifemora; genua; tibiae and tarsi with 3-2-3-3; 5-5-4-2; 8-7-6-7; 6-5-6-5 and 29-25-23-22 setae respectively as against 3-1-3-2; 4-5-3-1; 8-8-6-5; 5-6-5-5 and 15-12-8-9 in this new species.

This new species also relates to *A. heryfordi* Smiley in that:

1. Hysterosomal shield triangular in this new species while it is squarish in *A. heryfordi*.

2. Ventral hysterosoma with 5 pairs of simple setae between coxae II and genital region in *A. heryfordi* as against 6 pairs in this new species.

This new species can also be separated from *A. huysseeni* den Heyer and *A. taurus* (Kramer) by the setal counts of legs I-IV.

6. *Armascirus jasmina*, Bashir, Afzal & Khan, new species
(Figs 2 A – F)

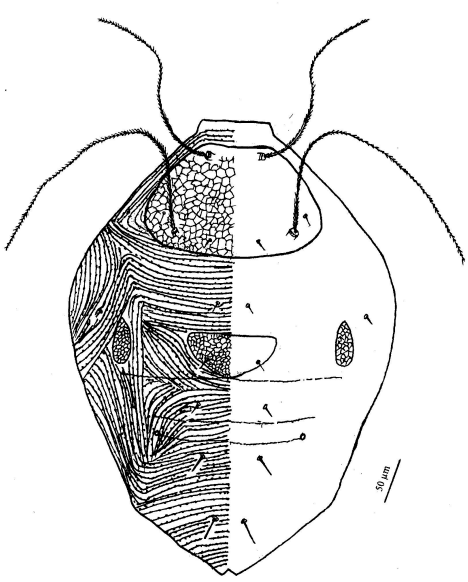


Fig. 2A. – *Armascirus jasmina*, Bashir, Afzal & Khan, new species, Dorsal Side

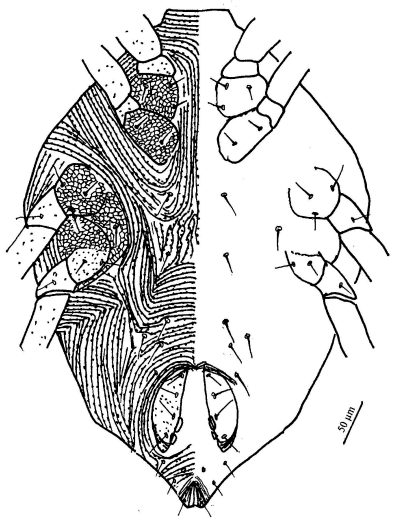


Fig. 2B. – *Armascirus jasmina*, Bashir, Afzal & Khan, new species, Ventral Side

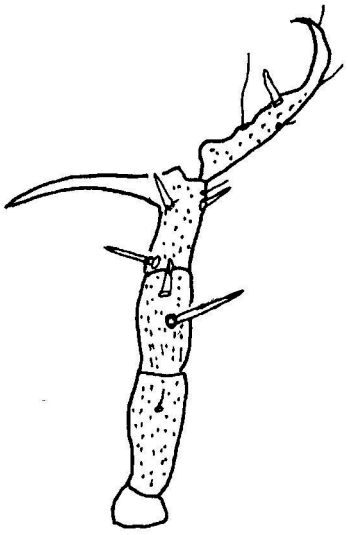


Fig. 2 C

Fig. 2 D

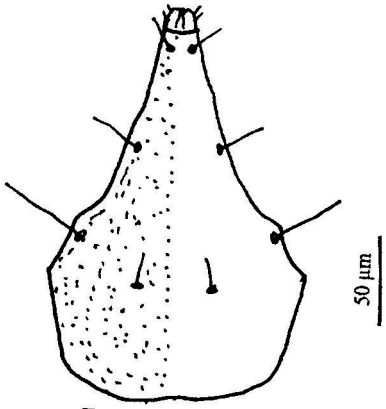


Fig. 2 E

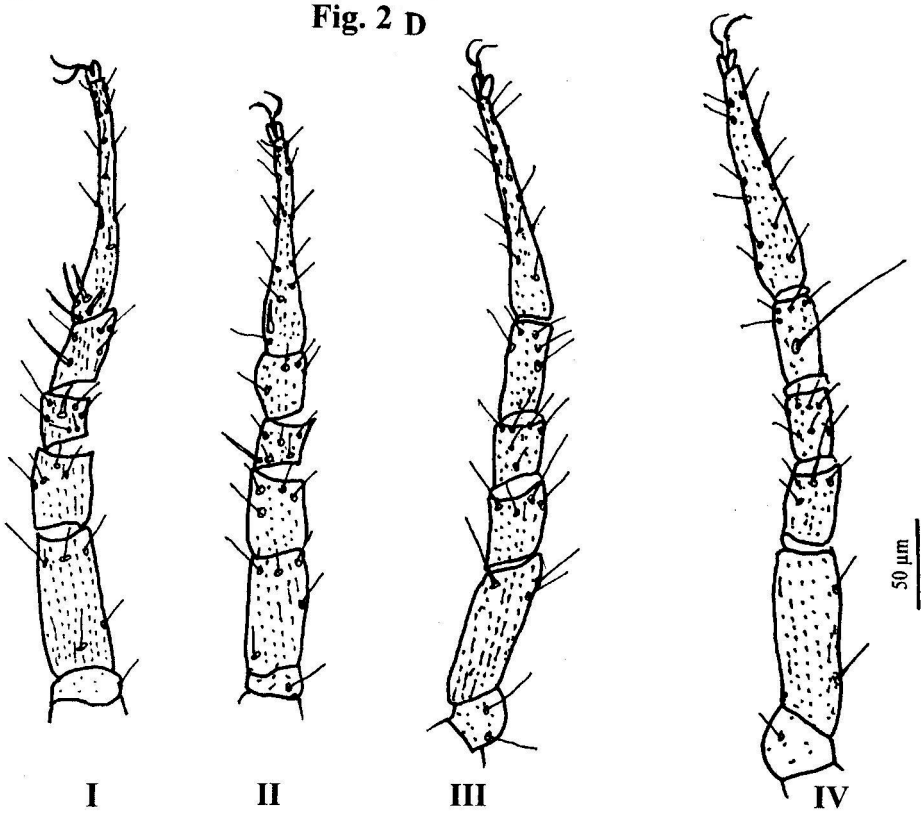


Fig. 2 F

Fig. 2C. *Armascirus jasmina*, Bashir, Afzal & Khan, new species, Palp
Fig. 2D. *Armascirus jasmina*, Bashir, Afzal & Khan, new species, Chelicera
Fig. 2E. *Armascirus jasmina*, Bashir, Afzal & Khan, new species, Hypostome
Fig. 2F. *Armascirus jasmina*, Bashir, Afzal & Khan, new species, legs I-IV

Female

Gnathosoma

Gnathosoma 421 (411-450) long and 117 (108-118) wide. Hypostome papillate, sub-rectangular in shape and cone-shaped distally; with 4 pairs simple hypognathal setae (hg_1-hg_4) and two pairs adoral setae (Fig. 2E).

Palp 5 segmented, all segments papillate, measuring 320 (310-320). Chaetotaxy of palp as follows: trochanter none; basifemur with one simple seta; telofemur with one apophysis and one spine-like seta; genu with one long triangular apophysis, one simple seta and 3 spine-like setae; tibiotarsus terminating in a small claw, with 4 (1 long + 3 small) simple setae, and one thick, stout spine-like seta (Fig. 2C).

Chelicera 180 (180-200) long, terminating in a claw, dorsal and ventral sides with lobes, with one dorsolateral simple seta (Fig. 2D).

Dorsum

Body 510 (510-588) long (without gnathosoma) and 402 (323-441) wide.

Propodosoma with reticulated subrectangular shield, originating behind the base of gnathosoma and extending to the anterior region of hysterosoma. Propodosomal shield with anterior and posterior sensillae PS_1, PS_2 measuring 284 (265-284), 411 (385-411) respectively and propodosomal setae P_1, P_2 both simple 12 (11-14) and 12 (11-12) long, respectively.

Hysterosoma separated from propodosoma by papillate striae. Hysterosoma with two lateral reticulated shields and one triangular reticulated median shield with setae D_2 measuring 12 (12-14). Setae L_1, D_1, D_3, D_4, D_5 present on dorsal hysterosomal membrane. Setae L_1 11 (11-12), D_1 10 (10-12), D_3 13 (13-15), D_4 36 (34-36) and D_5 36 (36-38) long, all simple. Hysterosoma with one pair of lateral pores between setae D_3 and D_4 (Fig. 2A).

Venter

Venter with papillate striations. Coxae I-II and coxae III-IV contiguous and reticulated. Hysterosoma with 7 pairs of simple setae between coxae II and genital region. Genital shield with two valves having random papillae, each valve with 4 simple genital setae (g_1-g_4) in a longitudinal row and 2 genital suckers. Anal setae (a) 1 pair, paranal setae (pa) 2 pairs. One pair of minute pores near anal shield (Fig. 2B).

Legs

Legs I-IV measuring (from trochanter base to the tip of tarsus) 402 (402-412), 352 (253-382), 411 (411-421) and 460 (460-490) respectively. All legs with papillae, tarsi I-IV long, slender and attenuated, terminating in conspicuous lateral bilobed flanges. Chaetotaxy of legs I-IV as follows: coxae 3-2-3-2; trochanters 1-1-2-1; basifemora 5-5-3-2; telofemora 4-4-4-4; genua 7 (1 asl + 6 sts)-6(1 asl + 5 sts)-6-7; tibiae 5(1 asl + 4 sts)-4-5-5(1 T + 4 sts) and tarsi 16(4 asl + 12 sts)-14(1 asl + 13 sts)-13-12 (Fig. 2F).

Male

Unknown.

Types

Holotype female, collected at Faisalabad from leaf debris on 02-08-2004 (Hamid). Five female paratypes were collected from Faisalabad on 13-06-2004, one from Multan on 17-06-2004, five from Faisalabad on 13-06-2004, two from Bahawalpur on 12-08-2004, six from Lahore on 28-08-2004, two from Sialkot on 11-07-2004 and three from Rahim Yar Khan on 16-08-2004. All were collected from leaf debris and deposited in Acarology Research Laboratory, Department of Agri. Entomology, University of Agriculture Faisalabad – Pakistan.

Etymology

This species epithet refers to the source of collection. i.e. dried leaves of jasmine (*Jasminum grandiflorum*: Jasminaceae)

Remarks

This new species is very similar to *A. heryfordi* Smiley but can be separated from it as followings:

1. Dorsal hysterosoma with rectangular reticulated shield in *A. heryfordi* while in *A. jasmina* n. sp. hysterosoma with triangular reticulated shield.

2. Venter with 5 pairs of simple setae between coxae II and genital region in *A. heryfordi* as against 7 pairs in *A. jasmina* n. sp.

3. Chaetotaxy of coxae; basifemora; genua; tibiae and tarsi of *A. heryfordi* with 3-2-3-3; 5-5-4-2; 8-7-6-7; 7-6-6-5 and 19-13-13-13 setae respectively but in new species with 3-2-3-2; 5-5-3-2; 7-6-6-7; 5-4-5-5 and 16-14-13-12 setae respectively.

This new species can also be separated from *A. gimpeli* Smiley by the following characters:

1. Lateral hysterosomal shields absent in *A. gimpeli* while they are present in this new species.

2. Venter with 6 pairs of simple setae between coxae II and genital region in *A. gimpeli* as against 7 pairs in this new species.

3. Both species differ in setal counts on legs I-IV.

This new species is also similar to *A. taurus* (Kramer), but can be separated from it by the following characters:

1. Palp genu with two simple setae in *A. taurus* while palp genu with one seta in this new species.

2. Venter with 6 pairs of simple setae between coxae II and genital region in *A. taurus* as against 7 pairs in this new species.

3. Both species differ in setal counts of legs.

7. *Armascirus sabrii*, Bashir, Afzal & Khan, new species
(Figs 3 A – F)

Female

Gnathosoma

Gnathosoma 265 (265-274) long and 78 (78-88) wide. Hypostome papillate, sub-rectangular in shape and cone-shaped distally with almost parallel sides, with 4 pairs simple hypognathal setae (hg_1-hg_4) and two pairs adoral setae (Fig. 3E).

Palp 5 segmented, all segments papillate, measuring 210 (205-210). Chaetotaxy of palp as follows: trochanter none; basifemur with one simple seta; telofemur with one apophysis and one spine-like seta; genu with one long triangular apophysis, one simple seta and 2 spine-like setae; tibiotarsus terminating in a small claw, with 4 (3 small and 1 long) simple setae and one thick spine-like seta (Fig. 3C).

Chelicera 110 (110-115) long, terminating in a claw, dorsal and ventral sides with lobes, with one dorsolateral simple seta (Fig. 3D).

Dorsum

Body 412 (392-412) long (without gnathosoma) and 303 (274-303) wide.

Propodosoma with reticulated sub-triangular shield, originating behind the base of gnathosoma and extending to the anterior region of hysterosoma. Propodosomal shield with anterior and posterior sensillae PS_1, PS_2 measuring 245 (196-245), 284 (265-284) respectively and propodosomal setae P_1, P_2 both simple 8 (7- 8) and 8 (7-8) long, respectively.

Hysterosoma separated from propodosoma by striations bearing papillae. Hysterosoma with only one triangular reticulated median shield with setae D_2 measuring 10 (8-10). Setae L_1, D_1, D_3, D_4 and D_5 present on dorsal hysterosomal membrane. Setae L_1 10 (8-10), D_1 7 (7-8), D_3 16 (14-16), D_4 24 (24-26) and D_5 25 (25-27) long, all simple. Hysterosoma with one pair of pores lateral in position, between setae D_3 and D_4 (Fig. 3A).

Venter

Venter with papillated striations. Coxae I-II and coxae III-IV contiguous and reticulated. Hysterosoma with 5 pairs of simple setae between coxae II and genital region. Genital shield with two valves having random papillae, each valve with 3 simple genital setae (g_1-g_3) in a longitudinal row and 2 genital suckers. Anal setae (a) 1 pair, paranal setae (pa) 2 pairs. One pair of minute pores near anal shield (Fig. 3B).

Legs

Legs I-IV measuring (from trochanter base to the tip of tarsus) 274 (265-274), 245 (225-245), 284 (265-284) and 294 (284-294) respectively. All legs with papillae, tarsi I-IV long, slender and attenuated, terminating in conspicuous lateral bilobed flanges. Chaetotaxy of legs I-IV as follows: Coxae 3-1-3-2; trochanters 1-1-2-1; basifemora 1-2-1-0; telofemora 4-4-4-4; genua 6-7-5-6; tibiae 5(1 asl + 4 sts)-5-5(1 bsl + 4 sts)-5(1 T + 4 sts) and tarsi 11(2 asl + 9 sts)-7(1 bsl + 6 sts)-5-5 (Fig. 3F).

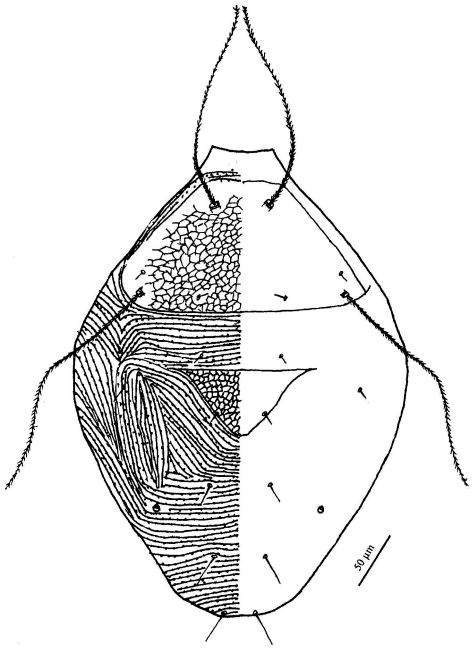


Fig. 3A. – *Armascirus sabrii*, Bashir, Afzal & Khan, new species, Dorsal Side

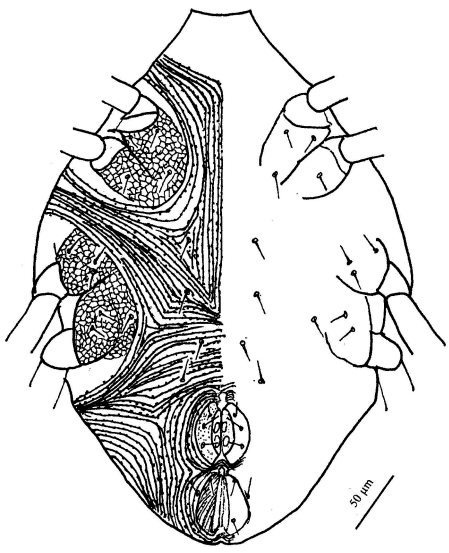


Fig. 3B. – *Armascirus sabrii*, Bashir, Afzal & Khan, new species, Ventral Side

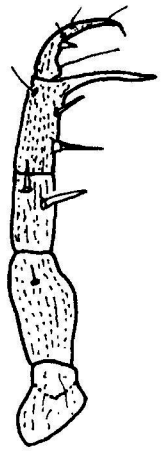


Fig. 3 C

Fig. 3 D

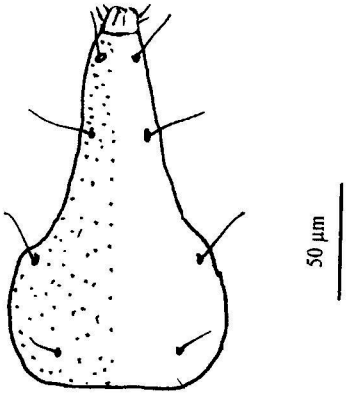


Fig. 3 E

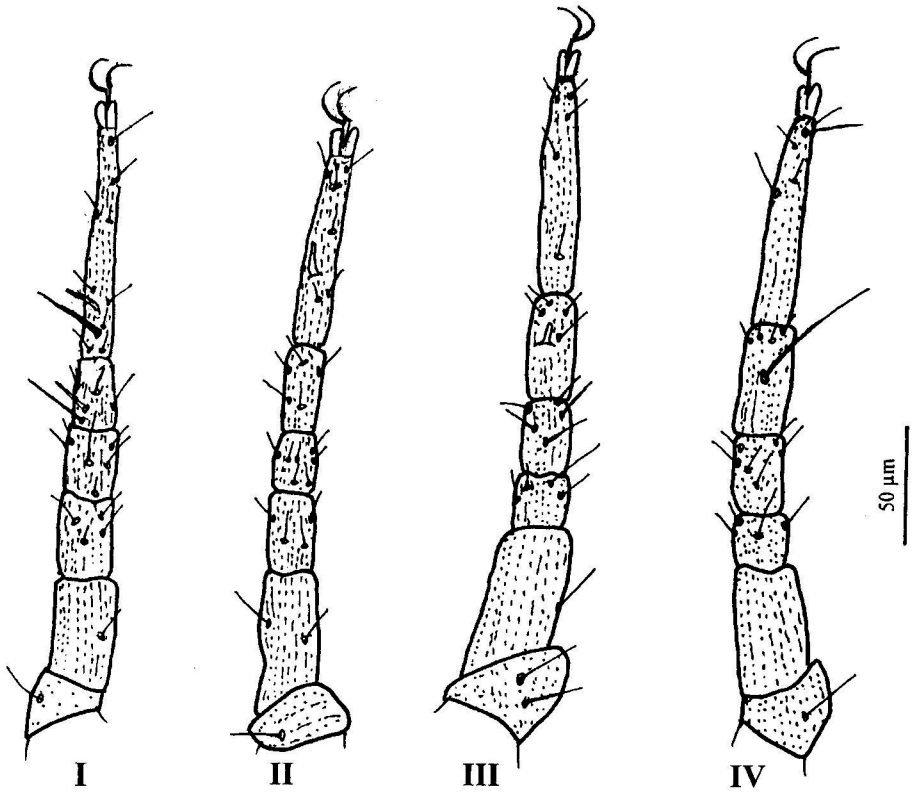


Fig. 3 F

Fig. 3C. *Armascirus sabrii*, Bashir, Afzal & Khan, new species, Palp
Fig. 3D. *Armascirus sabrii*, Bashir, Afzal & Khan, new species, Chelicera
Fig. 3E. *Armascirus sabrii*, Bashir, Afzal & Khan, new species, Hypostome
Fig. 3F. *Armascirus sabrii*, Bashir, Afzal & Khan, new species, legs I-IV

Male

Unknown.

Types

Holotype female, collected Faisalabad from leaf debris on 16-04-2004 (Hamid). Five female paratypes were collected from Faisalabad on 16-04-2004 and one was collected from Kahror Paka (Lodhran) on 20-07-2004. All were collected from leaf debris and deposited in Acarology Research Laboratory, Department of Agri. Entomology, University of Agriculture Faisalabad – Pakistan.

Etymology

This species is named for Mr. M. Altaf Sabry, a dedicated collector and researcher in the Department of Agri. Entomology, University of Agriculture, Faisalabad, Pakistan.

Remarks

This new species can be separated from *A. pluri* Muhammad & Chaudhri and *A. mactator* Muhammad & Chaudhri by the absence of a lateral reticulated shield.

This new species can also be separated from *A. lebowensis* den Heyer as follows:

1. Palp telofemur with two apophyses in *A. lebowensis* as against only one in *A. sabrii* n. sp.

2. Venter with 7 pairs of simple setae between coxae II and genital region in *A. lebowensis* where as in *A. sabrii* n. sp. venter with only 5 pairs of simple setae.

3. Chaetotaxy of legs I-IV in *A. lebowensis* as follows: basifemora 5-5-4-2; genua 9-7-6-6; tibiae 7-6-5-5 and tarsi 25-24-22-21 while chaetotaxy of legs I-IV in *A. sabrii* n. sp. as follow: basifemora 1-2-1-0; genua 6-7-5-6; tibiae 5-5-5-5 and tarsi 11-7-5-5.

This new species also closely resembles *A. gimpeli* Smiley, but can be separated from it by the following characters:

1. Palp genu with 3 spine-like setae in *A. gimpeli* while with two spine-like setae in this new species.

2. Venter with 6 pairs of simple setae between coxae II and genital region in *A. gimpeli* as against 5 pairs in this new species.

3. Both species differ in setal counts of legs.

4. Genital shield with 4 pairs of simple setae in *A. gimpeli* whereas genital shield with 3 pairs of simple setae in this new species.

8. *Armascirus gojraensis*, Bashir, Afzal & Khan, new species
(Figs 4 A – F)

Female

Gnathosoma

Gnathosoma 254 long and 78 wide. Hypostome flask-shaped, with 4 pairs simple hypognathal setae (hg_1-hg_4) and 2 pairs adoral setae (Fig. 4E).

Palp 5 segmented, all segments papillate, measuring 200. Chaetotaxy of palp as follows: trochanter none; basifemur with one simple seta; telofemur with one apophysis and one spine-like seta; genu with one long triangular apophysis, 3 spine-like setae and one simple seta; tibiotarsus terminating in a small claw; with 4 (1 long + 3 small) simple setae and one thick, stout spine-like seta (Fig. 4C).

Chelicera 140 long, terminating in a claw, dorsal and ventral sides with lobes, with one dorsolateral simple seta (Fig. 4D).

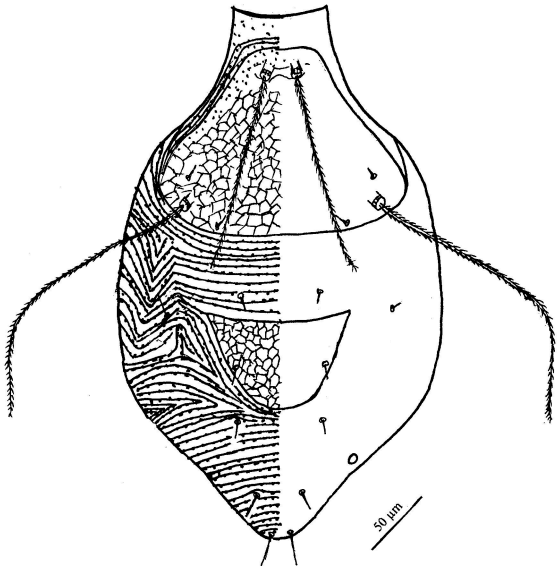


Fig. 4A. – *Armascirus gojraensis*, Bashir, Afzal & Khan, new species, Dorsal Side

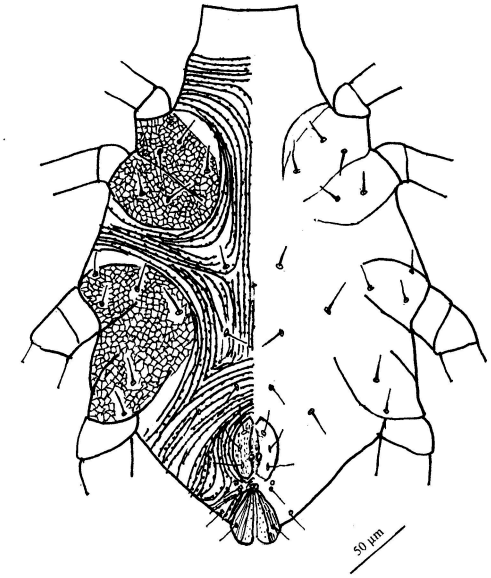


Fig. 4B. – *Armascirus gojraensis*, Bashir, Afzal & Khan, new species, Ventral Side

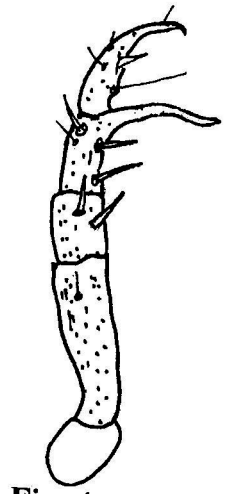


Fig. 4 C

Fig. 4 D

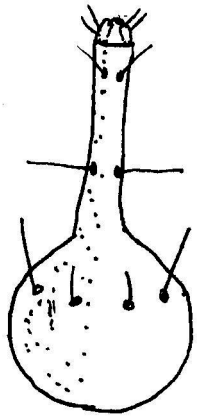


Fig. 4 E

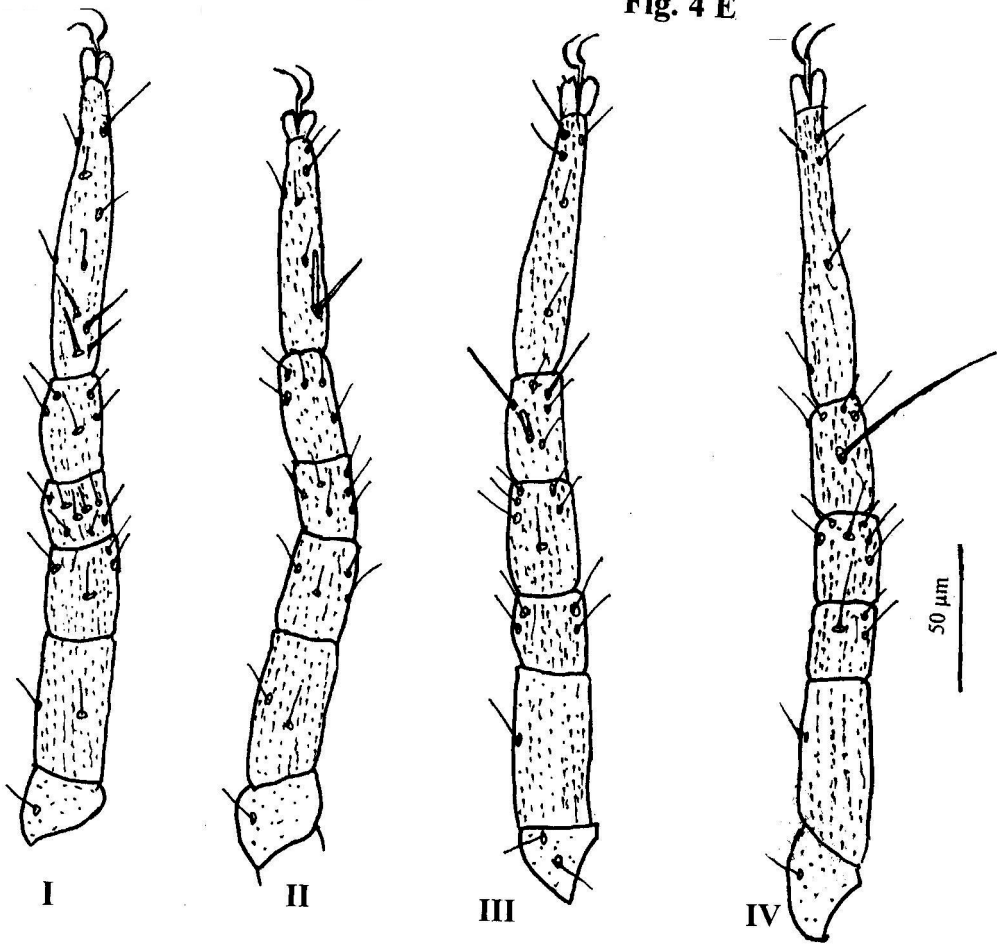


Fig. 4 F

Fig. 4C. – *Armascirus gojraensis*, Bashir, Afzal & Khan, new species, Palp
Fig. 4D. – *Armascirus gojraensis*, Bashir, Afzal & Khan, new species, Chelicera
Fig. 4E. – *Armascirus gojraensis*, Bashir, Afzal & Khan, new species, Hypostome
Fig. 4F. – *Armascirus gojraensis*, Bashir, Afzal & Khan, new species, legs I-IV

Dorsum

Body 362 long (without gnathosoma) and 235 wide. Propodosoma with reticulated sub rectangular shield, originating behind the base of gnathosoma and extending to the anterior region of hysterosoma. Propodosomal shield with anterior and posterior sensillae PS_1, PS_2 measuring 186, 264 respectively and propodosomal setae P_1 and P_2 both simple 8 and 10 long, respectively.

Hysterosoma separated from propodosoma by striae bearing papillae. Hysterosoma with only one triangular reticulated median shield with setae D_2 measuring 12. Setae L_1, D_1, D_3, D_4, D_5 present on dorsal hysterosomal membrane. Setae L_1 9, D_1 10, D_3 12, D_4 17 and D_5 27 long, all simple. Hysterosoma with one pair of pores between seta D_3 and D_4 (Fig. 4A).

Venter

Venter with papillated striations. Coxae I-II and coxae III-IV contiguous and reticulated. Hysterosoma with 4 pairs of simple setae between coxae II and genital region. Genital shield with two valves having lobe like dots arranged in rows, each valve with 3 simple genital setae (g_1-g_3) in a row and 2 genital suckers. Anal setae (a) 1 pair, paranal setae (pa) 2 pairs. One pair of minute pores near anal shield (Fig. 4B).

Legs

Legs I-IV measuring (from trochanter base to the tip of tarsus) 264, 254, 284 and 303 respectively. All legs with papillae, tarsi I-IV long, slender and attenuate, terminating into conspicuous lateral bilobed flanges. Chaetotaxy of legs I-IV as follows: Coxae 3-2-3-3; trochanters 1-1-2-1; basifemora 2-2-1-1; telofemora 4-4-4-3; genua 8-6-6-6; tibiae 5-5-6(1 bsl + 5 sts)-5(1 T + 4 sts) and tarsi 10(4 asl + 6 sts)-7(1 asl + 1 bsl + 5 sts)-5-5 (Fig. 4F).

Male

Unknown.

Type

Holotype female, collected Gojra (T.T. Singh), Pakistan, from leaf debris on 06-08-2004 (Hamid) and deposited in Acarology Research Laboratory, Department of Agri. Entomology, University of Agriculture Faisalabad - Pakistan.

Etymology

This species epithet is for the type locality.

Remarks

This new species can be separated from *A. limpopoensis* den Heyer as follows:

1. Palp telofemur in *A. limpopoensis* with one triangular apophysis but in this new species this triangular apophysis is absent.

2. Venter with 7 pairs of simple setae between coxae II and genital region in *A. limpopoensis* as against 5 pairs in this new species.

3 Chaetotaxy of legs I-IV in *A. limpopoensis* as follows: basifemora 5-5-4-2; telofemora 4-4-4-4; genua 8-7-6-8; tibiae 8-6-6-5 and tarsi 25-25-23-21 whereas in the new species chaetotaxy of legs I-IV as follows: basifemora 2-2-1-1; telofemora 4-4-4-3; genua 8-6-6-6; tibiae 5-5-6-5 and tarsi 10-7-5-5.

This new species can also be separated from *A. gimpeli* Smiley by the following characters:

1. Genital shield with 4 pairs of simple setae in *A. gimpeli* while genital shield with 3 pairs of simple setae in *A. gojraensis* n. sp.

2. Both species differ in setal counts of legs I-IV.

3. Venter with 6 pairs of simple setae between coxae II and genital region in *A. gimpeli* as against 4 pairs in *A. gojraensis* n. sp.

This new species also resembles to *A. taurus* (Kramer), but can be separated from it by the following characters.

1. Hysterosomal lateral shields are absent in this new species while lateral shields are present in *A. taurus*.

2. Venter with 6 pairs of simple setae between coxae II and genital region in *A. taurus* as against 4 pairs in this new species.

3. Genital shield with 4 pairs of simple setae in *A. taurus* while with 3 pairs in this new species.

4. Setal counts on legs I-IV differ in both species.

DISCUSSION

DEN HEYER erected the genus *Armascirus* in 1978 and designated *A. huyssteeni* as its type species. He placed this genus under the subfamily Cunaxinae, tribe Armascirini, which also includes the genus *Dactyloscirus* Berlese, 1916. Some important works on this genus were published by SEPASGOSARIAN (1984) and LIANG (1985), but major contributions are by SMILEY (1992) who added five new species and made a total of ten species in this genus. From Pakistan a total of eight species (including four new species herein described) have been recorded.

The dendrogram of genus *Armascirus* (Fig. 5) depicts the highest similarity (92.76%) between the species *mactator* and *pluri*: both share the same habitat (Faisalabad) in central Punjab and are exposed to similar environmental factors. The species *jasmina* and *asghari*, both from discrete localities in the Punjab, show a similarity of 90.53% with each other. The species *akhtari* and *satianaensis*, both from same locality and sharing 79.72% characters, join the *jasmina – asghari* pair at 74.50% affinity level, whereas *gojraensis – sabrii* pair having an affinity of 89.41% also from the same locality join this cluster at 69.91% affinity level, thus forming a big cluster. The species pair *mactator – pluri* join the large cluster sharing weak phenetic affinity of only 21.97%.

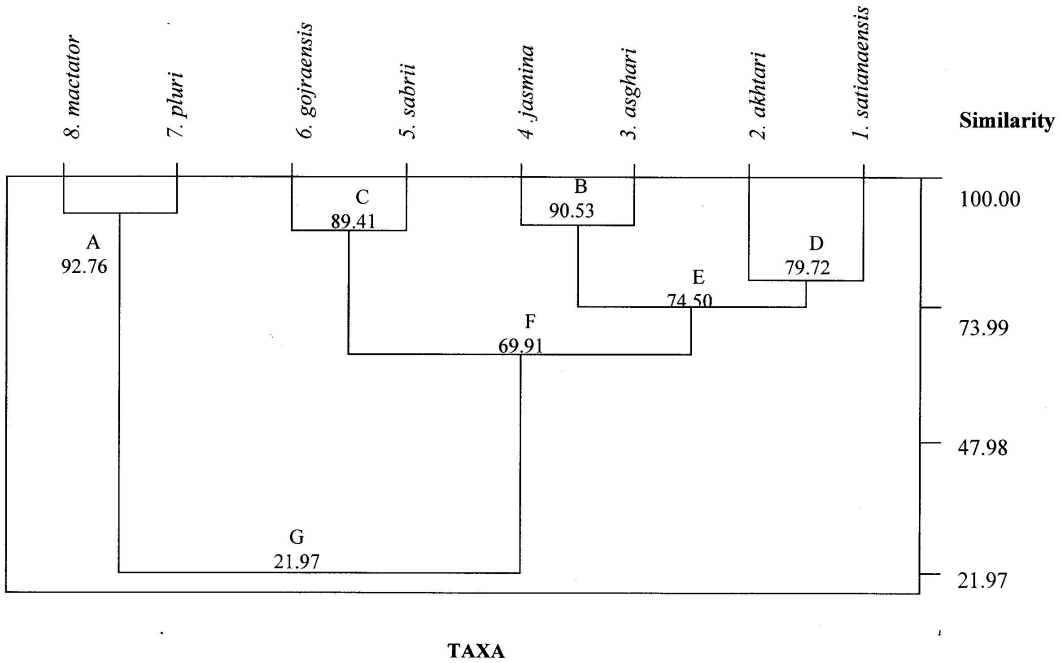


Fig. 5. – Dendrogram of 8 species of the genus *Armascirus* from Pakistan

TABLE 1

Prevalence of 24 characters in 8 species of the Genus *Armascirus* (Acari; Cunaxidae) from Pakistan.

Characters	Species							
	satianaensis	*akhtari*	*asghari*	*jasmina*	*sabrii*	*gojraensis*	*pluri*	*mactator*
I	3	3	3	3	2	3	3	3
II	0	1	1	1	1	1	1	1
III	1	1	1	1	0	0	1	1
IV	12	12	14	14	10	10	12	12
V	4	4	4	4	3	3	4	4
VI	4	4	3	3	3	4	3	4
VII	2	1	2	2	1	2	2	2
VIII	2	2	2	2	2	3	3	3
IX	4	4	4	5	1	2	5	5
X	4	5	5	5	2	2	5	5
XI	3	3	4	3	1	1	4	4
XII	3	1	2	2	0	1	2	2
XIII	4	4	4	4	4	3	4	4
XIV	8	8	8	7	6	8	8	9
XV	4	8	6	6	7	6	7	7
XVI	6	6	6	6	5	6	6	6
XVII	7	5	6	7	6	6	7	7
XVII	6	5	6	5	5	5	6	7
XIX	5	6	5	4	5	5	5	6
XX	6	5	6	5	5	6	6	6
XXI	11	15	18	16	11	10	29	29
XXII	10	12	15	14	7	7	25	24
XXIII	9	8	13	13	5	5	23	22
XXIV	7	9	12	12	5	5	22	21

I, Spines on palp genu; II, Setae on palp genu; III, Long triangular apophysis on palp genu (0, absent; 1, present); IV, Setae on ventral hysterosoma; V, Setae on genital shield; VI, Genital shield (1, smooth; 2, striated; 3, random dots; 4, with dots arranged in rows); VII, Setae on Coxa II; VIII, Setae on Coxa IV; IX, Setae on basifemur I; X, Setae on basifemur II; XI, Setae on basifemur III; XII, Setae on basifemur IV; XIII, Setae on telofemur IV; XIV, Setae on genu I; XV, Setae on genu II; XVI, Setae on genu III; XVII, Setae on genu IV; XVIII, Setae on tibia I; XIX, Setae on tibia II; XX, Setae on tibia III; XXI, Setae on tarsus I; XXII, Setae on tarsus II; XXIII, Setae on tarsus III; XXIV, Setae on tarsus IV.

The observed similarity might be due to the shared states of the chosen attributes, with the similarity increasing with the number of these shared attributes. Sometimes, however, we may restrict the attributes considered to only those that are relevant or similar in diagnostics in the sense that the characters chosen are shared only by some but not by all the species under consideration. According to JORDINE & SIBSON (1971), a similarity may thus be converted into dissimilarity for any fixed list of attributes in such cases.

KOHN & ORIONS (1962) have argued that closely related species could exploit different niches through adaptation not involving conspicuous morphological character differences. This point was made earlier by DOBZHANSKY (1940) and SIMPSON (1953; 1961) who advocated that the adaptive value – be it morphological, ecological or physiological – of a species complex is the property of genotype. This observation has been condensed by WILSON (1965) who argued, in relation to phenotypic plasticity, that behaviour (distribution) is the part of phenotype most likely to become modified in response to changes in the environment. Future studies may reveal that the evolutionary plasticity of mite behaviour (distribution) provides an excellent avenue of adaptation.

LITTLEJOHN (1981) concluded that a discontinuous array of biological diversity exists, particularly when viewed in a spatially restricted situation, and to understand the mechanisms and processes responsible for the origin and maintenance of this diversity one should look into the environment to formulate a combined ecological and genetic analysis. It is with this respect that some assumptions have been made to stimulate further studies.

Regardless of what has been presented in this paper, there remain many issues – ecological, physiological, and behavioural or genetics – for further study in Cunaxid mites.

REFERENCES

BASHIR MH & AFZAL M (2005). New cunaxid mites of the genus *Armascirus* from Punjab – Pakistan. Pakistan Journal of Agricultural Sciences, 42(3-4): 117-122.

DEN HEYER J (1978). Four new species of *Armascirus* gen. nov. (Prostigmata: Acari) from the Ethiopian Region. Journal of Entomological Society of South Africa, 41(2): 217-239.

DOBZHANSKY T (1940). Speciation as a stage in evolutionary divergence. American Naturalist, 74: 312-321.

JORDINE N & SIBSON N (1971). Mathematical Taxonomy. John Wiley and Sons, London. 286 pp.

KOHN AJ & ORIONS GH (1962). Ecological data in the classification of closely related species. Systematic Zoology, 11: 119-127.

LIANG GW (1985). New species and new record of cunaxid mites from China. Acta Zootaxonomica Sinica, 7: 79-81.

LITTLEJOHN MJ (1981). Reproductive Isolation: a critical review. In: ATCHLEY WR & WOODRUFF D (eds), Evolution and Speciation. Cambridge University Press. 436 pp.

MUHAMMAD T & CHAUDHRI WM (1991). Two new Cunaxid mites of the genus *Armascirus* (Acarina: Cunaxidae) from Pakistan. Pakistan Entomologist, 13(1-2): 50-55.

SIMPSON GG (1953). The major features of evolution. Columbia University Press. New York. 285 pp.

SIMPSON GG (1961). Principles of Animal Taxonomy. Columbia University Press. New York. 247 pp.

SMILEY RL (1975). A generic revision of the mites of the family Cunaxidae (Acarina). Annals of the Entomological Society of America, 68(2): 227-244.

SMILEY RL (1992). The Predatory Mite Family Cunaxidae (Acari) of the World with a New Classification. Indira Publishing House, 356 pp.

SEPASGOSARIAN H (1984). The world genera and species of the family Cunaxidae (Actinedida: Acarida). Zeitschrift für Angewandte Zoologie, 71: 135-150.

TAGORE A & PUTATUNDA BN (2003). Mites associated with some ornamental plants at Hisar, Haryana. Pest Management and Economic Zoology, 11(1): 37-44.

WALTER DE & KAPLAN DT (1991). Observations on *Coleoscirus simplex* (Acarina: Prostigmata), a predatory mite that colonizes greenhouse cultures of rootknot nematode (*Meloidogyne* spp.), and a review of feeding behaviour in the Cunaxidae. Experimental and Applied Acarology, 12(1-2): 47-59.

WILSON EO (1965). A consistency test of phylogenies based on contemporaneous species. Systematic Zoology, 14: 214-230.

Received: January 9, 2006
Accepted: March 11, 2008

Belg. J. Zool., 138 (2) : 128-131 July 2008

Feeding ecology of various age-classes of brown trout in River Nera, Central Italy

Romolo Fochetti[1], Roberto Argano[2] & José Manuel Tierno de Figueroa[3]

[1] **Dept. of Environmental Sciences, University of Viterbo, Largo dell'Università 01100, Viterbo, Italy. E-mail: fochetti@unitus.it**
[2] **Dept. of Animal and Human Biology, v.le Università, 32, 00185, Rome, Italy**
[3] **Dept. de Biología Animal. Facultad de Ciencias. Universidad de Granada, 18071, Granada, Spain. E-mail: jmtdef@ugr.es**

ABSTRACT. We studied the composition of the stomach contents of brown trout (*Salmo trutta trutta*) of different ages in the river Nera, Central Italy. Each age class of fish consumed significantly different prey taxa. Plecopteran nymphs tended to increase in percentage as the individuals became older, while ephemeropteran nymphs were always present in high percentage. Trichopteran larvae were the most abundant prey in trout younger than 2+, while their percentage decreased considerably in older fish. The remaining aquatic prey (except dipteran larvae) were scarce and, finally, terrestrial prey were consumed more by older individuals. Vanderploeg & Scavia's index shows a high preference for species of Trichoptera by trout younger than 3+, plecopteran species by those older than three years, and a general negative preference for species of Ephemeroptera by all age classes.

KEY WORDS : *Salmo trutta trutta*, diet, age, Italy.

INTRODUCTION

Salmonids are generally considered as opportunists (HUNT & JONES, 1972) or generalists (HYNES, 1970) since they are unselective on prey. However, the predatory activity of the brown trout (*Salmo trutta trutta* L., 1758) cannot be considered simply proportional to the environmental density of the prey, as shown by WARE (1972) for rainbow trout.

FOCHETTI et al. (2003) studied the stomach contents of brown trout from the Nera River (Central Italy), confirming the presence of selectivity in its feeding behaviour. In fact, the electivity index showed a negative selection for some species of Ephemeroptera and Diptera and positive selection for species of Trichoptera. Moreover, it was also found that ephemeropteran species dominated spring and summer diets, while trichopteran species prevailed in winter.

Since the diet of fish often changes with body size (ELLIOTT, 1967; WERNER & GILLIAM, 1984), and in salmonids older fish shift their preferences towards larger prey (KEELEY & GRANT, 1997), the aim of the present study was to analyse the possible changes in diet between the different age classes of individuals of brown trout in a river of Central Italy. Moreover, we wanted to verify the relationship between the availability of potential macrobenthic prey and their actual presence in the diet, and to compare the electivity values shown by brown trout of different ages.

MATERIALS AND METHODS

The study site was located along the River Nera. This river originates in the Sibilline Mountains (Central Italy) and flows for about 125km, before joining the River Tiber. The study area encompassed the middle 30km region of the river, a river stretch that has been declared Regional Park with the aim of protecting the fish community, which is mainly composed of brown trout, Italian roach (*Rutilus rubilio* Bonaparte, 1837), soufie (*Leuciscus souffia* Risso, 1826), chub (*Leuciscus cephalus* L., 1758) and bullhead (*Cottus gobio* L., 1758) (MEARELLI et al., 1996). The trout population is sustained by yearly reintroduction of fry and juveniles. In the study stretch the river has an average width of 6 meters, a depth of 80-90cm, and substratum is mainly composed of cobbles, pebbles and gravel. The river banks are flanked by willow (*Salix* spp.), alder (*Alnus glutinosa* L., 1758), elm (*Ulmus minor*, Miller 1768) and poplar (*Populus nigra* L., 1758) trees.

Macrobenthos was sampled by kicking method (3 minutes searching in all major microhabitats; mesh 0.47mm; see HELLAWELL, 1978) seasonally, from October 1996 to January 1998, at four sampling sites.

Brown trout were caught by angling along the same stretch where macrobenthos was collected. A preliminary electrofishing was unsuccessful, due to the river's depth, velocity, and bottom irregularities that prevented netting the fish. While angling is a selective method of collecting trout, the feeding habit of angled fish is generally considered to be representative of the feeding habits of the entire population for the size classes represented (ELLIOTT & JENKINS, 1972; HUNT & JONES, 1972; TIPPETS & MOYLE, 1978; MATHOOKO, 1996). The captures occurred at dawn and before dusk, from February to October 1997, excluding the breeding period in autumn.

A total of 56 trout were caught. After capture, standard length (SL) and total length (TL) of each fish were measured, and a scale sample (6-10 scales) was removed for age determination on the basis of NEEDHAM's criteria (1969) with the aid of a stereoscopic microscope. Because brown trout under 13cm are protected, all small individuals were released. Hence, the study included adult fish aged 2 years and older. Stomachs were removed in the

field and placed in 10% formalin solution. For each stomach both a qualitative and a quantitative evaluation of the contribution given to the trout's diet from every food item was made. The percentage in the gut content of each prey type for each age class of trout was calculated (%P=number of items of a prey type * 100/total number of items consumed).

VANDERPLOG & SCAVIA's (1979a; 1979b) electivity index was used to quantify prey selection. The index is defined as follows:

$$E = \frac{[W_i - (1/n)]}{[W_i + (1/n)]}$$

where W_i is CHESSON's (1978) coefficient:

$$W_i = \frac{(r_i/p_i)}{\Sigma(r_i/p_i)}$$

n is the number of different categories, r_i is the relative proportion in the diet of a food category and p_i is the proportion of the same category in the benthic community.

This index is used in most fish prey selection studies because it is relatively unbiased. The index can vary from –1 (greatest negative selection) to +1 (greatest positive selection). E gives a good estimate of the effort exerted by the predator in selecting a prey and gives high weight to the prey that, being rare in the environment, requires greater effort from the predator.

A non-parametric observed *versus* expected χ^2-test was employed to detect significant differences in the diet composition between age classes.

RESULTS

Stomachs were full in 51 out of 56 trout investigated; they contained 64 taxa of benthic or terrestrial macroinvertebrates and/or vegetation, which were grouped into 16 categories (Table 1). Aquatic insects were the most ingested prey, particularly trichopteran larvae and ephemeropteran nymphs, while non-insect aquatic animals were rarely eaten by the trout.

The percentage of plecopteran nymphs tended to increase with the individual's age; ephemeropteran nymphs were always present in high percentage (Table 2). Trichoptera larvae formed a high percentage (approximately 50%) in trout three and younger than three years old and after that age the percentage decreased considerably. The remaining aquatic prey (Crustacea, Gasteropoda, Nematoda and Annelida) were not abundant, and terrestrial prey (including adults of Plecoptera, Ephemeroptera and Trichoptera and other arthropods of terrestrial origin) were consumed more by older individuals. Ephemeropteran nymphs and trichopteran larvae were the most abundant prey and were always present in gut contents of trout in age classes 2 and 2+, trichopteran and dipteran larvae in age class 3, ephemeropteran nymphs in age class 3+, ephemeropteran nymphs, followed by dipteran larvae, terrestrial prey and plecopteran nymphs, in age class 4-5.

Differences in diet composition among different age classes were significant between every age class (χ^2 test is not significant only between individuals of age classes 2 and 2+) (Table 3).

If we consider only the aquatic prey (Tables 4 & 5), we obtain the same pattern described in Tables 2 and 3. The most abundant macrobenthic taxon was Ephemeroptera, followed by Diptera; they were generally present in a higher percentage in the macrobenthos than they were in the diet of any age class. Trichopteran species showed opposite tendency.

Vanderploeg & Scavia's index "E" (Table 6) shows high preference for trichopteran prey in trout younger than four, for plecopteran prey in trout older than three, and a general negative preference for ephemeropteran species as prey in all age classes.

TABLE 1

Gut content composition of brown trout showing the number of items found by age classes. n=number of individuals studied in each age class, A=adult, N=nymph, L=larva, Plecop=Plecoptera, Ephem=Ephemeroptera, Trichop=Trichoptera, Dipt=Diptera, Coleop=Coleoptera, Crust=Crustacea, Gast=Gastropoda, Nem=Nematoda, Annel=Annelida, Terr=other terrestrial prey, Veg=vegetation.

Age class	n	Plecop		Ephem		Trichop		Dipt		Coleop		Crust	Gast	Nem	Annel	Terr	Veg	Total
		A	N	A	N	A	L	A	L	A	L							
2	18	0	18	13	102	2	231	0	19	3	0	2	1	1	1	28	18	439
2+	11	0	5	5	89	1	112	0	20	0	3	2	0	0	0	5	23	265
3	10	0	14	2	26	0	119	0	51	0	0	3	0	0	1	1	37	254
3+	5	4	6	0	43	0	12	0	3	1	0	1	0	0	0	6	3	79
4-5	7	3	22	6	72	0	7	0	31	0	0	3	0	0	2	24	4	174

TABLE 2

Percentage in the gut content of each prey type (%P). For each age class of trout, %P =number of items of a prey type * 100/total number of items consumed. Vegetation was not included. Abbreviations as in Table 1.

Age class	n	Plecop		Ephem		Trichop		Dipt		Coleop		Crust	Gast	Nem	Annel	Terr
		A	N	A	N	A	L	A	L	A	L					
2	18	0	4.3	3.1	24.2	0.5	54.9	0	4.5	0.7	0	0.5	0.2	0.2	0.2	6.7
2+	11	0	2.1	2.1	36.8	0.4	46.3	0	8.3	0	1.2	0.8	0	0	0	2.1
3	10	0	6.5	0.9	12	0	54.8	0	23.3	0	0	1.4	0	0	0.5	0.5
3+	5	5.3	7.9	0	56.6	0	15.8	0	3.9	1.3	0	1.3	0	0	0	7.9
4-5	7	1.8	12.9	3.5	42.4	0	4.1	0	18.2	0	0	1.8	0	0	1.2	14.1

TABLE 3

Comparison of gut content of different age classes of brown trout with non-parametric $\chi 2$-test (df=14). In bold and italic when p>0.05.

	2+	3	3+	4-5
2	***21.85***	111.33	113.29	660.78
2+		72.68	86.08	463.85
3			241.70	670.70
3+				65.69

TABLE 4

Relative percentage (%) of aquatic macroinvertebrate in the gut content (aquatic components only) and in macrobenthos. Abbreviations as in Table 1 except Others=Gastropoda + Nematoda + Annelida.

Age class	Plecop N	Efem N	Trichop L	Dipt L	Coleop L+A	Crust	Others
2	4.8	27	61.1	5	0.8	0.5	0.8
2+	2.2	38.5	48.5	8.7	1.3	0.9	0
3	6.5	12.1	55.6	23.8	0	1.4	0.5
3+	9.1	65.2	18.2	4.5	1.5	1.5	0
4-5	16.1	52.6	5.1	22.6	0	2.2	1.5
Macrobentos	3.9	60.8	5.9	15.7	4.9	7.8	1.0

TABLE 5

Comparison of gut content (considering only the aquatic preys) of different age classes of brown trout with non-parametric $\chi 2$-test (df=6). In bold and italic when p>0.05.

	2+	3	3+	4-5
2	***11.72***	34.95	126.58	650.64
2+		71.61	70.80	395.92
3			205.13	537.98
3+				55.93

TABLE 6

VANDERPLOG AND SCAVIA's (1979a; 1979b) electivity index (E) for the eaten macrobenthic prey. n=number of individuals studied in each age class, A=adult, L=larva, Plecop=Plecoptera, Ephem=Ephemeroptera, Trichop=Trichoptera, Dipt=Diptera, Coleop=Coleoptera, Crust=Crustacea, Others=Gastropoda + Nematoda + Annelida.

Age class	n	Plecop (N)	Efem (N)	Trichop (L)	Dipt (L)	Coleop (L+A)	Crust	Others
2	18	-0.21	-0.65	0.69	-0.75	-0.87	-1	-0.40
2+	11	-0.47	-0.40	0.70	-0.47	-0.64	-0.87	- 1
3	10	- 0.08	- 0.87	0.67	- 0.12	- 1	-0.87	- 0.72
3+	5	0.39	- 0.07	0.50	- 0.55	-0.55	-0.65	- 1
4+5	7	0.52	-0.17	-0.22	0.07	- 1	-0.65	0.10

DISCUSSION

It is usually accepted that the salmonid diet changes with age (ELLIOTT, 1967). Some authors have not detected those differences in diet when trout are two years old or older (i.e. VOLLESTAD & ANDERSEN, 1985). Our results showed that, in fish older than two years, stomach contents differed significantly between the different age classes.

The changes in diet shown by our study cannot be explained by the fact that, in salmonid, prey size is known to change with body size [larger (and older) fish shift their preferences towards larger prey (KEELEY & GRANT, 1997)]. The relationship between prey size and body size has been established in salmonids smaller than 14.5cm (KEELEY & GRANT, 1997), which may simply have difficulties ingesting large macroinvertebrates. In our study all fish were longer than 13cm; moreover, they fed on prey that were smaller than the size predicted by BANNON & RINGLER's (1986) and WANKOWSKY`s (1979) foraging model for salmonids. This was also noted by STEINGRÍMSSON & GÍSLASON (2002) for *S. trutta* in Iceland. Nevertheless, the presence of limitations to ingestion of large prey by smaller fish could explain, in our results, the increase of plecopteran nymphs in the diet of older trout (particularly Plecoptera of the genera *Dinocras* and *Perla,* which can reach a length of 3-3.5cm), and the positive values of the electivity index recorded for this prey in trout older than three years.

FOCHETTI et al. (2003) stressed that trout show a positive selection towards trichopteran prey in the River Nera. Our detailed analysis of gut contents in each age class, even though based on the observation of reduced number of specimens, showed that this is only true for fish younger than four years. The choice of feeding mainly on trichopteran prey was explained by FOCHETTI et al. (2003) by the reduced mobility of brown trout in winter and by its habit of concealing itself in macrophyte beds in that season. In fact, the brown trout is a visual forager; it shifts to prey on the more available, sedentary trichopteran species in winter, when invertebrate drift is reduced. This shifting in habitat preferences and foraging strategies could be a more common habit in young brown trout than in more experienced and stronger older ones. The same explanation could account for the higher percentage of terrestrial prey in older trout found in our study. The preference for terrestrial prey by older trout has previously been reported for the same species by NEVEU & THIBAULT (1977) in the Pyrenees and MONTORI et al. (2006) in the Prepyrenees.

REFERENCES

BANNON E & RINGLER NH (1986). Optimal prey size for stream resident brown trout (*Salmo trutta*): test of predictive models. Canadian Journal of Zoology, 64: 704-713.

CHESSON J (1978). Measuring preference in selective predation. Ecology, 55: 622-629.

ELLIOTT GV & JENKINS TM (1972). Winter food of trout in three high elevation Sierra Nevada lakes. California fish and games, 58: 2213-2237.

ELLIOTT JM (1967). The food of trout (*Salmo trutta*) in a Dartmoor stream. Journal of Applied Ecology, 4: 60-71.

FOCHETTI R, AMICI I & ARGANO R (2003). Seasonal changes and selectivity in the diet of brown trout in the River Nera (Central Italy). Journal of Freshwater Ecology, 18(3): 437-444.

HELLAWELL JM (1978). Biological surveillance of rivers: a biological monitoring handbook. Water Research Centre, Stevenage Laboratory, Elder Waing, Herts.

HUNT PC & JONES JW (1972). The food of brown trout in Ilyn Alaw, Anglesey, North Wales. Journal of Fish Biology, 4: 333-352.

HYNES HBN (1970). The ecology of running waters. Liverpool University Press, Liverpool.

KEELEY ER & GRANT JWA (1997). Allometry of diet selectivity in juvenile Atlantic salmon (*Salmo salar*). Canadian Journal of Fisheries and Aquatic Sciences, 54: 1894-1902.

MATHOOKO JM (1996). Rainbow trout as a potential natural "drift sampler" in a tropical lotic ecosystem. Limnologica, 26(3): 245-254.

MEARELLI M, LORENZONI M, PETESSE ML, CAROSI A, GIOVINAZZO G, BEVAGNA D, CINGOLANI L, GHETTI L, MONTILLI G, NELLI P, RUCO P & ZAMPA O (1996). Carta ittica della Regione Umbria – Bacino del Fiume Nera. Giunta regionale, Servizio per gli intervent ittiofaunistici, Umbria.

MONTORI A, TIERNO DE FIGUEROA JM & SANTOS X (2006). The diet of the brown trout *Salmo trutta* (L.) during the reproductive period: size-related and sexual effects. International Review of Hydrobiology, 91(5): 438-450.

NEEDHAM PR (1969). Trout streams. Winchester Press, New York.

NEVEU A & THIBAULT M (1977). Comportament alimentaire d'une population sauvage de truite fario (*Salmo trutta* L.) dans un ruisseau des Pyrènèes atlantiques, Le Lissuraga. Annales Hydrobiologiques, 8: 111-128.

STEINGRÍMSSON SÓ & GÍSLASON GM (2002). Body size diet and growth of landlocked brown trout, *Salmo trutta*, in the subarctic river Laxá, North-East Iceland. Environmental Biology of Fisheries, 63: 417-426.

TIPPETS WE & MOYLE PB (1978). Epibenthic feeding by rainbow trout in the McCloud River, California. Journal of Animal Ecology, 47: 549-559.

VANDERPLOG HA & SCAVIA D (1979a). Two electivity indices for feeding with special reference to zooplankton grazing. Journal of the Fisheries Research Board of Canada, 36: 362-365.

VANDERPLOG HA & SCAVIA D (1979b). Calculation and use of selectivity coefficients of feeding: zooplankton grazing. Ecological modelling, 7: 362-365.

VOLLESTAD LA & ANDERSEN R (1985). Resource partitioning of various age groups of brown trout *Salmo trutta* in the littoral zone of lake Selura, Norway. Archive für Hydrobiologie, 105: 177-185.

WANKOWSKI JWJ (1979). Morphological limitations, prey size selectivity, and growth response of juvenile Atlantic salmon, *Salmo salar*. Journal of Fish Biology, 14: 89-100.

WARE DM (1972). Predation by rainbow trout: the influence of hunger, prey density and prey size. Journal of the Fisheries Research Board of Canada, 29(8): 1193-1201.

WERNER EE & GILLIAM JF (1984). The ontogenic niche and species interactions in size-structured populations. Annual Review of Ecology and Systematic, 15: 1042-1052.

Received: June 5, 2006
Accepted: April 16, 2008

Belg. J. Zool., 138 (2) : 132-134 July 2008

A new species of *Tephritis* Latreille (Diptera: Tephritidae) from Turkey

Murat Kütük

University of Gaziantep, Faculty of Science and Art, Department of Biology, 27310, Gaziantep, Turkey
Corresponding author : E-mail: mkutuk@gantep.edu.tr

ABSTRACT. *Tephritis erdemlii* n. sp., from Isparta province, Turkey, associated with *Cirsium vulgare* is described and illustrated. The new species is similar in wing pattern to *T. cometa israelis* Freidberg, and *T. acanthiophilopsis* Hering, differing in the details of wing pattern and the conspicuously longer aculeus.

KEY WORDS : *Tephritis*, new species, Tephritidae, Turkey.

INTRODUCTION

With about 170 species (NORRBOM et al., 1999; KORNEYEV & DIRLBEK, 2000), *Tephritis* Latreille is the sixth largest genus of Tephritidae and third largest genus in the Tephritinae. Although the genus is known from most zoogeographic regions, the majority of the species (about 120) are Palaearctic. The most complete key to species is HERING (1944), which is outdated. Modern keys to species for several countries are available e.g., WHITE (1988) for Great Britain; FREIDBERG & KUGLER (1989) for Israel and nearby areas; MERZ (1994) for North and Central Europe; WANG (1996) for China, KÜTÜK (2003) for Turkey, but the coverage, especially for west Asia is partial.

Most species of *Tephritis* infest the flowerheads of Asteraceae hosts, collectively belonging to several tribes, with or without the induction of galls. A few species induce the formation of galls in the upper or subterranean parts of stems of Asteraceae (FREIDBERG, 1984).

In summer 1999, the author collected a series of adults of an uncommon species of the genus *Tephritis* that infested the flowerheads of *Cirsium vulgare* (SAVI.-TEN.) in Turkey, and which is similar to *T. acanthiophilopsis* and *T. cometa israelis*. The new species is described and figured below.

MATERIALS AND METHODS

Terminology and morphological interpretations used in this paper follow WHITE et al. (1999). Type specimens are deposited in the Department of Biology, Faculty of Science & Art, Gaziantep University, Gaziantep, Turkey (GUGT). The possible host plant of the new species was identified by Prof. Şemsettin Civelek (Department of Biology, Fırat University, Elazığ, Turkey).

Tephritis erdemlii Kütük n. sp.
(Figures 1-5)

Type Materials

Holotype: ♀, Turkey: Isparta, Yalvaç, Sultan mountain, 38° 16' N, 31° 25' E, 1520 m, 08.VII.1999, on *Cirsium vulgare* (SAVI.-TEN.) (leg. M. Kütük). Paratypes; 7 ♂♂, 11 ♀♀, same data as holotype, collected on *Cirsium vulgare*. Additional paratypes; 2 ♂♂, Isparta, Yalvaç, 38° 15' N, 31° 22' E, 1570 m, 11.VI.1999, on *C. vulgare*; 2 ♂♂, 4 ♀♀, Antalya, Akseki, Göktepe plateau, 37° 40' N, 32° 00' E, 2100 m, 13.VII.1999, on *Cirsium vulgare*; 2 ♂♂, Isparta, Aksu, Çayır plateau, 37° 47' N, 31° 14' E, 1880 m, 14.VII.1999, on *C. vulgare*; 2 ♂♂, 2 ♀♀, Isparta, Yenişarbademli, 37° 43' N, 31° 18' E, 1780 m, 27.VI.2000, on *C. vulgare*. The holotype is in excellent condition, and deposited in the Department of Biology, University of Gaziantep, Gaziantep, Turkey, together with most paratypes.

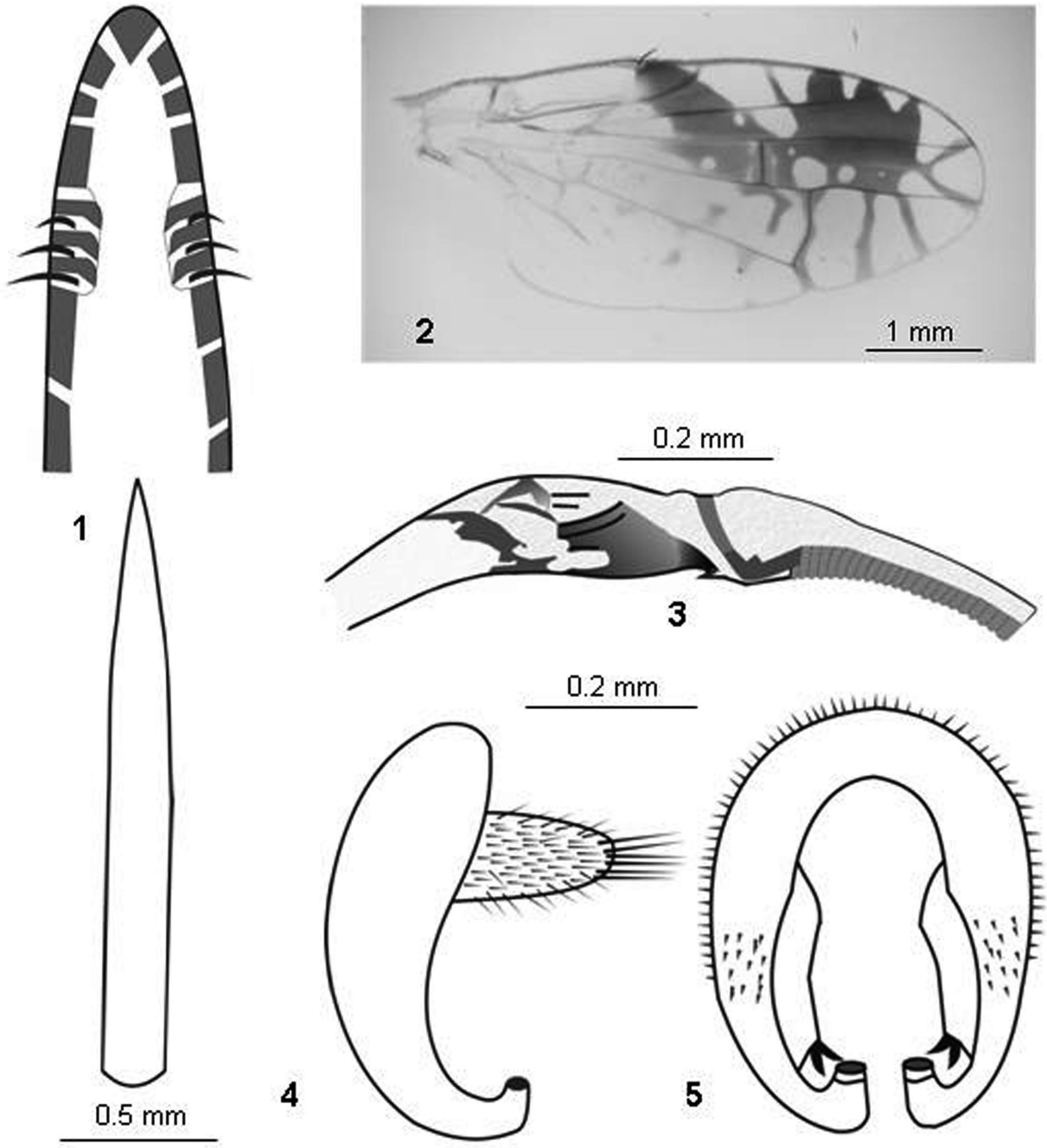
Description

Head: Mostly yellow to brown; blackish on occiput and ocellar tubercle; first flagellomere red to brown; basal half of arista brown, apical half black; pedicel 0.4-0.5 times as long as first flagellomere and with black setulae; palpus mostly red, brownish at apex; fronto-facial angle about 135°; genital seta distinct and whitish.

Thorax: Ground colour predominantly black; microtrichia dark gray, more silvery on scutum; most setae dark brown and acuminate; posterior notopleural seta yellowish and lanceolate; setulae whitish, 13-16 pairs present on margin of scutellum; basal scutellar seta 2 times as long as apical scutellar seta; halter yellow.

Legs: Setulae mostly yellowish, some black; tarsi dark red to brown.

Wing: Venation typical for genus. Vein R_{4+5} ventrally with 5-8 (usually 6) setulae, on basal section 0-5, (usually 4). Pattern: Basal half mostly hyaline, apical half brown stellate; wide hyaline costal indentation present distal to pterostigma (including apex of pterostigma), ending slightly distal to crossvein R-M and extending to vein R_{4+5}; cell r_1 with two additional hyaline areas (3 total) extending to vein R_{2+3} (Fig. 2) including one near apex of vein R_{2+3}; ultimate section of vein M 3.6 times as long as penultimate section.



Figs 1-5. – *Tephritis erdemlii* n. sp; 1- aculeus, 2- wing, 3- glans (dorsolateral view), 4- epandrium and proctiger (lateral view), 5- epandrium and surstyli (posterior view).

Female abdomen: Ground colour black; microtrichia dark gray; setulae whitish oviscape dark brown to black, 0.6 times as long as preabdomen; aculeus pointed (Fig. 1), with three pairs of hairs at apex.

Male abdomen: Epandrium (Figs 4; 5) yellow to brown; proctiger pale yellow with brown setulae (Fig. 5); glans sclerotized brownish and pointed at margin near base (Fig. 3).

Measurements (length in mm): Female body 5.8-7.7, wing 4.2-5.4; Male: body 4.7-5.4; wing 4.0-4.8.

Comparative notes

The new species is similar to *Tephritis. acanthiphilopsis* Hering from Turkey, and to *T. cometa israelis* (Loew), known from Greece, Syria and Israel, sharing with them similar wing patterns (basal half mostly hyaline, apical

half brown stellate, wide hyaline costal indentation present beyond pterostigma, including apex of pterostigma).

T. acanthiophilopsis has a similar wing pattern but the hyaline areas are more restricted, including the large hyaline costal indentation, which often does not extend to vein R_{4+5} and is often divided into anterior and posterior, rounded spots, the two other hyaline spots usually not extending to vein R_{2+3}; oviscape 0.4 times as long as preabdomen, length of female specimens less than 5.4 mm. *T. cometa israelis* has similar wing pattern but only one hyaline spot extending to vein R_{2+3}, two other hyaline areas not extending to vein R_{2+3} in cell r_1, oviscape 0.3 times as long as preabdomen, length of female specimens less than 4.5 mm. The new species differs from both species by the hyaline spots of the wing (three hyaline spots extending to or crossing vein R_{2+3} in cell r_1), oviscape 0.6 times as long as preabdomen, length of female specimens more than 5.8 (5.8-7.7) mm.

T. seperata is similar to the new species in having three hyaline spots extending to vein R_{2+3} in the cell r_1 but has three round hyaline spots present anterior of vein R-M in cell br (only one round hyaline spot present anterior of vein R-M in *T. erdemlii*.

All compared species are apparently associated with different host plants: *T. cometa israelis* - with *Cirsium gaillardotii* (Freidberg & Kugler, 1989), *T. acanthiophilopsis* - with unknown host (Hendel, 1927) Replace by: *Cirsium tuberosum* (A. Freidberg, pers. comm.). *T. erdemlii* n. sp. is believed to feed in the flower heads of *Cirsium vulgare* (Asteraceae).

Etymology

This species is named after Prof. Ümit Erdemli, a friend and excellent hydrobiologist, who has contributed much to the study of Pisces and Crustaceae in Turkey.

ACKNOWLEDGMENTS

We thank Dr. Amnon Freidberg (Tel Aviv University, Department of Zology, Tel Aviv, Israel) for reviewing the manuscript. We are grateful to Prof. Şemsettin Civelek (University of Fırat, Department of Biology, Elazığ, Turkey), who determined the host plant of the new species. This study was supported by the TUBITAK (project number: TBGA-1709, 1999) and İnönü University Project of Research Foundation (project number: 2004-3).

REFERENCES

Freidberg A (1984). Gall Tephritidae (Diptera). In: Ananthakrishnan TN (ed), Biology of Gall Insects, Oxford and IBH Publishing Co., New Delphi:1 29-167.

Freidberg A & Kugler J (1989). Fauna Palaestina. Insecta IV. Diptera: Tephritidae. Israel Academy of Sciences and Humanities, Jerusalem, 212 p.

Hendel F (1927). 49. Trypetidae. die Fliegen der Palaerktischen Region, Stuttgard, 5(1): 221 p.

Hering M (1944). Bestimmungtabelle der Gattung *Tephritis* Latreille, 1804. Siruna Seva, 5: 17-31.

Korneyev VA & Dirlbek J (2000). The Fruit Flies (Diptera: Tephritidae) of Syria, Jordan and Iraq. Studia Dipterologica, 7(2): 463-482.

Kütük M (2003). Güney Batý Anadolu Bölgesi Meyve Sinekleri (Diptera: Tephritidae) Faunasý ve Sistematiği Üzerine Araştırmalar. PhD Thesis No: 542, Adana, Turkey.

Merz B (1994). Diptera, Tephritidae. Insecta Helvetica Fauna, HGE press, Geneve.

Norrbom AL, Caroll LE, Freidberg A (1999). Status of Knowledge. In: Thompson FC (ed), Fruit Fly Expert Identification System and Systematic Information Database, Myia, 9: 9-49.

Wang X (1996). The Fruit Flies (Diptera: Tephritidae) of the East Asian Region. Acta Zootaxonomica Sinica 21:Supplement.

White IM (1988). Tephritid Flies (Diptera: Tephritidae). Handbooks for the Identification of British Insects, Dorset Press, London.

White IM, Headrick DH, Norrbom AL & Caroll LE (1999). Glossary. In: Aluja M & Norrbom AL (eds) Fruit flies (Tephritidae): phylogeny and evolution of behaviour. CRC Press, Boca Raton: 881-924.

Received: July 26, 2006
Accepted: April 16, 2008

Belg. J. Zool., 138 (2) : 135-139 July 2008

Development of the predatory pentatomid *Picromerus bidens* (L.) at various constant temperatures

Kamran Mahdian[1,2], Luc Tirry[1] & Patrick De Clercq[1*]

[1] **Laboratory of Agrozoology, Department of Crop Protection, Faculty of Bioscience Engineering, Ghent University, Coupure Links 653, B-9000 Ghent, Belgium**

[2] **Department of Crop Protection, Faculty of Agriculture, Vali-e-Asr University of Rafsanjan, Rafsanjan, Iran**

Corresponding author : * Patrick De Clercq, Department of Crop Protection, Faculty of Bioscience Engineering, Ghent University, Coupure Links 653, B-9000 Ghent, Belgium. E-mail: patrick.declercq@ugent.be

ABSTRACT. Development of the palearctic predatory heteropteran *Picromerus bidens* was assessed at constant temperatures (15, 18, 20, 23, 27, 32 and 35±1°C) using larvae of the cotton leafworm *Spodoptera littoralis* as prey. All experiments were done at a 12:12 (L:D) h photoperiod and 65±5% relative humidity. The predator developed to maturity between 18 and 32°C, but eggs failed to hatch at 15 and 35°C. Duration of development of the different immature stages of *P. bidens* decreased with increasing temperature from 18 to 32°C. The total development time for *P. bidens* ranged from 24.8 days at 32°C to 85 days at 18°C. The estimated lower thresholds for development varied between life stages and ranged from 8 to 16°C. The thermal requirements for development of the egg stage, completion of the nymphal period and total development of *P. bidens* were estimated to be 208, 270 and 500 degree-days with lower thresholds of 10.5, 13.8 and 12.2°C, respectively. Upper threshold temperature for development of eggs and nymphal stages was estimated to be between 32 and 35°C. Egg hatch percentage, nymphal survival and sex ratio were determined at each temperature. The data reported here should be helpful in predicting development of *P. bidens* populations in the field and offer valuable basic information for the use of this native predator in biological control programs.

KEY WORDS : *Picromerus bidens*, Pentatomidae, predator, temperature thresholds, thermal requirements

INTRODUCTION

Picromerus bidens (Linnaeus) is a predatory pentatomid, which is widely distributed in the western Palearctic region. In the Nearctic region this species has been recorded from more than 180 locations in North America since its introduction some time before 1932 (LARIVIÈRE & LAROCHELLE, 1989). This pentatomid is associated with a wide range of habitats including wet and dry areas such as bushes, fields and forests, where it prefers shrubby areas rich in woody plants (trees or bushes), but it is also found on herbaceous plants (SCHUMACHER, 1911; MAYNÉ & BRENY, 1948; SOUTHWOOD & LESTON, 1959). *P. bidens* is a polyphagous predator that preys on larvae of many Lepidoptera, Coleoptera and leaf-eating Hymenoptera, and more rarely on pupae and adults of soft-bodied insects (JAVAHERY, 1986; LARIVIÈRE & LAROCHELLE, 1989). Several authors have emphasized the potential of *P. bidens* for reducing populations of insect pests in a variety of ecosystems (see DE CLERCQ, 2000).

P. bidens may be a possible alternative for the exotic species *Podisus maculiventris*, (Say) which is indigenous throughout North America. There have been multiple introductions of this species in Europe since the 1930s for classical biological control of the Colorado potato beetle, *Leptinotarsa decemlineata* (Say) but the predator has never succeeded in establishing. From the late 1990s up to recently, *P. maculiventris* was commercially available in Europe for augmentative biological control of caterpillar pests in greenhouse crops. However, commercialization of this exotic polyphagous predator was discontinued as a result of growing environmental concerns (VAN LENTEREN et al., 2003). Considerable knowledge exists on the seasonal cycle of *Picromerus bidens* (MAYNÉ & BRENY, 1948; JAVAHERY, 1986; MUSOLIN, 1996; MUSOLIN & SAULICH, 2000) but information regarding the effect of climate on the bio-ecology of this species is still scarce. Understanding the relationship between temperature and development of arthropod predators is essential for accurately predicting natural enemy interactions with pests (ROSEN & HUFFAKER, 1983; OBRYCKI & KRING, 1998; NECHOLS et al., 1999). Although temperature is only one of the ecological factors in predator-prey dynamics, it is a primary factor affecting the ability of a predator to regulate pest populations. The objective of the current study was to determine the temperature-dependent development of the different immature stages of *P. bidens* at constant temperatures.

MATERIALS AND METHODS

A culture of *P. bidens* was started with eggs originating from a laboratory colony at the Department of Entomology and Biological Control, All-Russian Research Institute for Plant Quarantine, Moscow, Russia. The colony of *P. bidens* used in this study was maintained at 23±1°C, 65±5% relative humidity (RH), and a 12:12 (L:D)h photoperiod. The food of the stock colony of the predator consisted mainly of larvae of the cotton leafworm *Spodoptera littoralis* (Boisduval) reared on an artificial diet modified from POITOUT & BUES (1970).

Eggs of *P. bidens* undergo obligatory diapause and need to be stored at low temperatures (2-3°C) for at least one month to initiate embryonic development. Egg masses that underwent such cold treatment were used in

the experiments on development. Development of *P. bidens* was monitored under the following constant temperatures: 15, 18, 20, 23, 27, 32 and 35±1°C. All experiments were done at a 12:12 (L:D) h photoperiod and 65±5% RH. At each temperature, development of the egg stage was monitored using 100 eggs and development of nymphs was followed starting with 40 first instars. Egg batches were taken from cold storage, transferred to plastic Petri dishes (9cm diameter) and placed in incubators at each of the test temperatures. Development of incubated eggs was monitored daily and hatching recorded. Newly emerged nymphs (less than 12h old) of *P. bidens* were transferred to individual Petri dishes (9cm diameter) lined with absorbent paper. A moist paper plug fitted into a small plastic cup provided water. Nymphs were offered prey from the second instar onwards because, as in other asopines, first instars of *P. bidens* do not feed and only take up water (De Clercq, 2000). Upon reaching the second instar, the predator was fed daily with an excess of fifth instars of the cotton leafworm *S. littoralis*. Nymph development and survival of *P. bidens* were monitored on a daily basis. Sex was determined when the individuals reached the adult stage.

Development rate of each immature stage of *P. bidens* was calculated using the reciprocal of the average development duration (i.e., 1/d). The relationship between development rate and temperature was described by a linear regression model (Arnold, 1959) fit to the linear section of the data points. Temperature points above and below the linear portion of the development rate curve were not used to estimate thermal requirements (in degree-days or DD) or lower threshold temperatures. The lower temperature thresholds for each of the immature stages of *P. bidens* were determined as the *x*-intercept (t=-a/b) (Arnold, 1959) and the degree-day requirements (K) were determined as the inverse of the slope (k=1/b) of the regression lines (Campbell et al., 1974).

TABLE 1

Duration (days) of the immature stages of *P. bidens* at five constant temperatures

Stage	Temperature				
	18°C	20°C	23°C	27°C	32°C
Egg	32.62±0.48	22.70±0.30	14.44±0.34	12.78±0.15	10.14±0.10
First instar	8.35±0.20	5.56±0.07	4.61±0.08	3.43±0.09	2.00±0.00
Second instar	9.15±0.22	7.54±0.12	6.07±0.19	4.56±0.16	3.20±0.07
Third instar	8.52±0.27	7.50±0.23	6.02±0.28	4.56±0.16	2.82±0.09
Fourth instar	9.46±0.29	8.23±0.25	6.45±0.16	3.79±0.10	2.82±0.10
Fifth instar	13.13±0.24	11.23±0.12	9.84±0.14	6.30±0.28	4.18±0.08
Total nymph period	47.60±1.08	39.80±0.46	32.40±0.42	19.26±0.34	14.80±0.21
Total development	85.00±1.39	62.43±0.59	47.32±0.50	35.77±0.46	24.75±0.27

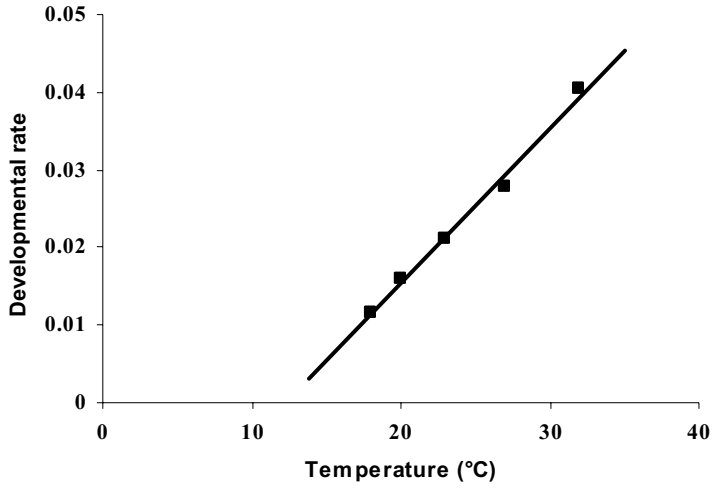


Fig. 1. – The relationship between temperature and development rate for total development (egg-adult) of *P. bidens* at constant temperatures. The line represents the linear regression of the data between 18 and 32°C.

RESULTS

Picromerus bidens developed to adulthood between 18 and 32°C, but eggs failed to hatch at 15 and 35°C, dying without any observed evidence of embryonic development. Therefore, the temperatures tested encompassed the range of constant temperatures allowing complete development of the predator. The total time for development of *P. bidens* ranged from 24.8 days at 32°C to 85 days at 18°C. Linear regression equations of development rate of each life stage versus temperature are given in Table 1. Fig. 1 shows the relationship between temperature and development rate for the total development (egg-adult) of *P. bidens*. High coefficients of determination ($r^2>0.97$, $P<0.001$) indicated a good linear model fit in all cases. The lower development threshold values and degree-day requirements for each life stage of *P. bidens* are presented in Table 2. The estimated lower thresholds for development varied between life stages and ranged from 8 to 16°C. The thermal requirement for development of the egg stage after cold storage was 208DD with a lower threshold of 10.5°C. The thermal requirement for completion of the nymphal period was 270DD with a lower threshold of 13.8°C. Total development of *P. bidens* required 500DD with a lower threshold of 12.2°C. Rapid decline of development rate between 32 and 35°C suggests that the upper threshold temperature for development (i.e. the temperature above which the rate of development starts decreasing) of eggs and nymphal stages was within this temperature range.

Egg hatch ranged from 65 to 86% at temperatures between 18 and 23°C and averaged 84 and 69% at 27 and 32°C, respectively (Table 3). Survival of nymphs increased from 28 to 69% with increasing temperature from 18 to 23°C, and subsequently decreased to 59% as temperature further increased up to 32°C (Table 3). Sex ratios of *P. bidens* adults ranged from 1 male: 0.6 female to 1 male: 1.7 female at the tested temperatures.

TABLE 2

Lower development thresholds (t), degree-day requirements (K) and linear regression equations and coefficients of determination for development of the immature stages of *P. bidens* at different constant temperatures

Stage	t (°C)	K (DD)	Regression equation	r^2	P
Egg	10.48	208.33	Y=-0.0503+0.0048X	0.97	<0.001
First instar	10.94	54.94	Y=-0.1991+0.0182X	0.98	<0.001
Second instar	9.12	81.96	Y=-0.1113+0.0122X	0.99	<0.001
Third instar	8.21	86.95	Y=-0.0945+0.0115X	0.99	<0.001
Fourth instar	14.48	49.26	Y=-0.2941+0.0203X	0.98	<0.001
Fifth instar	16.50	64.93	Y=-0.2541+0.0154X	0.99	<0.001
Total nymph period	13.76	270.27	Y=-0.0509+0.0037X	0.98	<0.001
Total development	12.25	500.00	Y=-0.0245+0.002X	0.99	<0.001

TABLE 3

Egg hatch, nymph survival, and sex ratio of adults of *P. bidens* at five constant temperatures

Temperature (°C)	Egg hatch (%)	Nymph survival (%)	Sex ratio (male: female)
18	65	28	1:0.6
20	71	48	1:0.9
23	86	69	1:1.7
27	84	60	1:1.0
32	69	59	1:0.9

DISCUSSION

MAYNÉ & BRENY (1948) reported that the second, third and fourth nymphal stadia of *P. bidens* are of equal length, about 12 to 14 days each, whereas the fifth stadium takes somewhat longer and total nymph time is about two months under summer field conditions in Belgium. These authors also noted that 30 to 35 days are required for development of *P. bidens* on larvae of *Ephestia kuehniella* Zeller, 1879, at a constant temperature of 25-26°C. JAVAHERY (1986) reported that development duration of the nymphal stage of the insect in Québec was 44 days in field cages, and development of the first, second, third, fourth and fifth instars took on average 8, 8, 9, 9 and 10 days, respectively. MUSOLIN & SAULICH (2000) noted that different photoperiods had no effect on nymph development of *P. bidens* at 24.5°C. Nymph development took 25 to 26 days at different photoperiods except for nymphs reared at a 20:4 (L:D) h photoperiod, which took 28 days to develop (MUSOLIN & SAULICH, 2000). These authors showed that nymph duration of this predator averaged 36 days under field conditions in the Belgorod region of Russia. The development durations found in the current study are comparable with those previously reported by above-mentioned authors.

We know of no other studies in which temperature thresholds and thermal requirements for development of *P. bidens* have been estimated. MAHDIAN et al. (2006) indicated that predation behaviour of *P. bidens* was affected by temperature; all nymphal stadia of *P. bidens*

from the second instar onward were able to prey successfully upon fourth-instar *S. littoralis* at temperatures between 18 and 27°C. Mean daily prey consumption by male and female adults of *P. bidens* increased with increasing temperature. Further, these authors showed that the type of functional response (i.e. the relationship between rate of prey consumption and prey density) of *P. bidens* switched from type II to type III as temperature increased from 18 to 27°C.

Several linear and nonlinear models have been proposed to describe the relationship between temperature and arthropod development (CAMPBELL et al., 1974; WAGNER et al., 1984; LACTIN et al., 1995; BRIÉRE et al., 1999). The linear equation has been documented as a suitable model for calculation of lower development thresholds and thermal constants in a partial temperature range (e.g., CAMPBELL et al., 1974; HONĚK, 1999; JAROŠIK et al., 2002; KONTODIMAS et al., 2004). The variability of the estimated thermal thresholds for the successive developmental stages of *P. bidens* suggests, however, that the linear degree-day model may not always yield accurate estimates. For instance, the linear model estimated the lower thermal threshold for egg development of *P. bidens* to be 10.5°C, whereas our observations showed that eggs did not develop successfully at a constant temperature of 15°C. Further, estimates of lower development thresholds of fourth and fifth instars were considerably higher than those of the earlier instars. Other models, including nonlinear regression, may enable a more accurate description of the relationship between development of immature stages of *P. bidens* and temperature (for a review, see KONTODIMAS et al., 2004); however, non-linear models do not enable the calculation of thermal constants. Also given its ease of use, the linear degree-day model has therefore been widely used as a phenological model (KONTODIMAS et al., 2004).

The results of the current study on *P. bidens*, together with reported high predation capacities of this predator against noctuid caterpillars within a wide range of temperatures, indicate that *P. bidens* may perform well when released as a biological control agent of defoliator pests both in open fields and heated glasshouses. Whereas temperature is one factor that is important for the establishment of the predator's population in the crop and that will determine its foraging and predation capacity, there are considerable environmental complexities that may affect the predator-prey system and will eventually determine the outcome of a biological control programme. Nevertheless, the current data will be useful in the selection of the most effective life stage of the predator that is best adapted to conditions favouring the target pest in a given crop situation.

ACKNOWLEDGEMENTS

The statistical suggestions of Mohammad Amin Jalali are gratefully acknowledged. The authors are grateful to the Ministry of Science, Research and Technology of Iran for financial support to K. Mahdian (Ph.D. grant no. 781153)

REFERENCES

ARNOLD CY (1959). The determination and significance of the base temperature in a linear heat unit system. Proceedings of the American Society for Horticultural Science, 74: 430-445.

BRIÉRE JF, PRACROS P, LE ROUX AY & PIERRE JS (1999). A rate model of temperature-dependent development for arthropods. Environmental Entomology, 28: 22-29.

CAMPBELL A, FRAZER BD, GILBERT N, GUTIERREZ AP & MACKAUER M (1974). Temperature requirements of some aphids and their parasites. Journal of Applied Entomology, 11: 431-438.

DE CLERCQ P (2000). Predaceous stinkbugs (Pentatomidae: Asopinae). In: SCHAEFER CW & PANIZZI AR (eds), Heteroptera of Economic Importance. CRC Press, Boca Raton: 737-789.

HONĚK A (1999). Constraints on thermal requirements for insect development. Entomological Science, 2: 615-621.

JAROŠIK V, HONĚK A & DIXON AFG (2002). Developmental rate isomorphy in insects and mites. American Naturalist, 160: 497-510.

JAVAHERY M (1986). Biology and ecology of *Picromerus bidens* (Hemiptera: Pentatomidae) in southeastern Canada. Entomological News, 97: 87-98.

KONTODIMAS DC, ELIOPOLOUS PA, STATHAS GJ & ECONOMOU LP (2004). Comperative temperature-dependent development of *Nephus includens* (Kirsch) and *Nephus bisignatus* (Boheman) (Coleoptera: Coccinellidae) preying on *Planococcus citri* (Risso) (Homoptera: Pseudococcidae): evaluation of linear and various nonlinear models using specific criteria. Environmental Entomology, 33: 1-11.

LACTIN DJ, HOLLIDAY NJ, JOHNSON DL & CRAIGEN R (1995). Improved rate model of temperature-dependent development by arthropods. Environmental Entomology, 24: 68-75.

LARIVIÈRE MC & LAROCHELLE A (1989). *Picromerus bidens* (Heteroptera: Pentatomidae) in North America, with a world review of distribution and bionomics. Entomological News, 100: 133-145.

MAHDIAN K, VANTORNHOUT I, TIRRY L & DE CLERCQ P (2006). Effects of temperature on predation by the stinkbugs *Picromerus bidens* and *Podisus maculiventris* (Heteroptera: Pentatomidae) on noctuid caterpillars. Bulletin of Entomological Research, 96: 489-496.

MAYNÉ R & BRENY R (1948). *Picromerus bidens* L.: Morphologie. Biologie. Détermination de sa valeur d'utilisation dans la lutte biologique contre le doryphore de la pomme de terre – la valeur économique antidoryphorique des Asopines indigènes belges. Parasitica, 4: 189-224.

MUSOLIN DL (1996). Photoperidic induction of aestivation in the stink bug *Picromerus bidens* (Heteroptera: Pentatomidae). A preliminary report. Entomological Review, 76: 1058-1060.

MUSOLIN DL & SAULICH AH (2000). Summer dormancy ensures univoltinism in the predatory bug *Picromerus bidens*. Entomologia Experimentalis et Applicata, 95: 259-267.

NECHOLS JR, TAUBER MJ, TAUBER CA & MASKAI S (1999). Adaptations to hazardous seasonal conditions: dormancy, migration, and polyphenism. In: HUFFAKER CB & GUTTIEREZ AP (eds), Ecological entomology. 2nd ed. Wiley, New York: 159-200.

OBRYCKI JJ & KRING TJ (1998). Predaceous Coccinellidae in biological control. Annual Review of Entomology, 43: 295-321.

POITOUT S & BUES R (1970). Elevage de plusieurs espèces de lepidoptères Noctuidae sur milieu artificiel riche et sur milieu artificiel simplifié. Annales de Zoologie, Ecologie Animale, 2: 79-91.

ROSEN D & HUFFAKER DR (1983). An overview of desired attributes of effective biological control agents, with particular emphasis on mites. In: HOY MA, CUNNINGHAM GL & KNUSTON L (eds), Biological control of pests by mites. University of California, Berkeley: 2-11.

SCHUMACHER F (1911). Beiträge zur Kenntnis der Biologie der Asopiden. Zeitschrift für wissenschaftliche Insektenbiologie, 7: 40-47.

SOUTHWOOD TRE & LESTON D (1959). Land and water bugs of the British Isles. Frederick Warne and Co., London, U.K.

VAN LENTEREN J, BABENDREIER D, BIGLER F, BURGIO G, HOKKANEN HMT, KUSKE S, LOOMANS AJM, MENZLER-HOKKANEN I, VAN RIJN PCJ, THOMAS MB, TOMMASINI MG, & ZENG QQ (2003). Environmental risk assessment of exotic natural enemies used in inundative biological control. BioControl, 48: 3-38.

WAGNER TL, WU HI, SHARP PJH, SCHOOLFIELD RM & COULSON RN (1984). Modeling insect development rates: a literature review and application of a biophysical model. Annals of the Entomological Society of America, 77: 208-225.

Received: August 8, 2006
Accepted: November 16, 2007

Belg. J. Zool., 138 (2) : 140-145 July 2008

Effect of two insect growth regulators on the ecdysteroid contents in eggs of the mealworm

Hinda Berghiche[1], Moufida Houamria[1], Sandrien Van de Velde[2], Noureddine Soltani[1] & Guy Smagghe[2*]

[1] **Laboratoire de Biologie Animale Appliquée Département de Biologie, Faculté des Sciences, Université d'Annaba, 23000 Annaba, Algérie**

[2] **Laboratory of Agrozoology, Department of Crop Protection, Faculty of Bioscience Engineering, Ghent University, B-9000 Ghent, Belgium**

Corresponding author : * Guy Smagghe, e-mail: guy.smagghe@ugent.be

ABSTRACT. RH-0345 (halofenozide), a bisacylhydrazine derivative, is a nonsteroidal ecdysteroid agonist that mimics the action of the moulting hormones, while KK-42, an imidazole compound, is a potent inhibitor of ecdysteroid biosynthesis. Previous results suggested that the observed reduction of ecdysteroid titre, leading to a reduction of reproductive capacity in the mealworm *Tenebrio molitor*, is due to the direct and rapid action of KK-42 on ecdysteroid biosynthesis. Moreover, RH-0345 was found to increase ecdysteroid production and partially reverse the effects on reproductive events induced by KK-42. Therefore, the present study evaluates the effects of these two insect growth regulators (IGRs) on egg ecdysteroids in mealworms. The IGRs were applied topically (10µg/insect) on newly-emerged adult females. A qualitative and quantitative analysis of ecdysteroids from pooled freshly laid eggs was made by an enzyme-immunoassay (EIA) using two specific antibodies, the rat monoclonal EC 19 antibody to measure for 20-hydroxyecdysone (20E) and the rabbit polyclonal B antibody for ecdysteroids. EIA measurements confirmed the presence of free ecdysteroid and 20E in control and treated series. In addition, the conjugated ecdysteroids were predominant in eggs of untreated mealworms. RH-0345 increased the amounts of 20E relative to the free ecdysteroids. In contrast, KK-42 reduced the amounts of total ecdysteroids, but had no significant effect on the relative hormonal composition of free and conjugated ecdysteroids.

KEY WORDS : Insects, Eggs, Ecdysteroids, Insect growth regulators, RH-0345, Halofenozide, KK-42.

INTRODUCTION

Agrochemical research has resulted in the discovery of novel insecticides that act on selective biochemical sites present in specific insect groups. Good examples are the insect growth regulators (IGRs) such as benzoylphenylurea, which inhibit chitin formation in insects, or other chemicals such as juvenile hormone analogues (JHA) and ecdysteroid agonists, which affect the hormonal regulation of moulting and development processes (ISHAAYA 1990; DHADIALLA et al., 1998; 2005; PALLI & RETNAKARAN 2001). The ecdysteroid agonist RH-0345 (halofenozide) represents a new class of IGRs, which has been developed to provide safe insecticides with high activity in Coleoptera (DHADIALLA et al., 1998; 2005; NAKAGAWA et al., 2001). Similar to the prototype RH-5849 and other ecdysteroid agonists like RH-5992 (tebufenozide) and RH-2485 (methoxyfenozide), this molecule caused in insects a premature and incomplete lethal moult (WING, 1988; WING et al., 1988; SMAGGHE & DEGHEELE, 1994a,b; CARLSON et al., 2001). Various studies revealed that the prototype ecdysteroid agonist RH-5849 presented high activity in species of Lepidoptera and Coleoptera, but later became superseded by RH-5992, RH-2485 and RH-0345, which have enhanced activity against lepidopteran and coleopteran pest insects. The ecdysteroid agonist compounds exert their toxicity by binding to the nuclear ecdysteroid receptor EcR as does the natural insect moulting hormone, 20-hydroxyecdysone (20E) (DHADIALLA et al., 1998; 2005; RIDDIFORD et al., 2000). KK-42 is an imidazole compound considered as an inhibitor of ecdysteroid biosynthesis. KK-42 reduces the ecdysteroid production by ovaries and interferes with reproductive processes in adult females of the mealworm *Tenebrio molitor* (KUWANO et al., 1983; 1985; AMRANI et al., 2004). However, the mode of action of these compounds on reproductive events in insects is not so well understood so far.

Data accumulated on the normal development of ovaries and their regulation provide an experimental basis for investigating IGRs that interfere in the insect's endocrine system, especially for ecdysteroids (SOLTANI-MAZOUNI et al., 1999). As reviewed by HAGEDORN (1985) and LAFONT et al. (2005), in the female adult ecdysteroids are synthesized by the follicle cells in the ovaries and play a major role in ovarian development, vitellogenesis and oocyte/egg maturation. Then the ovarian ecdysteroids, in both free and conjugated forms, are almost entirely taken up by and stored/concentrated in the maturing eggs and may serve as hormonal substrate for embryonic moults during embryogenesis. Many experiments confirm the presence of the free and conjugated forms of ecdysteroids in ovaries of *Schistocera gregaria* (REES & ISAAC, 1984), *Locusta migratoria* (LAGUEUX et al., 1984) and *Manduca sexta* (THOMPSON et al., 1987). It is here an attractive hypothesis that the maternal ecdysteroids are the source of hormone for the embryo during embryogenesis.

To date, few authors have examined the physiological reasons for sublethal effects of IGRs on suppression of the next generation. KOSTYUKOVSKY et al. (2000)

observed that RH-5849 could achieve almost complete control of F_1 adults of *Tribolium castaneum* and *Rhyzopertha dominica* when given to the F_0 generation at 10 ppm. MEDINA et al. (2002) reported that females of *Chrysoperla carnea* lacewings treated with three representative IGRs (the chitin synthesis inhibitor diflubenzuron, the JHA pyriproxyfen and the ecdysteroid agonist RH-5992), accumulated these compounds into the ovaries and eggs, but to a different extent, and although the amount of diflubenzuron was low (74-197pg/egg), it was sufficient to cause egg mortality. Also after treatment with RH-5992 in eggs there existed a reduction of viability (MEDINA et al., 2001). Recently it has been reported that RH-0345 interferes with the reproduction events of *T. molitor* (TAÏBI et al., 2003), and that ovarian follicle cells are sites of biosynthesis of ecdysteroids and protein for egg shell formation (SOLTANI-MAZOUNI & SOLTANI, 1995; SOLTANI-MAZOUNI et al., 1999). In another assay, it was shown that RH-5992 and RH-2485 were able to decrease the mean fecundity and/or fertility of important pest species from several different insect orders (SMAGGHE & DEGHEELE, 1994a,b; SUN & BARRETT, 1999). RH-0345 was the most potent stimulator of the release of hormone into the culture medium by pupal integument explants and by ovaries of *T. molitor* (SOLTANI et al., 1998; 2002). In addition, RH-0345 was able to modify the composition of ecdysteroid amounts in mealworms (TAÏBI et al., 2003). Recently, we reported that RH-0345 is able to partly reverse the depressive effects on reproductive events induced by KK-42 in *T. molitor* (AMRANI et al., 2004).

In this research we used the mealworm *T. molitor*, which is of worldwide importance in stored food products, as a model target beetle. Experiments were undertaken to evaluate the activity of RH-0345 and KK-42 on reproductive events. The compounds were applied topically to adults of *T. molitor* and their effects on ecdysteroid amounts in eggs/embryos were examined.

MATERIALS AND METHODS

Experimental animals

T. molitor pupae from a stock colony were sexed and kept separately until emergence. Adults were collected 0-4h following emergence and reared on wheat flour at 27°C and 80% relative humidity in almost continuous darkness (SOLTANI-MAZOUNI et al., 1999).

Chemicals and treatments

RH-0345 was kindly supplied by Rohm and Haas Research Laboratories (Spring House, PA, USA). KK-42 was kindly provided by Dr. E. Kuwano (Kyushu University, Japan). The compounds were dissolved in acetone (3µl/insect) and administrated topically at 10µg/female on the ventral side of abdomen of newly emerged adult females.

Extraction and enzyme immunoassay of ecdysteroids

EIA measurements have previously been described by TAÏBI et al. (2003). In brief, freshly-laid eggs were collected in control and treated series. Pooled samples, containing the first 30 freshly laid eggs, were submitted to extraction of free ecdysteroids on the one hand and total ecdysteroid on the other with methanol (100%) by sonication and centrifugation (5,000g, 10min). The supernatants containing free and total ecdysteroids were taken and evaporated separately. For determination of the total ecdysteroid amounts (free+conjugated), the samples were further submitted to an enzymatic hydrolysis with porcine liver esterase (EC3.1.1.1., 100,000 units, Sigma Co., St. Louis, IL, USA) (DE REGGI et al., 1992; TAÏBI et al., 2003). Subsequently the extracts were re-extracted using methanolic precipitation of enzymes. Then each individual sample of free and total ecdysteroids was analyzed in duplicate by an EIA using a conjugate of 20E coupled to peroxidase as enzymatic tracer and tetramethyl benzidine as a colour reagent. Quantitative and qualitative analysis of egg ecdysteroids was made by two specific antibodies kindly supplied by Dr. J.-P. Delbecque (University of Bordeaux, France): a rat monoclonal EC 19 antibody to measure 20E amounts, and a rabbit polyclonal B antibody to determine ecdysteroid amounts (DE REGGI et al., 1992). The conjugated amounts of ecdysteroids were deduced by subtracting the amounts of free ecdysteroids from that of total ecdysteroids, both determined with the polyclonal antibody. Data are expressed as pg ecdysteroid equivalents per egg in freshly laid eggs of *T. molitor* females.

Statistical analysis

Results are presented as means ± sd based on four replicates of 30 eggs each. Data were subjected to ANOVA and a *post hoc* LSD test was used to separate means. Statistical analyses were performed using MINITAB 12.21 software (PA Stat College, USA). The significance level was * $p<0.05$, ** $p<0.01$.

RESULTS

The quantitative and qualitative analysis of ecdysteroids in freshly laid eggs, before and after hydrolysis, has been tested by an EIA. The results confirmed the presence of free ecdysteroids with the polyclonal antibody and also of 20E with the monoclonal antibody in all extracts from control and treated series (Fig. 1). In addition, in eggs of untreated mealworms the presence of conjugated ecdysteroids was predominant (Fig. 2). After topical treatment with RH-0345 and KK-42 respectively (10µg/insect), ecdysteroid measurement on extracts of freshly laid eggs showed that RH-0345 increased significantly ($p<0.03$) the amounts of 20E as compared to control series. In contrast, KK-42 caused a significant reduction in ecdysteroid amounts in eggs ($p<0.01$) (Fig. 1).

It can be noted that RH-0345 increased significantly ($p=0.005$) the free ecdysteroids and decreased significantly ($p=0.023$) the conjugated ecdysteroids. However, this ecdysteroid agonist had no significant effect on total ecdysteroids in eggs. KK-42 reduced significantly

($p<0.01$) both total and conjugated ecdysteroids but not the free ecdysteroid form (Table 1). RH-0345 increased significantly the relative importance of free ecdysteroids and decreased significantly the relative importance of conjugated ecdysteroids ($p<0.05$). In contrast, KK-42 had no effect on free ecdysteroid proportions in relation to conjugated ecdysteroids (Fig. 2).

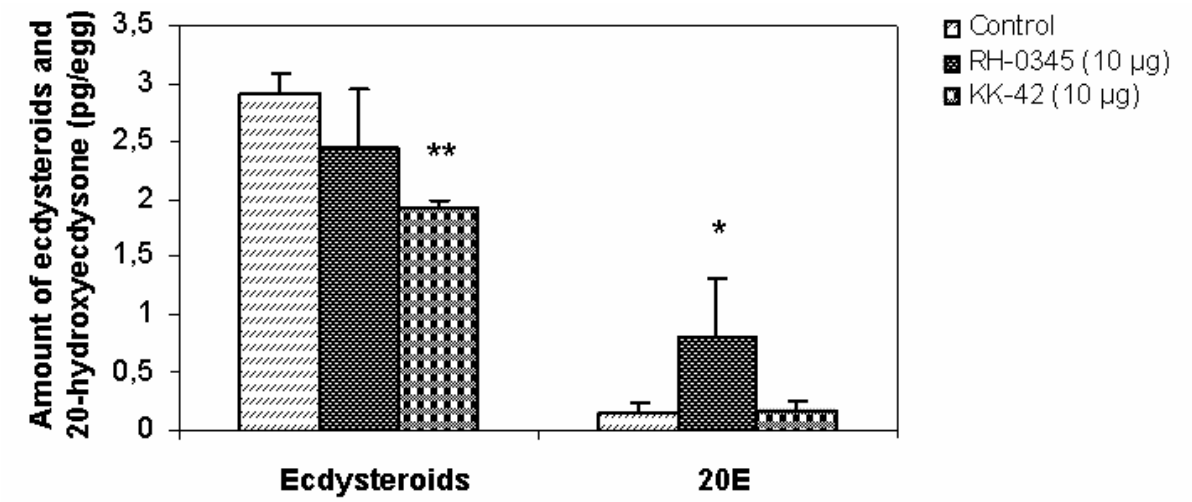


Fig. 1. – Amounts of ecdysteroids detected by polyclonal antibody, and 20-hydroxyecdysone (20E) detected by monoclonal antibody (both in pg/egg), in eggs freshly deposited by females of *Tenebrio molitor* after topical treatment with RH-0345 and KK-42 (10µg/insect). Data are expressed as means ± sd based on four replicates of 30 eggs each. Within the same group, significant differences are indicated by asterisks, * $p<0.05$, ** $p<0.01$.

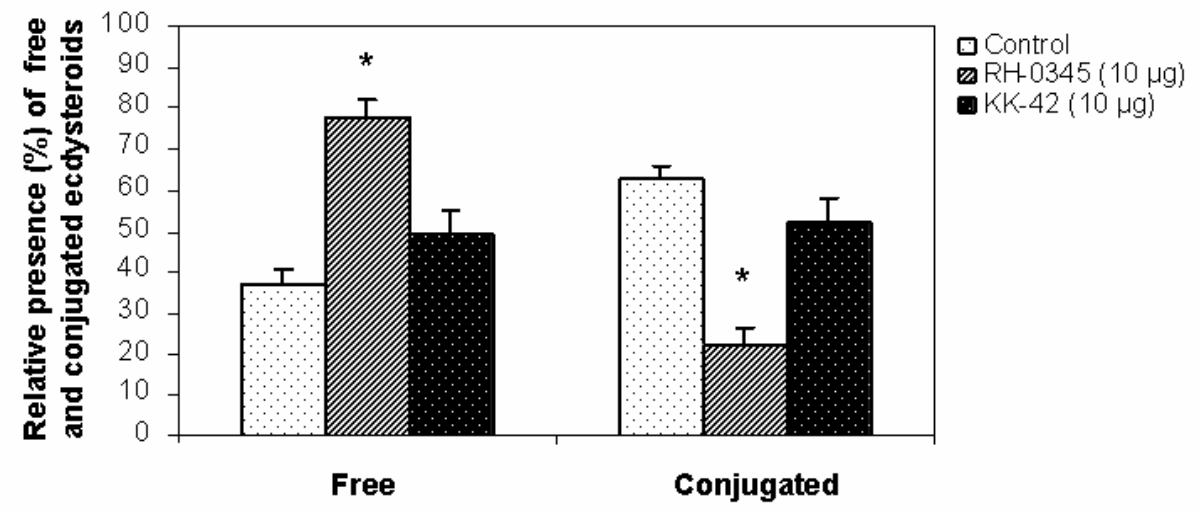


Fig. 2. – The relative presence (%) of free and conjugated ecdysteroids (pg/egg) in eggs freshly deposited by females of *Tenebrio molitor* after topical treatment with RH-0345 and KK-42 (10µg/insect). Data are expressed as means ± sd based on four replicates of 30 eggs each. Within the same group, significant differences are indicated by asterisks, * $p<0.05$.

TABLE 1

In vivo effect of RH-0345 and KK-42, when topically applied at 10µg per female adult, on the amounts of free, conjugated and total ecdysteroids (pg/egg) in eggs freshly laid by *T. molitor* females. Data are expressed as means ± sd based on four replicates of 30 eggs each. Within the same column, values followed by the same letter are not significantly different at the significance level of $p<0.05$

Treatments	Free	Conjugated	Total
Control	1.15±0.13 a	1.93±0.05 c	3.08±0.10 b
RH-0345	2.53±0.84 b	0.72±0.20 a	3.25±0.44 b
KK-42	1.01±0.14 a	1.09±0.13 b	2.10±0.08 a

DISCUSSION

In continuation of our research, the experiments reported here studied in more detail the ovicidal effects of two novel IGRs, the ecdysteroid agonist RH-0345 with a dibenzoylhydrazine structure and the anti-ecdysteroid KK-42 with an imidazole structure. In previous assays RH-0345 and KK-42 were applied at 10µg on female adults; these small amounts did not kill the adults but did cause a reduction in egg viability of about 15% (TAÏBI et al., 2003). We focus here on the significance that the two IGRs may interfere in the ecdysteroid amounts in eggs causing egg mortality through the female adult. In addition, we distinguished between free and conjugated ecdysteroid amounts. From the EIA measurements, we can demonstrate here for the first time that RH-0345 increased the amounts of free ecdysteroids in eggs and decreased that of the conjugated ecdysteroids. But on total ecdysteroid synthesis and accumulation in eggs it had no effect. In contrast, the imidazole compound KK-42 reduced the ecdysteroid amounts in the eggs of *T. molitor*. As reviewed in HAGEDORN (1985) and LAFONT et al. (2005), it is a well-known phenomenon that increases in free ecdysteroids in the eggs result from the release of free hormones from stored maternal ecdysteroids. In adults, free 20E, together with JH, plays an important role in oocyte development, maturation, vitellogenesis and accessory gland development. The concentration of free insect hormones can be modified through interference with the hormone biosynthesis, processing and degradation. As reviewed by LAFONT et al. (2005), free and conjugated forms of ecdysteroids are confirmed in the ovaries of many insects, and it is likely that these maternal ecdysteroids are the source of hormone for the embryo during embryogenesis. Indeed the embryonic ecdysial gland seems not to be necessary for the increase in free ecdysteroid titre or for the accompanying cycles of cuticulogenesis. So any interference in the ecdysteroid hormone response using an ecdysteroid agonist or antagonist would result in abnormal oocyte growth, egg formation and embryogenesis leading to loss of the progeny (SOLTANI et al., 1998; DHADIALLA et al., 1998; 2005). Indeed in previous studies ecdysteroid agonists caused a decline in fecundity and fertility in different insect orders such as Lepidoptera, Diptera, Coleoptera and Orthoptera (WING & RAMSAY, 1989; SMAGGHE & DEGHEELE, 1992; 1994a,b; DHADIALLA et al., 1998; 2005; SWEVERS et al., 1999; SUN et al., 2003; TAÏBI et al., 2003). Also LAWRENCE (1992) found a reduction of protein synthesis and/or incorporation into eggs due to RH-5849 application on *Anastrepha suspense* adults. RH-5992 also caused mortality and the treated females of *Plodia interpunctella* showed smaller ovaries with fewer eggs (SALEM et al., 1997). FARINOS et al. (1999) confirmed the negative effects of RH-0345 on yolk protein accumulation and egg formation in beetle adults (*Leptinotarsa decemlineata*). The latter study also revealed the presence of RH-0345 in the eggs and a reduction in fecundity and/or decrease in progeny survival.

On the mechanisms that may underlie ecdysteroidogenesis and the changes in ecdysteroid amounts, existing data indicate that different factors, hormones and peptides, may influence ecdysteroid biosynthesis (SMAGGHE et al., 1995; GÄDE et al., 1997; GÄDE & HOFFMANN, 2005; LAFONT et al., 2005). Also ecdysteroids themselves play a role in their own biosynthesis via feedback regulation of the PTTH axis in the insect brain, and also via direct action on the ecdysteroid-producing prothoracic glands in the larval stages or the endocrine organs (ovaries, testis) in adults. E and 20E have been reported to stimulate PTTH synthesis and secretion. So for an ecdysteroid agonist compound like RH-0345 we may expect stimulation of free ecdysteroid amounts after treatment. Indeed, SOLTANI-MAZOUNI et al. (2004) reported that the ecdysteroid agonists RH-0345, RH-5849 and RH-5992 increased the ovarian ecdysteroid amounts in adult females after *in vivo* treatment. Also in pupae of *T. molitor*, it was evident that RH-0345 could increase the ecdysteroid titre under *in vivo* conditions and with the use of integument *in vitro* cultures (SOLTANI et al., 2002; TAÏBI et al., 2003). However, as the positive feedback of ecdysteroids happens through an as yet unknown pathway, it also remains a mystery how exactly an ecdysteroid agonist like RH-0345 may have provoked the increase in 20E and free ecdysteroid amounts.

A crucial question that also arises from these studies is why and how the profiles of ecdysteroid titres, free and conjugated, are punctuated with high peaks and deep troughs at specific times during development. Many experiments confirm the presence of the free and conjugated forms of ecdysteroids in ovaries, and it is proposed that these maternal polar conjugated compounds represent storage forms of the hormone, and hydrolysis of these in the developing egg would result in the embryo being exposed to free active hormone (HAGEDORN, 1985; LAFONT et al., 2005). However the mechanisms behind these hormone dynamics are not yet clear, and so the relative reduction of conjugated ecdysteroids due to treatment RH-0345 also cannot yet be explained.

The imidazole compound KK-42 has also been found to reduce the hormonal production in hormone biosynthesis sources from female adult crickets (LORENZ et al., 1995). Interestingly, SOLTANI et al. (1997) also reported a few years later that KK-42 disturbed the growth and the development of oocytes, and in parallel this IGR was found to reduce the amounts of ecdysteroids released into the culture medium by ovaries. The inhibitory action of KK-42 on ecdysteroid production was also observed in prothoracic glands and ovaries under *in vivo* and *in vitro* conditions with the silkmoth *Bombyx mori* (KUWANO et al., 1985). To date several authors have proposed some hypotheses on the mode of action of KK-42. It has been reported to act as an anti- JH, in lowering ecdysteroid levels and/or to induce precocious metamorphosis or diapause termination. The anti-JH activity is more likely interference with the JH synthesis rather than destruction of corpora allata tissue (KUWANO et al., 1983). Herewith, HIRAI et al. (2002) recently reported that the precocious metamorphosis induction by KK-42 correlated with an enhanced expression of BmJH esterase in the fat body, suggesting that KK-42 enhances BmJHE gene expression in the fat body inducing hemolymph JH esterase activity. In addition, to explain the mode of action of KK-42 in ecdysteroidogenesis, SHIOTSUKI & KUWANO (2004) designed an affinity chromatography column system to

detect proteins with high affinity from insect tissues. Although they detected a single receptor protein in the prothoracic glands, the exact underlying mechanism remains unknown. Here, we believe that further research with the availability of the recently characterized and identified Halloween genes, encoding the ecdysteroid-producing cytochrome P450 enzymes, and microarray technologies (WILLIAMS et al., 2002; LAFONT et al., 2005) will provide opportunities to unravel the mechanisms of imidazoles in the hierarchy of ecdysteroid biosynthesis.

In conclusion, the results obtained in this study are the first demonstrating that RH-0345 increased the amounts of both free ecdysteroids and 20E in eggs. In contrast KK-42 reduced the amounts of total and conjugated ecdysteroids. These reported hormonal disturbances in eggs of mealworms help to explain the reduction in egg viability that we scored in previous experiments using RH-0345 and KK-42 on female adults (TAÏBI et al., 2003). However, further research, for instance on the function and dynamics of free and conjugated ecdysteroids in the embryo, is required to better understand the mechanism underlying the ovicidal toxicity of such novel IGRs.

ACKNOWLEDGEMENTS

This research was supported by the National Fund for Scientific Research of Algeria (FNR, Algeria) and the Fund for Scientific Research (FWO-Vlaanderen, Belgium).

REFERENCES

AMRANI L, ZERGUINE K, FARINE JP, SMAGGHE G & SOLTANI-MAZOUNI N (2004). Imidazole derivative KK-42 reduces ecdysteroid titers and interferes with reproductive processes in adult females of *Tenebrio molitor*. Pesticide Biochemistry and Physiology, 80: 163-172.

CARLSON GR, DHADIALLA TS, HUNTER R, JANSSON RK, JANY CS, LIDERT Z et al. (2001). The chemical and biological properties of methoxyfenozide, a new insecticidal ecdysteroid agonist. Pest Management Science, 57: 115-119.

DE REGGI ML, PITOIZET N, GHARIB B & DELBECQUE JP(1992). New enzyme immunoassay for ecdysteroids using peroxydase enzyme and polyclonal or monoclonal antibodies. In: Xth ecdysone workshop, Liverpool, 6-7th April, Abstract, pp. 9.

DHADIALLA TS, CARLSON GR & LE DP (1998). New insecticides with ecdysteroidal and juvenile hormone activity. Annual Review of Entomology, 43: 545-569.

DHADIALLA TS, RETNAKARAN A & SMAGGHE G (2005). Insect growth- and developmental-disturbing insecticides. In: GILBERT LI, IATROU K & GILL SK (eds), Comprehensive Molecular Insect Science, vol. 6. Elsevier, Oxford, pp. 55-116.

FARINOS GP, SMAGGHE G, TIRRY L & CASTAÑERA P (1999). Action and pharmacokinetics of a novel insect growth regulator, halofenozide, in beetles of *Aubeonymus mariaefranciscae* and *Leptinotarsa decemlineata*. Archives of Insect Biochemistry and Physiology, 41: 201-213.

GÄDE G, HOFFMANN KH & SPRING JH (1997). Hormonal regulation in insects: Facts, Gaps and Future directions. Physiological Reviews, 77(4): 263-1032.

GÄDE G & HOFFMANN KH (2005). Neuropeptides regulating development and reproduction in insects. Physiological Entomology, 30: 103-121.

HAGEDORN HH (1985). The role of ecdysteroids in reproduction. In: KERKUT GA & GILBERT LI (eds), Comprehensive Insect Physiology, Biochemistry, and Pharmacology, vol 8. Pergamon Press, Oxford, pp. 205-262.

ISHAAYA I (1990). Benzoylphenyl-ureas and other selective control agents, mechanism and application In: CASIDA JE (ed), Pesticides and alternatives. Elsevier Sciences, Amsterdam, pp. 365-376.

KOSTYUKOVSKY M, CHEN V, ATSMI S & SHAAYA E (2000). Biological activity of two juvenoids and two ecdysteroids agonist three stored product insects. Insect Biochemistry and Molecular Biology, 30: 891-897.

KUWANO E, TAKEYA R & ETO M (1983). Terpenoid imidazoles: New anti-juvenile hormones. Agricultural and Biological Chemistry, 47: 921-923.

KUWANO E, TAKEYA R & ETO M (1985). Synthesis and anti-juvenile hormone activity of 1-Substituted-5-((E) – 2,6 – dimethyl – 1,5 – heptadienyl) imidazoles. Agricultural and Biological Chemistry, 49: 483-486.

HIRAI M, KAMIMURA M, KIKUCHI K, YASUKOCHI Y, KIUCHI M, SHINODA T & SHIOTSUKI T (2002). cDNA cloning and characterization of *Bombyx mori* juvenile hormone esterase: an inducible gene by the imidazole insect growth regulator KK-42. Insect Biochemistry and Molecular Biology, 32: 627-635.

LAFONT R, DAUPHIN-VILLEMANT C, WARREN JT & REES H (2005). Ecdysteroid chemistry and biochemistry. In: GILBERT LI, IATROU K & GILL SK (eds), Comprehensive Molecular Insect Science, vol. 3. Elsevier, Oxford, pp. 125-195.

LAGUEUX M, HOFFMANN JA, GOLTZENÉ N, KAPPLER C, TSOUPRAS G, HETRU C & LUU B (1984). Ecdysteroids in ovaries and embryos of *Locusta migratoria*. In: HOFFMANN JA & PORCHET M (eds), Biosynthesis, metabolism and mode of action of invertebrate hormones. Springer, Berlin, pp. 168-180.

LAWRENCE PO (1992). Egg development in *Anastrepha suspense*: influence of the ecdysone agonist, RH-5849. In: ALUJA M & LIEDO P (eds), Fruit Flies: Recent advances in Research and Control Programs. Springer, Berlin, pp. 51-56.

LORENZ J, LENZ M & HOFFMANN KH (1995). Effects of pharmacological agents on ecdysteroid synthesis *in vitro* in ovaries and abdominal integument from female adult crickets, *Gryllus bimaculatus* de Geer (Ensifera, Gryllidae). Zeitschrift fur Naturforschung C-A Journal of Biosciences, 50C: 286-293.

MEDINA P, BUDIA F, TIRRY L, SMAGGHE G & VIÑUELA E (2001). Compatibility of spinosad, tebufenozide and azadirachtin with eggs and pupae of the predator *Chrysoperla carnea* (Stephens) under laboratory conditions. Biocontrol Science and Technology, 11: 597-610.

MEDINA P, SMAGGHE G, BUDIA F, DEL ESTAL P, TIRRY L & VIÑUELA E (2002). Significance of penetration, excretion, and transovarial uptake to toxicity of three insect growth regulators in predatory lacewing adults. Archives of Insect Biochemistry and Physiology, 51: 91-101.

NAKAGAWA Y, SMAGGHE G, VAN PAEMEL M, TIRRY L & FUJITA T (2001). Quantitative structure-activity studies of insect growth regulators: XVIII. Effects of substituent on the aromatic moiety of dibenzoylhydrazines on larvicidal activity against the Colorado potato beetle *Leptinotarsa decemlineata*. Pest Management Science, 57: 858-865.

PALLI SR & RETNAKARAN A (2001). Ecdysteroid and juvenile hormone receptors: properties and importance in developing novel insecticides. In: ISHAAYA I (ed), Biochemical Sites of insecticide Action and Resistance. Springer, New York, pp. 107-132.

REES HH & ISAAC RE (1984). Biosynthesis of ovarian ecdysteroid phosphates and their metabolic fate during embryogenesis in *Schistocerca gregaria*. In: HOFFMANN JA & PORCHET

M (eds), Biosynthesis, metabolic and mode of action of invertebrate hormones. Springer, Berlin, pp. 181-185.

RIDDIFORD LM, CHERBAS P & TRUMAN JW (2000). Ecdysone receptors and their biological actions. Vitamins and Hormones-Advances in Research and Applications, 60: 1-73.

SALEM H, SMAGGHE G & DEGHEELE D (1997). Effects of tebufenozide on oocyte growth in *Plodia interpunctella*. Mededelingen van de Faculteit Landbouwkundige en Toegepaste Biologische Wetenschappen, Universiteit Gent, 62(1): 9-13.

SHIOTSUKI T & KUWANO E (2004). Detection of proteins with a high affinity for imidazole insect growth regulator, KK-42. Journal of Pesticide Science, 29: 121-123.

SMAGGHE G & DEGHEELE D (1992). Effect of nonsteroidal ecdysteroid agonist RH-5849 on reproduction of *Spodoptera littoralis* (Boisd.) (Lepidoptera: Noctuidae). Parasitica, 48: 23-29.

SMAGGHE G & DEGHEELE D (1994a). Action of a novel nonsteroidal ecdysteroid mimic, tebufenozide (RH 5992), on insects of different orders. Pesticide Science, 42: 85-92.

SMAGGHE G & DEGHEELE D (1994b). The significance of pharmacokinetics and metabolism to the biological activity of RH 5992 (tebufenozide) in *Spodoptera exempta*, *Spodoptera exigua* and *Leptinotarsa decemlineata*. Pesticide Biochemistry and Physiology, 49: 224-234.

SMAGGHE G, BÖHM G.-A., RICHTER K & DEGHEELE D (1995). Effects of nonsteroidal ecdysteroid agonists on the ecdysteroid titre in *Spodoptera exigua* and *Leptinotarsa decemlineata.* Journal of Insect Physiology, 41: 971-974.

SOLTANI N, PITOIZET N, SOLTANI-MAZOUNI N, DELACHAMBRE J & DELBECQUE JP (1997). Activity of an anti ecdysteroid compound (KK-42) on ovarian development and ecdysteroid secretion in mealworm. Mededelingen van de Faculteit Landbouwkundige en Toegepaste Biologische Wetenschappen, Universiteit Gent, 62(2): 531-537.

SOLTANI N, SMAGGHE G & SOLTANI-MAZOUNI N (1998). Evaluation of the ecdysteroid agonist RH-0345 on the hormonal production by integumental explants and ovaries in mealworms. Mededelingen van de Faculteit Landbouwkundige en Toegepaste Biologische Wetenschappen, Universiteit Gent, 63(2): 547-554.

SOLTANI N, ARIBI N, BERGHICHE H, LAKBAR S & SMAGGHE G (2002). Activity of RH-0345 on ecdysteroid production and cuticle secretion in *Tenebrio molitor* pupae *in vivo* and *in vitro*. Pesticide Biochemistry and Physiology, 72: 83-90.

SOLTANI-MAZOUNI N & SOLTANI N (1995). Effet du diflubenzuron en traitement *in vivo* et *in vitro* sur la morphométrie de l'ovaire de *Tenebrio molitor*. Mededelingen van de Faculteit Landbouwkundige en Toegepaste Biologische Wetenschappen, Universiteit Gent, 65(2): 453-460.

SOLTANI-MAZOUNI N, KHEBBEB MEH & SOLTANI N (1999). Production d'ecdystéroïdes ovariens durant la maturation des oocytes chez *Tenebrio molitor*. Annales de la Société Entomologique de France, 35: 82-86.

SOLTANI-MAZOUNI N, BOUKACHABIA A & SMAGGHE G (2004). Biological activity of some moulting hormone agonists in mealworms: ecdysteroid and protein analysis in ovaries. Revue Synthèse, 13: 7-10.

SUN X & BARRETT BA (1999). Fecundity and fertility changes in adult codling moth (Lepidoptera: Tortricidae) exposed to surfaces treated with tebufenozide and methoxyfenozide. Journal of Economic Entomology, 92: 1039-1044.

SUN X, SONG Q & BARRETT BA (2003). Effect of ecdysone agonist on vitellogenesis and the expression of EcR and USP in codling moth *Cydia pomonella*. Archives of Insect Biochemistry and Physiology, 52: 115-129.

SWEVERS L & IATROU K (1999). The ecdysone agonist tebufenozide (RH-5992) blocks the progression into the ecdysteroid-induced regulatory cascade and arrests silkmoth oogenesis at mid-vitellogenesis. Insect Biochemistry and Molecular Biology, 29: 955-963.

TAÏBI F, SMAGGHE G, AMRANI L & SOLTANI-MAZOUNI N (2003). Effect of ecdysone agonist, RH-0345, on reproduction of mealworm, *Tenebrio molitor*. Comparative Biochemistry and Physiology C, 135: 257-267.

THOMPSON MJ, SVOBODA JA, REES HH & WILZER KR (1987). Isolation and identification of 26-hydroxyecdysone-2-phosphate: An ecdysteroid conjugate of eggs and ovaries of tobacco hornworm, *Manduca sexta*. Archives of Insect Biochemistry and Physiology, 4: 183-190.

WILLIAMS DR, FISHER MJ, SMAGGHE G & REES HH (2002). Species specificity of changes in ecdysteroid metabolism in response to ecdysteroid agonists. Pesticide Biochemistry and Physiology, 72: 91-99.

WING KD (1988). RH-5849, a nonsteroidal ecdysone agonist: effects on a *Drosophila* cell line. Science, 241: 467-469.

WING KD, SLAWECKI RA & CARLSON GR (1988). RH-5849, a nonsteroidal ecdysone agonist: effects on larval Lepidoptera. Science, 241: 470-472.

WING KD & RAMSAY JR (1989). Other hormonal agents: ecdysone agonists. In: Progress and Prospects in Insect Control, BCPC Monograph 43. British Crop Protection Council, London, pp. 107-118.

Received: December 22, 2006
Accepted: January 18, 2008

Belg. J. Zool., 138 (2) : 146-148 July 2008

Feeding behaviour of the Columbretes lizard *Podarcis atrata*, in relation to *Isopoda* (*Crustaceae*) species: *Ligia italica* and *Armadillo officinalis*

Aurora M. Castilla[1,2*], Bieke Vanhooydonck[3] & Alessandro Catenazzi[4]

[1] **Estación Biológica de Sanaüja, Museo Nacional de Ciencias Naturales (CSIC). Ap. Correos nº 35, E-25280 Solsona, Lleida, España. e-mail: aurora@mncn.csic.es**

[2] **Dept. Biodiversity and Evolutionary Biology, National Museum of Natural Sciences (CSIC), C/ José Gutiérrez Abascal 2, E-28006 Madrid, Spain. e-mail: aurora@mncn.csic.es**

[3] **Laboratory of Functional Morphology, Department of Biology, University of Antwerp, Campus Drie Eiken, B-2610 Antwerp, Belgium e-mail: bieke.vanhooydonck@ua.ac.be**

[4] **Integrative Biology, University of California Berkeley, 3060 Valley Life Sciences Bldg #3140, Berkeley CA 94720, USA e-mail: acatenazzi@gmail.com**

Corresponding author : * Aurora M. Castilla. Estación Biológica de Sanaüja, Museo Nacional de Ciencias Naturales (CSIC). Ap. Correos nº 35, E-25280 Solsona, Lleida, España. e-mail: aurora@mncn.csic.es

ABSTRACT. The lizard *Podarcis atrata*, endemic to the Columbretes archipelago (Mediterranean, Spain) occupies very small islands characterized by extreme aridity and a reduced availability of potential insect prey. The results of an experimental study have shown that adult lizards offered individuals of *Ligia italica* and *Armadillo officinalis* (marine and terrestrial isopods, respectively) consumed at high frequencies; with males consuming them at significantly higher frequencies than females. The results are discussed in light of the importance of the inclusion of marine prey into the diet in arid insular ecosystems.

KEY WORDS : island, intertidal trophic resources, endemic lizard, arid zones

INTRODUCTION

The endemic lizard *Podarcis atrata* from the Columbretes archipelago (Mediterranean, Castellón, Spain), inhabits very small islands ranging from 0.5 to 13 hectares. The islands are characterized by extreme aridity and by the scarcity of terrestrial insects (see CASTILLA & BAUWENS, 1991). Adult lizards have been observed near shore feeding on seabird regurgitates, and scavenging on carcasses and gull eggs on several islands of the archipelago (CASTILLA et al., 1987). Lizards from arid coastal areas across the world are important consumers of marine and intertidal trophic resources (amphipods, crabs, shells, flies, etc) (Table 1). It is generally assumed that the inclusion of marine resources into the diet is promoted by the lack of other food resources (POLIS & HURD, 1996; BARRET el al., 2005; CATENAZZI et al., 2005; CATENAZZI & DONNELLY, 2007).

The isopod *Ligia italica* is very abundant on Columbretes, and inhabits rocky substrates from 0 to 12 meters above sea level. In some areas, lizards and isopods utilize the same rocks, thus facilitating predator-prey interactions. However, observations of the consumption of *L. italica* by *P. atrata* individuals under natural conditions are difficult because both species are very wary on exposed rocks without vegetation cover and a high abundance of bird predators. Moreover, lizards are very difficult to capture on vertical, eroded cliffs. Consequently, no data are available on stomach contents for animals living near shore. We decided to use an experimental approach similar to that employed in a previous study to examine the cannibalistic propensities of lizards on the same island (CASTILLA & VAN DAMME, 1996). We measured the willingness of *P. atrata* individuals to consume the marine isopod *Ligia italica* and the terrestrial one *Armadillo officinalis*, which is morphologically similar and has been found in the stomach contents of *P. atrata* (CASTILLA et al., 1987).

MATERIALS AND METHODS

The study was conducted on the island Columbrete Grande during the fist week of June 2006. We captured adult isopods of *L. italica* at the sea shore and offered them to free ranging adults of *P. atrata* living in vegetated areas of the island near human habitation where the lizards are abundant and used to human presence. Observations were conducted between 0900-1200 hours, with ambient temperature ranging from 20–22ºC, and relative humidity from 72-84%.

To be sure lizards were hungry at the time of the experiment we first offered them a mealworm (a preferred prey) attached to a noose. If the reaction of the lizard to the mealworm was positive (e.g. direct attack), we removed the mealworm from the noose and replaced it with an individual of *L. italica* (length x width ca. 15 x 6mm; 0.15g). We tested only adult lizards (svl=60-70mm, mass=5-9g) of both sexes. After each presentation we observed the reaction of the lizard and registered its behaviour (Table 2). We also recorded the handling time, as the time from the moment the lizard captured its prey to the moment the lizard completed ingestion. After three days we repeated the same experiment using terrestrial isopods, *A. officinalis* (length x width ca. 10x5mm; 0.05g) that were captured under rocks near human habitation.

TABLE 1

Insular and coastal lizards that include marine input in their diet. Note that there are only data for one species from the Mediterranean (present study).

Species (Reptilia: Squamata)	Zone	Reference
Amblyrhynchus cristatus	Galapagos	Dunson, 1969
Callisaurus draconoides	Baja California	Quijada-Mascareñas, 1992
Cryptoblepharus bautony	Madagascar	Fricke, 1970
Leiolopisma suteri	New Zealand	Towns, 1975
Microlophus peruvianus	Peru	Vogt, 1939
Phyllodactilus angustidigitu	Peru	Catenazzi & Donnelly, 2007
Uta spp.	Gulf of California	Soulé, 1966
Uta antiqua	Gulf of California	Ballinger & Tinkle, 1972
Uta encantadae	Gulf of California	Grismer, 1994
Uta lowei	Gulf of California	Grismer, 1994
Uta palmeri	Gulf of California	Wilcox, 1980
Uta stansburiana	Gulf of California	Barrett et al., 2005
Uta tumidarostra	Gulf of California	Grismer, 1994
Podarcis dugesii	Atlantic, Madeira	Davenport & Dellinger, 1995
Podarcis atrata	Mediterranean, Columbretes	present study

TABLE 2

Behavioural response of free living adult males and females of *Podarcis atrata* following presentation of individuals of two different *Isopoda (Crustacea)* prey attached to a noose. For each different lizard the first immediate reaction was recorded. The results are expressed in percentages (%), and sample size given (n).

***P. atrata* response**	***Ligia italica***				***Armadillo officinalis***			
	Males (n=18)		**Females (n=10)**		**Males (n=20)**		**Females (n=18)**	
	n	**%**	**n**	**%**	**n**	**%**	**n**	**%**
Ignore	0	0	4	40	2	10	5	28
Observe-ignore	1	6	2	20	1	5	2	11
Observe-attack-eat	3	17	1	10	5	25	1	6
Direct attack- eat	13	72	3	30	11	55	10	56
Direct attack- not eat	1	6	0	0	1	5	0	0

Differences in response (consumption of the prey or not) towards the two different prey items (*L. italica* or *A. officinalis*) by lizards of both sexes were analysed with a binomial regression (i.e., generalized linear model) with a probit link function to examine the putative effects of prey, sex and their interaction, using the program S-Plus. We also tested for differences in handling time between prey species using an analysis of variance. Prior to performing the one way ANOVA's we logarithmically transformed (log10) handling times.

RESULTS

In all cases (n=66) males and females reacted to the mealworm by directly attacking it. After that, most males consumed the isopod *L. italica* (89%), 72% attacking the prey directly without previous observation (Table 2). Most females (60%) ignored the specimen of *L. italica* offered, and only 40% of the females actually consumed them. Most males consumed the offered individuals of *A. officinalis* (80%), 55% attacking them directly without previous observation. Some females (39%) ignored this prey while 61% consumed the terrestrial isopod (Table 2).

We did not find a significant interaction effect between sex and prey ($t=1.2909$; $P>0.05$). However, the analysis showed a significant effect of sex ($t=2.8711$; $P<0.01$): males consumed both isopods species at higher proportions (*L. italica*=89%, *A. officinalis*=80%) than did females (*L. italica*=40%, *A. officinalis*=61%).

Handling time for consumption of the isopod *L. italica* was only measured for male lizards, and ranged from 22 seconds to 1 minute (mean=40.1sec; sd=13.6; n=13). Handling time of specimens of *A.officinalis* by male lizards ranged from 12 to 38sec (mean=23.9sec; sd=7.7; n=9), and the difference was significant ($F1, 26=9.38$; $p=0.005$). The mean ingestion time of specimens *A. officinalis* was longer for female lizards (mean=27.6sec; sd=14.1; n=7) than it was for males (23.9sec) but the difference was not significant ($F1, 14=0.11$; $p=0.75$).

DISCUSSION

The results of our study show that, in our experimental conditions, both sexes of the lizard *P. atrata* consumed the marine isopod *L. italica* in the same proportion as the terrestrial *A. officinalis*. However, males were more inclined to consume both prey types than females. In June, 80% of the females were pregnant, and they may be more selective for food during that stage. Additional experiments outside the breeding season would help to better interpret our results.

Handling time for males was lower for specimens of *A. officinalis* (24sec) than those of *L. italica* (40sec). In addition we observed one male ingesting three specimens of

A. officinalis, one after the other. For this individual, ingestion time was variable (22.41sec; 31.91sec and 24.64sec), suggesting that a larger sample size may be needed.

A longer handling time for specimens of *L. italica* could be related to the larger size of this species (ca. 5mm larger than individuals of *A. officinalis*). However, we believe that these differences were mainly due to the fact that a specimen of *L. italica* was sometimes thrown to the ground and picked up again by both males and females. Unfortunately, we could not quantify the "rejection behaviour" shown by six individuals. Interestingly, rejection behaviours were not observed with offered individuals of *A. officinalis*. Terrestrial isopods such as *A. officinalis* are abundant species in the vegetated and humid zones of the island, and are frequently consumed by *P. atrata* (CASTILLA et al., 1987) and other lizards (CARRETERO, 2004).

To test the importance of marine prey resources in the diet of *P. atrata*, future studies should include the analysis of stomach contents of lizards living near the seashore. Alternatively, stable isotope analysis could be used (e.g., CATENAZZI & DONNELLY, 2007) to test for the importance of the marine subsidies into the terrestrial ecosystem at the Columbretes islands. The only other data on European lizards utilising marine resources are for *Podarcis dugesi* on Madeira Island (Atlanic, Portugal; see DAVENPORT & DELLINGER, 1995). However, as there are many islands in the Atlantic and the Mediterranean seas holding lizard populations, the use of marine trophic resources to survive or complement the diet of lizards is likely more common. Indeed, our observations suggest that marine invertebrates may be an important food source for lizards living in harsh conditions on small islands.

ACKNOWLEDGEMENTS

We would like to thank the Generalitat Valenciana and the Secretaria General de Pesca Marítima (Ministerio de Agricultura, Pesca y Alimentación) for permission to work on the islands. Thanks to Diego Kersting (MAPA), Marta Aguiló (GV) and Guim Llacuna (Escuela de Capacitación Agraria de Solsona) for their help during the field work and to Pep Perolet and the boats CAT-CAT, Clavel I and Super Bonanza for transportation. We thank A. Herrel and D. Bauwens for comments. This paper is a contribution of the Bio-Ecological Station of the Museum of National Sciences (CSIC). This work was conducted on a contract "Ramón and Cajal" from the Spanish National Science Foundation (CSIC, Ministerio de Educación y Ciencia) (to AMC), and the Project MEC CGL2005-00391/ BOS (J. Martín & P. López, MNCN-CSIC).

REFERENCES

BALLINGER RE & TINKLE DW (1972). Systematics and evolution of the genus *Uta* (Sauria: Iguanidae). Miscellaneous Publications of the Museum of Zoology at the University of Michigan, 145: 1-83.

BARRETT K, ANDERSON WB, WAIT AD, GRISMER LL, POLIS GA & ROSE MD (2005). Marine subsidies alter the diet and abundance of insular and coastal lizard populations. Oikos, 109: 145-153.

CASTILLA AM, JIMÉNEZ J & LACOMBA I (1987). Los reptiles de Columbretes. In: ALONSO MATILLA LA, CARRETERO JL & GARCIA-CARRASCOSA AM (eds), Islas Columbretes. Contribución al estudio de su medio natural, Generalitat Valenciana, Valencia: 181-194.

CASTILLA AM & BAUWENS D (1991). Observations on the natural history, present status, and conservation of the insular lizard *Podarcis hispanica atrata*. Biological Conservation, 58: 69-84.

CASTILLA AM & VAN DAMME R (1996). Cannibalistic propensities in the lizard *Podarcis hispanica atrata*. Copeia, 1996: 991-994.

CARRETERO MA (2004). From set menu to a la carte. Linking issues in trophic ecology of Mediterranean lacertids. Italian Journal of Zoology, 2: 121-133.

CATENAZZI A, CARRILLO J & DONNELLY MA (2005). Seasonal and geographic eurythermy in a coastal Peruvian lizard. Copeia, 4: 713-723.

CATENAZZI A & DONNELLY MA (2007). The Ulva connection. Marine green algae subsidize terrestrial consumers in coastal Peru. Oikos, 116: 75-86.

DAVENPORT J & DELLINGER T (1995). Melanism and foraging behavior in an intertidal population of the Madeiran lizard *Podarcis* (=*Lacerta*) *dugesii* (Milne-Edwards, 1829). Herpetological Journal, 5: 200-203.

DUNSON WA (1969). Electrolyte excretion by the salt gland of the Galapagos marine iguana. American Journal of Physiology, 216: 995-1002.

FRICKE HW (1970). Die ökologische Spezialisierung der Eidechse *Cryptoblepharus boutoni cognatus* (Boettger) auf das Leben in der Gezeitenzone (Reptilia, Skinkidae). Oecologia, 5: 380-391.

GRISMER LL (1994). Three New Species of Intertidal Sideblotched Lizards (Genus *Uta*) from the Gulf of California, Mexico. Herpetologica, 50: 451-474.

POLIS GA & HURD SD (1996). Linking marine and terrestrial food webs: allochthonous input from the ocean supports high secondary productivity on small islands and coastal land communities. American Naturalist, 147: 396-423.

QUIJADA-MASCAREÑAS A (1992). Feeding and foraging of *Callisaurus draconoides* (Sauria: Phrynosomatidae) in the intertidal zone of coastal Sonora. Southwestern Naturalist, 37: 311-314.

SOULÉ M (1966). Trends in the insular radiation of a lizard. American Naturalist, 100: 47-64.

TOWNS DR (1975). Ecology of the black shore skink, *Leiolopisma suteri* (Lacertilia: Scincidae), in boulder beach habitats. New Zealand Journal of Zoology, 2: 389-407.

VOGT W (1939). Las lagartijas y las aves guaneras. Boletín de la Compañía Administradora del Guano [VOLUME?]: 346-348.

WILCOX BA (1980). Species number, stability, and equilibrium status of reptile faunas on the california Islands. In: POWER DM (ed), The California Islands. Proceedings of a multidisciplinary symposium, Museum of Natural History, Santa Barbara, California: 551-564.

Received: March 31, 2007
Accepted: April 16, 2008

Belg. J. Zool., 138 (2) : 149-153 July 2008

Protective action of peanut oil in rats exposed to gamma-rays

Edrees GMF, El-Kholy WM, El. Habiby EM & El-Sherbiny SA

Faculty of Science, Mansoura University, Mansoura, Egypt

Corresponding author : e-mail: gamaledrees600@yahoo.com

ABSTRACT. The present study aims to clarify the role of peanut oil as a radioprotector in male albino rats against oxidative stress and bone injury induced by ã-radiation. Rats were subjected to a dose of 5Gy, over an exposure time of 133sec, at a dose rate 3.759rad/sec. Prior to irradiation, rats received peanut oil subcutaneously, (0.75mL/kg) over a one month period, on three days/ week. Serum and bone mineral contents were estimated, and serum protein, cholesterol and creatinine concentrations were determined. We also investigated some enzyme activities as well as hormonal calcium control. It seems that the deleterious effects of exposure to ã-radiation on most estimated parameters affecting Ca metabolism can be controlled to some extent by peanut oil administration prior to irradiation.

KEY WORDS : Radiation, Peanut oil, Calcium metabolism.

INTRODUCTION

Exposure to ionizing radiation represents a genuine, increasing threat to mankind and our environment. The steadily increasing applications of radiation in clinical practice, industrial and agricultural activities, on top of residual radio-activity resulting from nuclear test explosions, have a measurable impact contributing to possible radiation hazards in humans. Control of radiation hazards is considered as one of the most important challenges in order to protect our lives from radiation damage.

Calcium is one of the essential elements for normal functioning of an organism, and its concentration in serum is kept within the narrow range of 8.7-10.7mg/dL (BROZOSKA & MONIUSZKO-JAKONIUK, 1996). Because of the importance of calcium in regulating vital cellular and tissue functions, the concentration of calcium ions in body fluids is regulated by an effective feedback control system including a Ca^{++} transporting subsystem (bone & kidney), Ca^{++} sensing receptors, and calcium regulating hormones: parathormone, calcitonin and 1,25-dihydroxy vit D_3, (HURWITZ, 1996). Parathormone and calcitonin positively regulate renal 1,α-hydroxylase gene expression (a key enzyme for $1,25(OH)_2D_3$ synthesis) which is found mainly in the kidneys (MURAYAMA et al., 1999). ENDO et al., (2000) suggested that $1,25(OH)_2D_3$ has the potential to alleviate hypocalcemia, through the inhibition of bone resorption. COLMAN et al., (2002) reported that calcitonin inhibits bone resorption by acting directly on osteoblasts, as it binds to high affinity osteoclastic receptors, and inhibits osteoclastic activity.

Ionizing radiations interact with biological systems through free radicals generated by water radiolysis. This indirect action plays an important role in the induction of oxidative stress leading to cellular damage and organ dysfunction (BERROUD et al., 1996). SHFRANOVSKAIA (2002) found that the combined effects of acute gamma-irradiation and thyroparathyroidectomy change the structure-functional state of sarcoplasmic reticulum membranes of rat skeletal muscles conditioned by the disturbance of hormonal regulation of molecular-cellular mechanisms of Ca^{++} exchange. There is evidence that gamma radiation damages bone tissue via free radical attack on the collagen (AKKUS et. al., 2005). Therapeutic doses of radiation have been shown to have deleterious consequences on bone health, occasionally causing osteoradionecrosis and spontaneous fractures HAMILTON et al. (2006). JUAN et al. (2002) reported that trans-3,4,5-trihydroxystilbene is a phytochemical present in peanuts, grapes and wine with beneficial effects such as protection against cardiovascular disease and cancer prevention. We measured several parameters in male albino rats subjected to ã-radiation, in order to clarify the role of peanut oil in protection against oxidative stress and bone injury.

MATERIALS AND METHODS

Male albino rats weighing 150±20g were divided into four categories each with six animals:

- (i) Normal control.
- (ii) γ-irradiated group [^{60} Co (5Gy) exposure time 133sec, at a dose rate 3.759rad/sec] at the Middle Eastern Regional Radioisotope centre for Arab countries.
- (iii) Peanut oil treated group (0.75mL/kg) for one month, 3 days/week.
- (iv) Peanut oil followed by γ-irradiation.

After the experimentation period (1 month), animals were placed in metabolic cages for 24h. For urine collection a few drops of HCl were added to avoid fermentation. Rats were then decapitated, blood samples collected, urine 4-hydroxyproline determined, and sera were separated for estimation of Ca, P, Mg, creatinine, total protein, cholesterol, alkaline phosphatase, acid phosphatase, $1,25(OH)_2D_3$, PTH, calcitonin (BERG, 1982). The right femur of each animal was cleaned from surrounding tissues, weighed and crushed, then completely homogenated in 3mL distilled water, and kept frozen at -20°C till examination. A known volume of the homogenate was used for estimation of Alkaline and Acid phosphatises (KIND & KING, 1954). Samples digested with concentrated nitric

acid were used for mineral estimation using an atomic absorption spectrophotometer.

RESULTS

As shown in Table (1), exposure to γ-radiation caused significant increases in serum Ca, P, and Mg concentrations. Meanwhile a decline was detected in estimated bone minerals.

Table (2) shows that irradiation increased serum and bone acid phosphatase activity as will as serum creatinine levels, and decreased that of alkaline phosphatase. In addition, Table (3) shows that levels of parathormone, calcitonin, $1,25(OH)_2D_3$ as well as serum cholesterol were elevated in the irradiated group. Also urine hydroxyproline significantly increased following irradiation, but serum total protein declined significantly. In the peanut oil pre-irradiated group all these parameters tended to approach the values found in the controls.

TABLE 1

Serum and bone mineral content in control and treated rats ($\bar{x}$±SE).

Estimated / Animal group	Serum Ca mg/dL	Bone Ca g/g wt.	Serum P mg/dL	Bone P g/g wt.	Serum Mg mg/dL	Bone Mg g/g wt.
Control	8.62±0.22	0.59±0.013	3.9±0.22	87.0±1.6	3.3±0.14	104.4±5.4
Peanut oil	8.94±0.11	0.63±0.014*	5.1±0.1***	72.0±1.4***	3.1±0.04	95.9±1.1
Irrad.	12.9±0.14***	0.35±0.014***	6.3±0.18***	32.3±1.3***	5.6±0.3***	68.2±1.9***
Peanut oil + irradiated	°°°9.74±0.12***	°°°0.47±0.011***	°°°5.2±0.25***	°°°39.7±0.7***	°°°3.1±0.2	°°°82.7±1.4**

* Significant at P<0.05
** Highly significant at P<0.02
*** Very highly significant P<0.01 relative to control group
°° Highly significant at P≤0.02
°°° Very highly significant P≤0.01 relative to irradiated group

TABLE 2

Metabolic and enzymatic activity in serum or bone in control and treated rats.

Estimated / Animal group	Serum creatinine mg/dL	Serum alk. pase KAU/dL	Bone alk. pase KAU/ g wt.	Serum acid pase KAU/dL	Bone acid pase KAU/ g wt.
Control	0.7±0.03	57.8±0.6	44.8±0.5	3.1±0.3	9.8±0.3
Peanut oil	0.6±0.02**	59.4±0.7	47.5±0.7*	3.7±0.2	9.8±0.2
Irrad.	2.1±0.11***	26.0±0.4***	23.8±1.05***	7.0±0.4***	12.2±0.25***
Peanut oil + irradiated	°°°0.8±0.02*	°°°37.8±0.4***	°°°37.5±0.3***	°°4.9±0.3**	°°°8.7±0.3*

* Significant at P<0.05
** Highly significant at P<0.02
*** Very highly significant P<0.01 relative to control group
°° Highly significant at P≤0.02
°°° Very highly significant P≤0.01 relative to irradiated group

TABLE 3

Serum cholesterol, total protein and some hormonal content in control and treated rats.

Estimated / Animal group	Serum Cholesterol mg/dL	Serum total protein g/dL	Parath ornone PTH (pg/mL)	1.25 $(OH)_2$- D_3 pg/ml	Urine Hydroxyproline (µg/mL)	Calcitonin pg/mL
Control	203±5.8	7.1±0.3	22.9±0.15	19.8±0.8	5.13±0.08	3.7±0.06
Peanut oil	211±3.5	6.6±0.15	22.4±1.2	20.8±0.6	5.3±0.15	3.9±0.07
Irrad.	509±7.2***	4.5±0.3***	27.8±0.42***	32.2±1.4***	7.25±0.11***	4.0±0.04**
Peanut oil + irradiated	°°°364±4.5***	°°6.1±0.12*	°°°22.8±0.08	°°°23.1±0.4**	°°°5.8±0.1***	4.1±0.12*

* Significant at P<0.05
** Highly significant at P<0.02
*** Very highly significant P<0.01 relative to control group
°° Highly significant at P≤0.02
°°° Very highly significant P≤0.01 relative to irradiated group

DISCUSSION

Gamma rays act either directly or by secondary reactions to produce biochemical lesions that initiate series of physiological symptoms. Ionizing radiation is known to induce oxidative stress through the generation of reactive oxygen species (ROS) resulting in imbalance of the pro-oxidant and antioxidant activities, ultimately resulting in cell death (SRINIVASAN et al., 2006). Numerous attempts have been made to investigate different means for controlling and protection from radiation hazards using chemical, physical and biological means.

In our experiments, a marked increase was noted in serum calcium content with concomitant decrease in the bone calcium content of irradiated groups. The observed increase may be attributed to an increase in parathyroid hormone as mentioned by FUJIWARA et al. (1994), and/or to an increase in the intestinal brush border membrane cation permeability (HIZHNYAK, 1997). On the other hand, the decline of bone calcium content may be due to bone demineralization after irradiation as reported by FUKUDA & LIDA (1999). The reduction in the calcium disturbances following irradiation in the peanut pre-irradiated group may be due to the vitamin E content of peanut oil providing a suitable level of zinc (FARAG, 1999), which enhances $1,25(OH)_2D_3$-stimulated bone metabolism and/or due to protection against free radicals generated by irradiation, (GLASCOTT et al., 1996).

An increase in serum inorganic phosphorus concomitant with a decrease in bone phosphorus in irradiated groups (Table 1) is in accordance with the results of FILIPOV et al. (1991), and may be due to bone demineralization after irradiation, and/or to the destruction or arrest of the activities of bone cells such as osteoblasts. The administration of peanut oil pre-irradiation seems to reduce radiation damage possibly due to its antioxidant effect (CHEN et al., 2002).

The increase in serum content and concomitant decrease in bone content of magnesium may be a result of increased levels of parathyroid hormones, which stimulate magnesium absorption from the gut (HULTER & PETERSON, 1984) and release of magnesium ion from bone (ZOFOKOVA & KANCHEVA, 1995), as well as acceleration of bone resorption (CHAVELLY & RIZZOLI, 1999). Retention of magnesium levels to near normal in the peanut oil pre-treated group may be attributed to the protection of the sulfhydral group (SH) from oxidative damage through inhibition of peroxidation of membrane lipids in the liver and kidney of rats (UPASANI & BALARMAN, 2001).

The increased serum creatinine in the irradiated group indicates development of nephritis and renal dysfunction, a result in agreement with BORG et al. (2002). This result may be attributed to impairment of glomerular selective properties caused by irradiation (BERRY et al., 2001). In the peanut oil protected group, serum creatinine level remained close to normal; this may be explained by the ability of some antioxidant in peanut oil to scavenge free radicals generated by irradiation, which would otherwise cause kidney damage (NATH et al., 1994).

The observed decline in serum and bone alkaline phosphatase in the irradiated group may be due to early decline in the intestinal alkaline phosphatase isoenzyme activity (STEPHAN et al., 1977). This decrease may also be attributed to a transitory reduction in the release of alkaline phosphatase to the enzymatic circulation by rapidly metabolizing cells, (GERACI et al., 1991), and/or injury to the intestinal mucosa after irradiation as mentioned by FAHIM et al. (1993). The decrease in bone alkaline phosphatase in the irradiated group implies bone deformity resulting from an excess of resorption over formation (AITSULA, 1986), as bone alkaline phosphatase is more specific as an important bone formation marker than is total alkaline phosphatase (KHOSLA et al., 1999).

The amelioration in alkaline phosphatase activity resulting from peanut oil pre-irradiation may be due to a beneficial effect on membrane permeability leading to the maintenance of a higher level in serum (JUAN et al., 2002). In addition the presence of the strong antioxidant resveratrol may increase alkaline phosphatase in osteoblastic cells to stimulate bone resynthesis, a view which is in accordance with MIZUTANI et al. (1998).

The elevated serum and bone acid phosphatase levels in the irradiated group may be attributed to the breakdown of lysosomal membranes by the lipid peroxidation effect of radiation, resulting in release of the enzyme (KUMAR et al., 2003). In addition, irradiation may lead to lesions in the developing lysosomal membrane, through the action of oxygen free radicals, increasing membrane permeability and allowing acid phosphatase to escape (BECCIOLINI et al., 1982). The elevated bone acid phosphatase may also be due to the release of enzyme from osteoclast lysosomes as a result of bone resorption after irradiation (AITSULA, 1986). The maintenance of more normal serum and bone acid phosphatase levels in the peanut oil protected group should be attributed to the free radical scavenging ability of vitamin E, which can suppress bone resorption and prevent membrane lesions.

A decline was registered in serum protein of the ã-irradiated group (Table 3), possibly caused by DNA damage after irradiation, resulting in subsegment changes in m-RNA causing impairment in gene transcription that could inhibit protein synthesis (LAI & SINGH, 1996). In addition, the formation of free radicals can cause breakage of chemical bonds and destruction of protein molecules (TENG & MOFFAT, 2000). The maintenance of more normal protein levels in the peanut oil pre-irradiation group may be due to trapping of free radicals by reservatrol, thus preventing DNA damage (CADENAS & BARJA, 1999).

Elevated serum cholesterol after irradiation (Table 3) may be due to the increased ability of the liver to biosynthesise cholesterol (CHEN & THACKER, 1985), as well as to the decreased activity of cholesterol 7-hydroxylase, the key enzyme involved in degradation of cholesterol in the liver, as mentioned by CHUPUKCHAROEN et al. (1985).

In addition, irradiation caused increases in serum LDLc by lipid peroxidation (NATH, 1996), leading to transport of cholesterol to extrahepatic tissue through LDLc receptor as mentioned by ABD-EL MOMEIN et al. (1989).

The considerably lower level of cholesterol in the peanut pre-irradiated group compared to the irradiated group may be attributed to the monounsaturated fatty acids (MUFA) present in peanut oil, which lowered the concen-

tration of circulating triglycerides and cholesterol (FELDMAN, 1999).

As presented in Table (3), whole body gamma irradiation produced elevation in the level of rat serum parathyroid hormone relative to the control group, a result which may indicate parathyroid adenoma and carcinoma caused by irradiation (CHRISTMAS et al., 1988). This increase may also be attributed to defective calcium absorption mechanism resulting from impaired hepatic and renal function, and consequently the formation of the active metabolite, vitamin D (SOTORNIK, 1997). The reduction in parathyroid damage following radiation in the peanut oil pretreated group could be due to presence in the oil of phytosterols, which have an anticancer effect (AWAD et al., 2000).

The increased calcitonin in the irradiated group may result from a feedback mechanism to overcome the increase in calcium and parathormone levels (FUJIWARA et al., 1994). The alteration of calcitonin level in the peanut oil protected group may be due to antioxidant activity, which tends to improve bone formation and decrease bone resorption and hence reduce serum calcium levels (ARJMANDI et al., 2002).

The increase in $1,25(OH)_2D_3$ seen in the irradiated groups may be an indication of increased half life of the compound due to disturbances in lipid solubility, rather than to an increase in its secretion (AMIZUKA et al., 1999). The increase in serum parathormone level after irradiation may also have led to this result (CHAVELLY & RIZZOLI, 1999). The increase in serum magnesium ion may also have contributed to the increase in $1,25(OH)_2D_3$ (RUDE et al., 1985). The amelioration in the serum level of $1,25(OH)_2D_3$ in the peanut oil protected group may be due to improvement in parathyroid activity assisted by the antioxidant properties of peanut oil (AWAD et al., 2000) and/or the restoration of the process of bone formation (ARJMANDI et al., 2002).

Hydroxyproline is the specific amino acid of collagen and is considered as a suitable marker for bone metabolism, reflecting its resorption. The increase in urinary hydroxyproline observed in the irradiated group is expected, and may be related to the destruction of bone collagen and bone resorption following irradiation (LIESESGANG et al., 1998). In addition, wounds caused by irradiation may also play a role in increasing hydroxyproline as reported by YANG et al. (2001). The lack of elevation in hydroxyproline levels seen in the peanut oil pretreated group may be due to inhibition of collagenase genes, by the vitamin E component of peanut oil, a view which is in accordance with (AZZI et al., 2001).

In conclusion single whole body Gamma irradiation of rats resulted in disturbances in calcium metabolism and the hormones influencing it. Peanut oil treatment pre-irradiation may play a protective role against these abnormalities.

REFERENCES

ABD-EL MONEIM AE, SHOUSHA MA, SALEM OA, EZZAT LE & HAMDY SM (1989). Effect of dietary vitamin E on some metabolic changes in whole body γ-irradiation rats. Egyptian Journal of Biochemistry, 7: 153-172.

AITSULA K (1986). Bone tissue response to irradiation and treatment model of mandibular irradiation injury. An experimental and clinical study Acta. Oto-laryngologica supplementum, 428: 1-54.

AKKUS O, BELANEY RM & DAS P (2005). Free radical scavenging alleviates the biomechanical impairment of gamma radiation sterilized bone tissue. Journal of. Orthopaedic Research, 23(4): 838-845.

AMIZUKA N, KWAN MY, GOLTZMAN D, OZAWA H & WHITE JH (1999). Vitamin D_3 differentially regulates parathyroid hormone/parathyroid hormone-related peptide receptor expression in bone and cartilage. Journal of Clinical Investigation, 103(3): 373-381.

ARJMANDI B, JUMA S, BEHARKA A, BAPNA M, AKHTER M & MEYDANI S (2002). Vitamin E improves bone quality in the aged but not in young adult male mice. Journal of Nutrtion and Biochemistry, 13(9): 543-545.

AWAD AB, CHAN KC, DOWNIE AC & FINK CS (2000). Peanuts as a source of beta-sitosterol, a sterol with anticancer properties. Nutrition and Cancer, 36(2): 238-241.

AZZI A, BREYER J, FEHER M, RICCIARELLI R, STOCKER A, ZIMMER S & ZINGG J (2001). Non antioxidant functions of alpha-tochopherol in a mooth muscle cells. Journal of Nutrition, 131(2): 378-381.

BECCIOLINI A, BENUCCI A, PORCIANI S, NARDINO A & LANINI A (1982). Dipeptidase activity in the small intestine after irradiation at different times of day. Strahlentherapie, 158(6): 368-374.

BERG RA(1982). Determination of 3-and 4-hydroxyproline. Methods of Enzymology, 82: 372-398.

BERROUD A, LE-ROY A & VOISIN P (1996). Membrane oxidative damage induced by ionizing radiation detected by fluorescence polarization. Radiation Environmental Biophysics, 35: 289-295.

BERRY C, BROSMAN MJ, FENNELL J, HAMILTON CA & DOMINIC AF (2001). Oxidative stress and vascular damage in hypertension. Nephrol and Hypertension, 10: 247-255.

BORG M, HUGHES T, HORRATH N, RICE M & THOMAS AC (2002). Renal toxicity after total body irradiation. International Journal of Radiation Oncology and Biological Physics, 54(4): 1165-1173.

BROZOSKA MM & MONIUSZKO-JAKONIUK J (1996). Homeostasis of calcium in the organism. Polski Merkuriusz Lekaraski, I(5): 363-365.

CADENAS S & BARJA G (1999). Resveratrol, melatonin, Vitamin E and PBN protect against renal oxidative DNA damage by the kidney carcinogen K Br Os. Free Radical Biology and Medicine, 26(11-12): 1531-1537.

CHAVELLY T & RIZZOLI R (1999). Bone and hormones effect of parathyroid hormone on the bone. Presse Médicale, 28(10): 547-553.

CHEN LH & THACKER RR (1985). Effect of dietary vitamin E and high supplementation of vitamin C on plasma glucose and cholesterol levels. Nutrition Research, (5): 527-531.

CHEN RS, WU PL & CHIOU RY (2002). Peanut roots as a source of resveratol. Journal of Agriculture Food Chemistry, 50(60): 1665-1669.

CHRISTMAS TJ, CHAPPIE CR, NOBBLE JG, MILORY EJ & COWIE AG (1988). Hyperparathyroidism after neck irradiation. British Journal of Surgery, 75(9): 873-874.

CHUPUKCHAROEN N, HOMARATAT P & WILAIRAT P (1985). Effects of vitamin E deficiency on the distribution of cholesterol in plasma lipoproteins and the activity of cholesterol 7-hydroxylare in the rabbit liver. Journal of Nutrition, 115: 468-472.

COLMAN E, HEDIN R, SWANN J & ORLOFF D (2002). A brief history of Calcitonin. Lancet, 359: 885-886.

ENDO K, KATSUMATA K, HIRATA M, MASSAKI T, KUBODERA N, NAKAMAURA T, IKEDA K & OGATA E (2000). 1,25-dihydroxy vitamin ds as well as its analogue OCT lower blood calcium through inhibition of bone resorption in hypercalcemic rats with continous parathyroid hormone-related peptide infusion. Journal of Bone Mineral Research, 1: 175-181.

FAHIM FA, RUSHDY HM, YOUSRI RM & ABADY MI (1993). Some biochemical aspects of the protective effect of strontium chloride on gamma irradiated rats. Biometals, 6(3): 163-170.

FARAG EK (1999). Interaction between supplemented selenium and/or vitamin E and Mn, Zn, Fe and Cu in schistosoma infected mice. Journal of Egyptian Society of Parasitolog, 29(2): 517-529.

FELDMAN BE (1999). Assorted monounsaturated fatty acids promote healthy hearts. American Journal of Clinical Nutrition, 70: 953-954.

FILIPOV ZH, BODUROV N & BOCHUKOV A (1991). Effect of UV-irradiation autologous blood on various morphological biochemical blood indices and phagocytic activity of the leukocytes in cattle. Experimental Hematology, 19(8): 742-748.

FUJIWARA S, SPOSTO R, SHIRAKI M, YOKOYAMA N, SASAKI H, KODAMA K & SHIMAKOKAK (1994). Levels of parathyroid hormone and calcitonin in serum among atomic bomb survivors. Radiation Research, 137: 96-103.

FUKUDA S & LIDA H (1999). Effect of clinostat-microgravity and heavy particle radiation in bone and calcium metabolism in rats. Journal of Japanese Bone Morphormetry, 9: 35-44.

GERACI JP, MARIANO MS & JACKSON KL (1991). Hepatic radiation injury in rat. Radiation Research, 125(1): 65-72.

GLASCOTT PA, GILFOR E, SERRONI A & FARBER JL (1996). Independent antioxidant action of vitamins E and C in cultured rat hepatocytes intoxicated with allyl alcohol. Biochemical Pharmacology, 52: 1245-1252.

HAMILTON SA, PECAUT MJ, GRIDLEY DS, TRAVIS ND, BANDSTRA ER, WILLEY JS, NELSON GA & BATEMAN TA (2006). A murine model for bone loss from therapeutic and space-relevant sources of radiation. 1: Journal of Applied Physiology, 101(3): 789-793.

HIZHNYAK SV (1997). The effect of x-ray irradiation on ionized calcium concentration in Enterocytes of rat small intestine. Journal Data of Scientific Research, 4: 101-105.

HULTER HN & PETERSON JC (1984). Renal and Systemic magnesium metabolism during chronic continuous parathormone infusion in normal subjects. Metabolism, 33: 662-666.

HURWITZ S (1996). Homeostatic control of plasma calcium concentration. Critical Reviews in Biochemistry and Molecular Biology, 31(1): 41-100.

JUAN ME, VINADRADELL MP & PLANASS JM (2002). The daily oral administration of high doses of transresveratol to rats for 28 days is not harmful. Journal of Nutrition, 132(2): 257-260.

KHOSLA S, MELTOO L3RD & RIFTGFC$_R$ BX (1999). Osteoporosis: gender differences and similarities. Lupus, 8(5): 393-396.

KIND PRN & KING EJ (1954). Estimation of plasma phosphatase by determination of hydrolysed phenol with amino antipyrine. Journal of Clincal Pathology, 7: 322-326.

KUMAR M, SHARMA KM, SAXENA SP & KUMAR A (2003). Radioprotective effect of panax gingseng on the phosphatases and lipid peroxidation level in testes of Swiss Albino Mice. Biological Pharmacy Bulliten, 26(3): 308-312.

LAI H & SINGH N (1996). Single and double strand DNA Breaks in rat brain cells after acute exposure to radio-frequency electromagnetic radiation. International Journal of. Radiation Biology, 69(4): 513-521.

LIESEGANG A, EICHER R, KRAENZLIN M, RUSCH P, WANNER M & RRIOND JI (1998). Determination of bone markers in dairy cows with parturient paresis. Schweizer Archiv Für Tierheilkunde, 140(10): 405-411.

MIZUTANI K, IKEDA K, KAWAI Y & YAMORI Y (1998). Resveratrol stimulates the proliferation and differentiation of osteoblastic MC_3 Ts-Ei cells. Biochemical and Biophysical Research Community, 253(3): 859-863.

MURAYAMA AM, MAKEYOMA K, KITANAKA S, KODERA Y, KAWAGUCHI Y, HOSOYA T & KATO S (1999). Positive and negative regulations of renal 25 hydroxyvitamin D_3 1 alpha hydroxylase gene by parathyroid hormone, calcitonin, and $1,25(OH)_2$ D_3 in intact animals. Endocrinology, 140(5): 2224-2231.

NATH KA, FISCHEREDER M & HOSTETTER TH (1994). The role of oxidants in progressive renal injury. Kidney International supplement, 45: 11-15.

NATH RL(1996). A text book of medical biochemistry, new age international limited. Publishers: 416-540.

RUDE RK, ADAMS JS, RYZEN E, ENDRES DB, NIIMI H, HOREST RL, HADAD JGJR & SINGER FR (1985). Low serum concentrations of 1.25-dihydroxyvitamin D in human magnesium deficiency. Journal of Clinical Endocrinology and Metabolism, 61(5): 933-940.

SHFRANOVSKAIA EV (2002). Effect of gamma-irradiation and thyro-parathyroidectomy on structural-functional state of membranes of sarcoplasmic reticulum of skeletal muscle. Radiatsionnia Biologiia Radioecologiia, 42(l): 44-47.

SOTORNIK I (1997). Relation between parathormone and 1,25-dihydroxy vitamin D_3 in chronic Kidney failure. Vnitrni Lekarstvi, 43(9): 616-619.

SRINIVASAN M, SUDHEER AR, PILLAIL KR, KUMAR PR, SUDHAKARAN PR, MENON VP (2006). Influence of ferulic acid on gamma-radiation induced DNA damage, lipid peroxidation and antioxidant status in primary culture of isolated rat hepatocytes. Toxicology, 228(2-3): 249-258.

STEPHAN J, HAVRANEK T & JOJKOVA K (1977). "Serum alkaline phosphatase as indicators of radiation damage in rats. Radiation Research, 70: 406-414.

TENG TY & MOFFAT K (2000). Primary radiation damage of protein crystals by an intense synchrotron X- ray beam. Journal of Synchrotron Radiation, 7: 313-317.

UPASANI CD & BALARMAN R (2001). Effect of vitamin E, vitamin C and spirulina on the levels of membrane bound enzymes and lipids in some organs of rats exposed to lead. Indian Journal of Pharmacology, 33: 185-191.

YANG S, MDYASTHA P & BINGEL (2001). A new superoxide Generating oxidase in murine osteoclasts. Journal of Biological Chemistry, 276(8): 5452-5458.

ZOFOKOVA I & KANCHEVA RL (1995). The relation between magnesium and calciotropic hormones. Magnesium Research, 8: 77-84.

Received: May 16, 2007
Accepted: January 4, 2008

Length-weight and length-length relationships of the bogue *Boops boops* (Linneaus, 1758) in Izmir Bay (Aegean Sea of Turkey)

Ali Kara* & Bahar Bayhan

*** Ege University, Faculty of Fisheries, Department of Fishing and Processing Technology, 35100 Bornova-Izmir, Turkey**
Ege University, Faculty of Fisheries, Department of Hydrobiology, 35100 Bornova, Izmir-Turkey.

Corresponding author : * Ali Kara, Ege University, Faculty of Fisheries, Department of Fishing and Processing Technology, 35100 Bornova-Izmir, Turkey; Tel: 00 90 232 3884000/1302; Fax: 00 90 232 3883685; e-mail (1): ali.kara@ege.edu.tr; e-mail (2): bahar.bayhan@ege.edu.tr

ABSTRACT. A total of 1190 specimens of bogue, *Boops boops*, were collected by gillnet and trammel net fishing between January 2005 and December 2005 in Izmir Bay (Aegean Sea of Turkey). Fish size in total length ranged from 9.2cm (minimum) in September to 27.6cm (maximum) in January. The length-weight relationships were determined for males, females, hermaphrodites and combined sexes as $W=0.0021L^{3.522}$, $W=0.0044L^{3.272}$, $W=0.0037L^{3.350}$ and $W=0.0035L^{3.419}$ respectively. The results indicated further that the length-length relationships were highly correlated ($r^2>0.990$, $P<0.001$).

KEY WORDS : *Boops boops,* length-weight relationship, length-length relationship, Izmir Bay, Aegean Sea.

INTRODUCTION

The length-weight (LWR) and length-length (LLR) relationships have been applied for basic uses for assessment of fish stocks and populations (RICKER, 1968). The length-weight relationships also helps to figure out the condition, reproduction history, life history and the general health of fishing species (NIKOLSKY, 1963; WOOTTON, 1992; PAULY, 1993; ERKOYUNCU, 1995; AVSAR, 1998) and is also useful in local and interregional morphological and life historical comparisons in species and populations.

It is necessary to use standard measures for all populations to render the results more reliable when making comparisons between populations. Therefore, the length-length relations of species under various environmental conditions should be known. The length-length relationship is also of great importance for comparative growth studies (MOUTOPOULOS & STERGIOU, 2002). In fisheries studies, fish length can often be measured more rapidly and easily than mass. The knowledge of the length-weight relationship makes it easier to determine the mass where only the length is known. In the field, the tail flukes are often cut, which makes it difficult to measure the total length accurately. Knowing the standard length will enable us figure out the total length.

The aim of the present study is to determine the length-weight and length-length relationships of females, males and hermaphrodites of *Boops boops,* the species caught in Izmir Bay in 2005.

MATERIALS AND METHODS

The samples were obtained monthly during commercial fishing trials conducted with gillnets and trammel nets during 2005 in Izmir Bay (Fig. 1).

The specimens were taken from commercial boats, kept in wooden boxes and brought to the laboratory as soon as possible (within two hours). Fish were measured in the laboratory for total length (TL), fork length (FL) and standard length (SL) to the nearest cm and weighed (W, wet weight) to the nearest g.

The length-weight relationships for weight were calculated using the equation, $W=aL^b$ (RICKER, 1979) where a is a coefficient related to body form and b is an exponent indicating isometric growth when equal to 3. The statistical significance level of r^2 was estimated by linear regressions on the transformed equation, LogBW=loga +b.logSL. Moreover, (1) TL vs FL; (2) FL vs SL; and (3) SL vs TL relationships were calculated by linear regressions. In order to test for likely significant differences in both slope and intercept, covariance analysis was performed. All statistical analyses were evaluated at $P<0.05$ significance level.

RESULTS AND DISCUSSION

A total of 1190 individuals were sampled during the study period. The shortest individual, 9.2cm, was obtained in September and the longest, 27.6cm TL, in January. It was determined that 54% of the samples were females (n=640), 43% males (n=516) and 3% hermaphrodites (n=34).

Seasonal length-weight relationships for males, females, hermaphrodites and the total sample population were determined as $W=0.0021L^{3.522}$, $W=0.0044L^{3.272}$, $W=0.0037L^{3.350}$ and $W=0.0035L^{3.419}$ respectively (Table 1).

Analysis of covariance revealed significant differences between sexes for the slopes (b) of the regression lines ($P<0.001$).

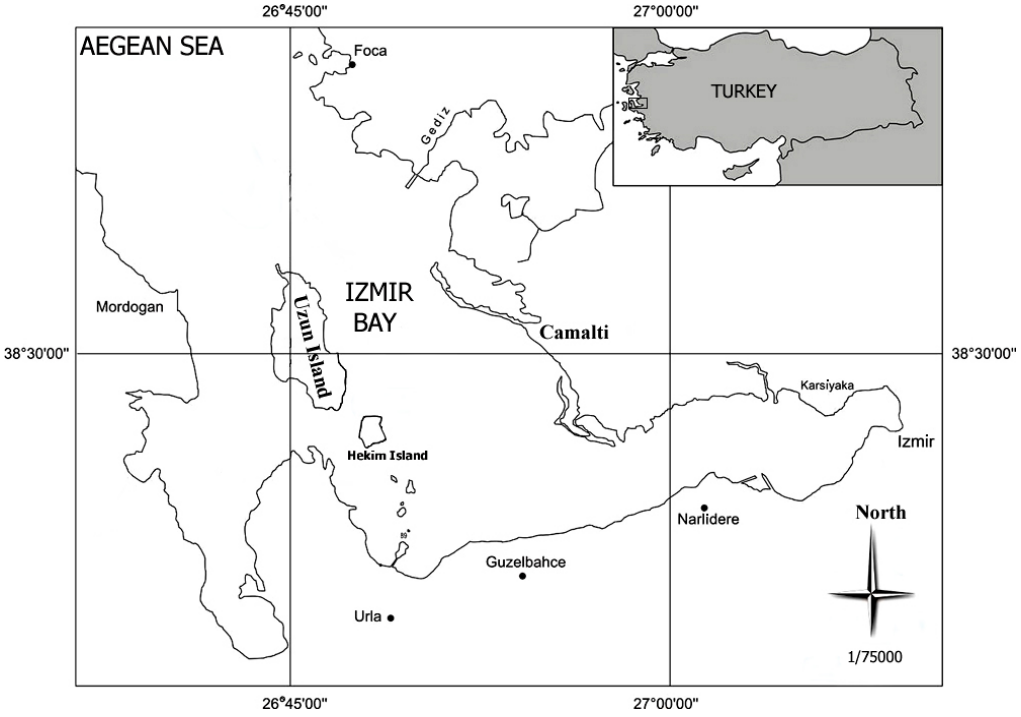


Fig. 1. – Study area (Izmir Bay)

TABLE 1

Monthly descriptive statistics and estimated parameters of length-weight relationships for both sexes of *Boops boops* in Izmir Bay (Aegean Sea) from January 2005 to December 2005 (M: male, F: female, H: hermaphrodite, A: all sexes, n: number of individuals, a: intercept, b: slope, CI: confidence limits, r^2: coefficient of determination).

Months	Sex	n	Length characteristics		Weight characteristics		Relationship parameters			
			TL Range (cm)	Mean TL (±SD)	W Range (g)	Mean W (±SD)	a	b	SE of b	r^2
January	M	20	22.0-27.0	24.42±1.63	150.35-243.96	191.68±33.38	0.0699	2.475	0.264	0.917
	F	40	21.8-27.6	24.20±1.42	149.99-250.21	184.44±27.91	0.1188	2.304	0.204	0.876
February	F	78	16.0-21.1	19.25±1.50	36.47-98.67	69.87±17.14	0.0062	3.145	0.136	0.935
March	M	180	13.6-22.9	19.14±2.20	22.44-117.68	69.87±22.26	0.0106	2.965	0.121	0.873
	F	96	15.0-21.6	19.21±1.80	32.11-123.92	72.72±23.90	0.0078	3.076	0.259	0.854
April	M	32	15.4-19.5	17.55±1.08	34.57-66.28	49.20±9.23	0.0118	2.901	0.010	0.889
	F	28	14.7-20.0	18.04±1.46	30.20-74.87	53.68±12.19	0.0124	2.887	0.159	0.965
May	M	40	22.2-26.6	24.65±1.07	151.96-281.67	260.64±26.96	0.0485	2.606	0.320	0.887
June	M	76	18.0-22.0	19.74±1.13	51.84-106.36	69.04±13.20	0.0088	3.002	0.182	0.884
	F	80	18.2-22.9	20.54±1.30	54.28-117.35	80.46±15.87	0.0142	2.854	0.179	0.870
July	M	14	18.4-23.5	21.26±1.80	69.88-128.65	97.31±23.75	0.0201	2.770	0.365	0.920
	F	126	11.3-23.3	17.79±2.72	12.10-132.86	60.70±27.92	0.0065	3.148	0.050	0.989
August	M	68	15.4-23.4	20.57±1.60	38.32-143.31	91.39±22.81	0.0059	3.184	0.149	0.935
	F	88	14.8-25.8	17.34±1.78	29.21-98.90	53.00±19.01	0.0046	3.263	0.070	0.979
	H	6	10.9-16.0	12.80±2.79	11.41-41.01	22.17±16.37	0.0044	3.298	0.080	0.999
September	F	48	15.6-23.3	19.56±2.39	38.48-124.17	77.06±27.28	0.0106	2.976	0.114	0.967
	H	8	9.2-18.2	14.35±3.76	7.18-57.30	33.24±20.52	0.0078	3.083	0.080	0.997
October	M	12	10.2-19.2	16.25±2.33	17.70-68.24	42.23±17.00	0.0101	2.973	0.157	0.989
	F	30	15.2-20.2	17.01±1.40	29.73-82.60	48.25±15.39	0.0024	3.487	0.198	0.960
	H	20	15.3-19.7	16.59±1.21	32.10-69.65	43.68±10.24	0.0122	2.909	0.113	0.938
November	M	38	18.3-25.6	22.03±2.35	54.78-169.26	118.08±35.62	0.0055	3.194	0.137	0.970
	F	18	17.1-26.7	22.07±3.79	46.69-191.75	117.10±62.47	0.0037	3.316	0.178	0.980
December	M	36	16.0-24.5	20.97±2.57	35.99-148.33	93.82±32.65	0.0057	3.177	0.080	0.991
	F	8	15.5-24.2	21.43±3.99	32.83-145.87	106.92±50.52	0.0033	3.363	0.020	0.999
Overall	M	516	13.6-27.0	20.28±2.69	17.70-281.67	91.41±48.80	0.0021	3.522	0.062	0.926
	F	640	11.3-27.6	19.14±2.73	12.10-261.76	75.62±40.83	0.0044	3.272	0.044	0.947
	H	34	9.2-24.9	16.84±4.30	7.18-222.75	62.82±40.28	0.0037	3.350	0.089	0.988
	A	1190	9.2-27.6	19.55±2.86	7.18-281.67	81.74±45.63	0.0035	3.419	0.016	0.948

Monthly LWRs of *Boops boops* presented in Table 1 show that the calculated allometric coefficients vary between 2.475 (January) and 3.194 (November) in males, between 2.304 (January) and 3.487 (October) in females and between 2.909 (October) and 3.298 (August) in hermaphrodites. All LLRs presented in Table 2 were highly significant (P<0.001), with all coefficient of determination values being greater than 0.990.

The LWR can be obtained from the length and weight measurements of the same fishes throughout their lives or from a sample of fish taken at a given time (WOOTTON, 1990). The parameters of the fish, LWRs are affected by a series of factors including season, habitat, gonad maturity, sex, diet, stomach fullness, health and preservation techniques (TESCH, 1971; BAGENAL & TESCH, 1978; HOSSAIN et al., 2006).

All allometric coefficients (b) estimated in this study were within the expected range 2.3-3.5, and according BAGENAL & TESCH (1978); KOUTRAKIS & TSIKLIRAS (2003), allometric coefficients (b) may range from 2 to 4.

There have been some other studies on the length-weight and length-length relationships of *B.boops* L., in Turkish Seas and other localites (MENNES, 1985; ALGERIA-HERNANDEZ, 1989; MERELLA et al., 1997; GONÇALVES et al., 1997; ABDALLAH, 2002; VALLE et al., 2003; OZAYDIN & TASKAVAK, 2006; KARAKULAK et al., 2006) and the b values reported in these studies are presented in Table 3.

The values of b found in studies conducted on *B. boops* in Turkish Seas indicate positive allometry of growth. Table 3 shows that in other parts of the Mediterranean Sea fish of this species exhibit isometric growth values as well as those positively approaching isometry (more positively).

TABLE 2

Length-length relationships between total length (TL), fork length (FL) and standard length (SL) of *Boops boops* in Izmir Bay (Aegean Sea) from January 2005 to December 2005 (n: number of individuals, a: intercept, b: slope, r^2: coefficient of determination).

Sex	Equation	n	a	b	r^2
	TL=a+bFL		-0.2165	1.1362	0.991
Male	FL=a+bSL	516	0.9006	1.0152	0.994
	SL=a+bTL		-05657	0.8616	0.990
	TL=a+bFL		-0.0405	1.1277	0.995
Female	FL=a+bSL	640	0.5915	1.0332	0.997
	SL=a+bTL		-0.7071	0.8683	0.992
	TL=a+bFL		-0.1872	1.1339	0.997
Hermaphrodite	FL=a+bSL	34	0.6199	1.0255	0.999
	SL=a+bTL		-0.4361	0.8595	0.999
	TL=a+bFL		-0.0877	1.1299	0.995
All	FL=a+bSL	1190	0.6866	1.0270	0.997
	SL=a+bTL		-0.6295	0.8644	0.994

TABLE 3

Length-weight relationships of *Boops boops* L. from different localities.

Author(s)	Area	Sex	Length range (cm)	Length type	a	b
MENNES, 1985	Western Sahara, Morocco	unsexed	-	-	0.0145	3.000
ALGERIA-HERNANDEZ, 1989	Central Adriatic Sea	female	13.50-23.00	TL	0.0056	3.088
		male	12.80-22.30	TL	0.0087	3.000
DJABALI et al.,1993	Bou-Ismail, Algeria	unsexed	-	-	0.0097	3.000
PETRAKIS & STERGIOU, 1995	G.S. Evvoikos, Greece	mixed	9.60-24.30	FL	0.0149	3.093
MERELLA et al., 1997	Balearic Islands, Spain	unsexed	12.40-26.60	TL	0.0082	3.000
GONÇALVES et al., 1997	South coast, Portugal	unsexed	15.80-35.50	TL	0.0083	3.037
ABDALLAH, 2002	Alexandria, Egypt	unsexed	3.70-14.60	TL	0.0070	3.130
VALLE et al., 2003	East coast, Spain	unsexed	9.70-16.70	SL	0.0161	2.812
KARAKULAK et al., 2006	Gökceada, Turkey	mixed	10.20-32.10	TL	0.0048	3.258
		female	15.40-32.10	TL	0.0032	3.390
		male	15.30-27.60	TL	0.0074	3.116
OZAYDIN & TASKAVAK, 2006	Izmir Bay, Turkey	mixed	10.70-23.50	FL	0.0003	3.033
This study	Izmir Bay, Turkey	mixed	9.20-27.60	TL	0.0035	3.419
		female	11.30-27.60	TL	0.0044	3.272
		male	13.60-27.00	TL	0.0021	3.522
		hermaphrodite	9.20-24.90	TL	0.0037	3.350

REFERENCES

Abdallah M (2002). Length-weight relationship of fishes caught by trawl off Alexandria, Egypt. Naga, the ICLARM Quarterly, 25(1): 19-20.

Algeria-Hernandez V (1989). Study on the age and growth of bogue (*Boops boops* L.) from the central Adriatic Sea. Cybium, 13: 281-288.

Avsar D (1998). Fisheries biology and population dynamics. University of Cukurova, Faculty of Fisheries, Adana, Turkey, p. 303 (in Turkish).

Bagenal TB & Tesch FW (1978). Age and growth. In: Bagenal T (ed), Methods for assessment of fish production in fresh waters, 3 rd edn. IBP Handbook No. 3, Blackwell Science Publications, Oxford, 101-136.

Djabali F, Mehailia A, Koudil M & Brahmi B (1993). Empirical equations for the estimation of natural mortality in Mediterranean teleosts. Naga, the ICLARM Quarterly, 16(1): 35-37.

Erkoyuncu I (1995). Fisheries biology and population dynamics. University of Ondokuz Mayis, Faculty of Fisheries, Sinop, Turkey, p. 256 (in Turkish).

Gonçalves JMS, Bentes L, Lino PG, Ribeiro J & Canario AVM (1997). Weight-length relationships for selected fish species of the small-scale demersal fisheries of the south and south and southwest coast of Portugal. Fisheries Research, 30: 253-256.

Hossain MY, Ahmed ZF, Leunda PM, Roksanul Islam AKM, Jasmine S, Oscoz J, Miranda R & Ohtomi J (2006). Length-weight and length-length relationships of some small indigenous fish species from the Mathabhanga River, southwestern Bangladesh. Journal of Applied Ichthyology, 22: 301-303.

Karakulak FS, Erk H & Bilgin B (2006). Length-weight relationships for 47 coastal fish species from the northern Aegean Sea, Turkey. Journal of Applied Ichthyology, 22: 274-278.

Koutrakis ET & Tsikliras AC (2003). Length-weight relationships of fishes from three northern Aegean estuarine systems (Geece). Journal of Applied Ichthyology, 19: 258-260.

Merella P, Quetglas A, Alemany F & Carbonell A (1997). Length-weight relationship of fishes and cephalopods from the Balearic Islands (western Mediterranean). Naga, the ICLARM Quarterly, 20(3/4): 66-68.

Mennes F (1985). Multispecies assessment of fish stocks off the western Sahara region with emphasis on the family Sparidae. Fishbyte, 3(3): 5-10.

Moutopoulos DK & Stergiou KI (2002). Length-weight and length-length relationships of fish species from the Aegean Sea (Greece). Journal of Applied Ichthyology, 18: 202-203.

Nikolsky GW (1963). The ecology fishes. Academic Press, London and New York. 352 pp.

Ozaydin O & Taskavak E (2006). Length-weight relationships for 47 fish species from Izmir Bay (eastern Aegean Sea, Turkey). Acta Adriatica, 47(2): 211-216.

Pauly D (1993). Fishbyte section editorial. Naga, the ICLARM Quarterly, 16: 26.

Petrakis D & Stergiou KI (1995). Weight-length relationships for 33 fish species in Greek waters. Fisheries Research, 21: 465-469.

Ricker WE (1968). Methods for Assessment of Fish Production in Freshwaters. Blackwell Scientific Publications, Oxford. 313 p.

Ricker WE (1979). Growth rates and Models. In: Hoar WS, Randall DJ & Brett JR (eds), Fish Physiology Vol. VIII, Bioenergetics and Growth, Academic Press, 677-743.

Tesch W (1971). Age and growth. In: Ricker WE (ed), Methods for assessment of fish production in fresh waters, 2nd edn. International Biological Programme, Oxford and Edinburgh, 97-130.

Valle C, Bayle JT & Ramos AA (2003). Weight-length relationships for selected fish species of the western Mediterranean Sea. Journal of Applied Ichthyology J, 19: 261-262.

Wootton RJ (1990). Ecology of teleost fishes. Chapman and Hall, London.

Wootton JT (1992). Indirect effect, prey susceptibility, and habitat selection: impacts of birds on limpets and algae. Ecology, 73(3): 981-991.

Received: June 8, 2007
Accepted: November 15, 2007

Belg. J. Zool., 138 (2) : 158-169 July 2008

Croissance et variations saisonnières de la composition en acides gras de l'huître *Crassostrea gigas* cultivée dans la lagune de Bizerte, Tunisie

Salwa Dridi[a,b*], Mohamed Salah Romdhane[b] & M'hamed El Cafsi[a]

[a] **Faculté des Sciences de Tunis, Département de Biologie, Unité de Physiologie et d'Ecophysiologie des Organismes Aquatiques, Campus Universitaire, 2092, El Manar II, Tunis, Tunisia.**

[b] **Institut National Agronomique de Tunisie, Département des Sciences de la Production Animale et de la Pêche, Unité d'écosystèmes et ressources aquatiques, 43 Av. Charles Nicole, 1082 Tunis, Tunisia.**

Corresponding author : * salwadridi@yahoo.fr

RÉSUMÉ. Ce travail, réalisé de janvier 2002 à janvier 2003, a pour principal objectif de suivre la croissance d'une population de l'huître creuse *Crassostrea gigas* dans la lagune de Bizerte en parallèle à, d'une part, la détermination de la composition en acides gras de l'ensemble gonade-glande digestive de chaque individu et, d'autre part, l'analyse des paramètres physiques du milieu d'échantillonnage. La séparation entre les individus en périodes d'activité gonadique (de mars à septembre) et les individus en repos sexuel (novembre à février) déduite d'une étude parallèle sur *C. gigas* de la lagune de Bizerte, nous a permis d'observer l'effet du cycle sexuel sur la croissance linéaire et pondérale. Ainsi, nous avons remarqué que durant le repos sexuel, la corrélation entre les différents paramètres des modèles de croissance est plus étroite que celle correspondant au reste du cycle. L'accélération de la croissance durant la phase d'inactivité sexuelle, est démontrée par l'augmentation de la longueur, de la largeur et de l'épaisseur de la coquille, respectivement de 2.04cm±0.18; 0.58cm±0.08 et 0.9cm±0.06 de janvier 02 à février 02 et 1.25cm±0.33; 0.65cm±0.11 et 0.28cm±0.07 de novembre 02 à janvier 03. L'accélération de la croissance durant le repos sexuel est également déduite du profil d'acide gras déterminé sur l'ensemble gonade-glande digestive. Ce dernier montre des teneurs maximales en acides gras totaux (7.97±1.31% de chair sèche) en automne. De plus, les acides gras saturés (représentés majoritairement par le C16:0), monoinsaturés [représentés majoritairement par C16:1(n-7), C18:1(n-7) et C20:1(n-7)] et polyinsaturés [représentés majoritairement par C20:5 (n-3) et C22:6 (n-3)] montrent des valeurs maximales durant le repos sexuel du bivalve (automne et hiver) dont les valeurs sont respectivement de 26.38%±1.78; 15.84%±0.56 et 65.22%±15.49 des acides gras totaux. Ces acides gras essentiels représentent dans ce cas une source d'énergie majeure pour le bivalve lui permettant une croissance remarquable des valves en longueur, largeur et épaisseur durant cette phase du cycle sexuel. L'augmentation des teneurs en acides gras durant le repos sexuel de *C. gigas* est favorisée par l'élévation de la production photosynthétique automnale (1.2µg/L).

MOTS CLÉS : *Crassostrea gigas*, croissance, acides gras, chlorophylle a, cycle sexuel.

Growth and seasonal variations of fatty acid composition of the Pacific oyster *Crassostrea gigas* from the Bizert lagoon, Tunisia

ABSTRACT. Growth and fatty acid composition of the gonad-digestive gland of the oyster *Crassostrea gigas* was studied from January 2002 to January 2003. Different measurements of oyster shell length and weight were discussed in relation to the environmental conditions, especially food availability. Separation between oysters in the two phases of the gametogenic cycle demonstrated that during sexual resting, the correlation between the various growth parameters is higher than those obtained during the gonadal activity, including ripeness and spawning. The total lipids and seasonal variations in the fatty acids of the gonad-visceral mass of the cupped oyster suggests a cycle of energy storage and utilization in the Bizert lagoon. Total fatty acid content (7.97±1.31–4.87±1.60% of dry tissue) varied significantly during the year, reaching the highest value in autumn in coincidence with the increase of nutrient availability demonstrated by the elevation in Chlorophyll a level in November. However the lowest values of total fatty acids occurred in summer which corresponded to the intense gametogenic activity and the decrease of the correlations observed. The 16:0, 18:1(n-7), 18:2(n-6), 18:3(n-3), 20:5(n-3) and 22:6(n-3) fatty acids predominated and presented maximal levels during winter and autumn (sexual resting stage) and contributed to the increasing shell length, width and thickness (2.04±0.18cm; 0.58±0.08cm and 0.90±0.06cm from January 02 to February 02 and 1.25±0.33cm; 0.65±0.11cm and 0.28±0.07cm from November 02 to January 03, respectively. *Crassostrea gigas* was characterized by a relatively high n-3/n-6 PUFA ratio (2.45–3.15) which was particularly high in winter. Fluctuations in the fatty acid composition of oysters between seasons are dependent upon the gonadic stages, temperature and the dietary resources available in their immediate habitat.

KEY WORDS : *Crassostrea gigas*, growth, fatty acids, chlorophyll a, gametogenic cycle.

INTRODUCTION

L'ostréiculture en Tunisie est une activité récente. L'huître creuse *Crassostrea gigas* (Thunberg, 1793) est exploitée dans la lagune de Bizerte depuis 1972 suite à l'importation de naissains du Japon puis de France (MEDHIOUB, 1993). La production d'huîtres en Tunisie a augmenté durant ces dernières années: elle est passée d'une tonne en 2000 à deux tonnes en 2001 et 16 tonnes en 2003 représentant ainsi 0.14% de la production totale des mollusques en Tunisie (FAO, 2006). L'ostréiculture tunisienne dépend, jusqu'à nos jours, de l'importation de naissains de l'étranger, ce qui représente un handicap économique majeur. Ce travail constitue une première contribution à l'étude de la croissance de *C. gigas* en fonction des variations saisonnières de ses teneurs en acides gras et de son cycle sexuel, afin de cibler les périodes durant lesquelles *C. gigas* de la lagune de Bizerte est vulnérable et sa résistance aux stress environnementaux plus limitée.

GIESE (1969), BAYNE (1976) et SASTRY (1979) ont montré que le stockage et l'utilisation des réserves lipidiques varient selon les espèces mais aussi selon les individus d'une même espèce. Par ailleurs, GABBOTT (1983); RUIZ et al. (1992); PAZOS et al. (1997); BERTHELIN et al. (2000) et OJEA et al. (2004) ont précisé que ces variations résultent des interactions complexes entre les conditions du milieu (notamment sa richesse trophique) et la physiologie des bivalves (notamment la croissance et la reproduction).

Considérant ces paramètres, nous nous sommes attachés, dans ce travail, à suivre la croissance de l'huître *C. gigas* en parallèle avec le suivi saisonnier de la composition en acides gras de l'ensemble gonade-glande digestive des individus analysés. Cette étude a été complétée par la détermination du cycle gamétogénique des huîtres et des paramètres physiques du milieu d'élevage.

MATÉRIEL ET MÉTHODES

1. Site et stratégie de prélèvement

Le site d'étude est localisé dans la lagune de Bizerte, située au nord de la Tunisie (Fig. 1). Les échantillons d'huîtres, cultivées en suspension dans la colonne d'eau, sont prélevés (N=40) mensuellement durant la période allant de janvier 2002 à janvier 2003, sur des cordes suspendues dans la ferme marine de Bizerte (FMB; 37° 09.38' N et 9° 53.55' E) (Fig. 1). Au début de l'expérimentation les huîtres âgées de 12 mois mesuraient 6.69cm±0.75 (longueur antéro-postérieure) et pesaient 30.94g±10.82.

2. Paramètres physico-chimiques

Les mesures de température et de salinité sont effectuées *in situ* tous les mois, à 1 mètre de profondeur dans le milieu d'échantillonnage des huîtres, respectivement à l'aide d'un thermomètre électronique de précision graduée à 1/10ème°C et d'un salinomètre du type WTW.LF 191 préalablement étalonné à l'eau de mer standard. Les mesures de la chlorophylle a sont réalisées tous les mois selon la méthode fluorimétrique qui consiste à mesurer la fluorescence avant (F_O) et après acidification (F_a) d'un extrait méthanolique du pigment (AMINOT & CHAUSSEPIED, 1983).

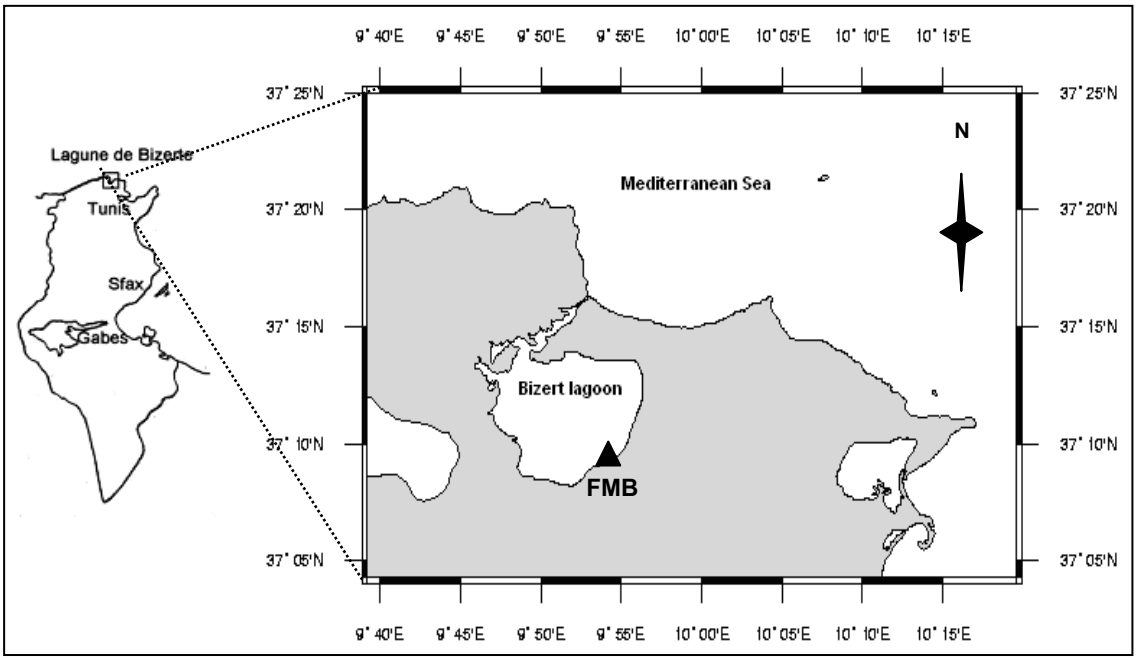


Fig. 1. – Localisation géographique du site d'étude (Ferme Marine de Bizerte, FMB) dans la lagune de Bizerte (▲).

3. Étude de la croissance

Les différentes mesures de taille ont été effectuées à l'aide d'un pied à coulisse avec une précision de 1/ 100ème cm, et les pesées au moyen d'une balance électronique de précision à 0.001g près.

Cette étude ayant pour objectif d'établir les relations allométriques liant les paramètres métriques et pondéraux, les paramètres suivants ont été déterminés pour chaque individu:

- Longueur (L): Dimension séparant le bord antérieur du bord postérieur.
- Largeur (l): Dimension séparant le bord dorsal du bord ventral.
- Epaisseur (E): Dimension donnée par la convexité des deux valves réunies.
- Masse totale fraîche (Ptot): Masse de l'individu entier dont la coquille a été débarrassée des sédiments et des organismes épibiontes.
- Masse de la chair fraîche (Pchf): Masse viscérale fraîche égouttée pendant au moins 30 minutes sur papier filtre.
- Masse de la chair sèche (Pchs): Masse viscérale après dessiccation pendant 72 heures à l'étuve à 60°C.
- Masse de la coquille sèche (Pcoqs): Masse de la coquille séchée (60°C pendant 72h).

Les relations allométriques obtenues ont obéi à la loi d'allométrie suivante:

$$Y=aX^b$$

Où:

- Y est la variable aléatoire dépendante représentant la dimension ou le poids d'une partie ou de la totalité du corps.
- X est la variable dépendante représentant le paramètre de référence.
- a est une constante caractéristique de l'espèce représentant l'indice de l'origine ou coefficient de condition c'est-à-dire la valeur de Y quand X=1.
- b est un coefficient d'allométrie ou coefficient de croissance. Il représente la pente dans la forme linéarisée de l'équation.

Cette équation est une fonction curviligne, pour la rendre linéaire nous la transformons en une relation logarithmique qui s'écrit sous la forme:

$$\text{Log } Y=\text{Log } a + b \text{ Log } X$$

4. Composition en acides gras

La composition en acides gras a été étudiée trimestriellement (n=6). Après dissection des huîtres et isolement de l'ensemble gonade-glande digestive, nous avons réalisé l'extraction des lipides totaux de ces tissus selon la méthode de Folch et al. (1957), modifiée par Bligh & Dyer (1959). L'obtention des esters méthyliques d'acides gras a été effectuée selon le protocole de Metcalfe et al. (1966). Les esters méthyliques sont analysés par chromatographie en phase gazeuse à l'aide d'un chromatographe HP modèle 4890 D équipé d'une colonne capillaire HP Innowax de 30m X 0.25mm de diamètre interne et dont l'épaisseur du filon est de 0.25µm. L'étalon interne utilisé est un acide gras à nombre impair de carbones absent dans nos échantillons. L'identité de chaque acide gras représenté par un pic sur les chromatogrammes expérimentaux est obtenue par comparaison avec une analyse dans les mêmes conditions d'un mélange témoin d'acides gras (esters méthyliques standard PUFA (n-1) de SUPELCO).

5. Analyses statistiques

Des données obtenues à partir des mesures de coquille ont été employées pour déterminer des rapports morphométriques des axes de croissance de la coquille en longueur, en largeur et en épaisseur dont les mesures ont subi une transformation logarithmique. Pour les rapports allométriques, nous avons utilisé les tests de régression linéaire pour l'obtention d'équations descriptives et la qualité de l'ajustement a été évaluée par le coefficient de corrélation (r). Les rapports de croissance allométriques entre d'une part la longueur, l'épaisseur et la largeur et d'autre part le poids total frais ont été estimés en utilisant la régression linéaire. La qualité de l'ajustement a été décrite en utilisant le coefficient de corrélation (r), (Sokal & Rohlf, 1969). Le test t de Student et les tests de comparaisons multiples de Duncan à $P<0.05$ ont été utilisés pour la comparaison des pentes des régressions linéaires.

Pour les données d'acides gras, les analyses de variance à deux facteurs ont permis de tester l'effet de la saison sur l'élévation ou la diminution des teneurs moyennes en acides gras. Ces dernières ont été comparées à l'aide du test de Duncan à $p<0.05$. Toutes les analyses statistiques ont été réalisées à l'aide du logiciel STATISTICA 6.0.

RÉSULTATS

Pendant la période d'étude, la température de l'eau mesurée dans le milieu d'élevage des huîtres varie entre un minimum de 10.9°C enregistré au mois de janvier et un maximum de 28°C (août) (Fig. 2). La température moyenne relevée s'établit à 19.7°C±5.8°C et les mesures de salinité oscillent entre 38‰ et 33‰. La salinité atteint son maximum suite aux évaporations estivales causées par l'accroissement thermique, puis la salinité diminue dès l'arrivée de la saison pluviale. La détermination de la concentration du milieu en chlorophylle a, a mis en évidence deux pics de production photosynthétique, un printanier au mois de mai (2.4µg/L) et un moins important automnal au mois de novembre (1.2µg/L). La moyenne calculée des douze mois de l'étude est de 0.7µg/L±0.6.

La croissance des huîtres a été étudiée en fonction du cycle sexuel de *C. gigas* qui, selon Dridi et al. (2006), est composé de 6 stades sur l'échelle de Lubet (1959). Ce cycle a commencé par le stade 0 (repos sexuel) qui s'est étendu de novembre à février, suivi du stade I (mars–avril 2002). La croissance des gamètes a été observée essentiellement en avril et la maturation gonadique, qui se compose des stades III A et III B, a débuté pour les huîtres de la lagune de Bizerte au mois de mai et s'est poursuivie jusqu'en août. Les émissions des gamètes (stade III D) ont été partielles au mois de Juin et totales au mois de septembre.

Les moyennes des mesures de tailles de *C. gigas* (longueur, largeur, épaisseur et poids total frais), ont varié considérablement durant la période de l'étude, allant de janvier 2002 à janvier 2003. Les différences de tailles calculées selon les différentes phases du cycle sexuel sont illustrées dans le Tableau 1. Nous avons observé deux diminutions remarquables du poids total frais aux mois de juin et septembre qui correspondent selon DRIDI et al. (2006) aux émissions gamétiques (Fig. 3).

Des relations allométriques significatives ont été mises en évidence pour les trois paramètres linéaires mesurés sur l'ensemble de la période d'étude (cycle annuel) (Tableau 2). En effet, les valeurs des coefficients de corrélation r de la période annuelle qui relient les différentes variables linéaires de l'huître sont comprises entre 0.57 ($p<0.05$) et 0.75 ($p<0.05$) témoignant d'une corrélation significative entre la longueur et la largeur, la longueur et l'épaisseur ainsi que la largeur et l'épaisseur. Pendant la phase d'activité sexuelle, les valeurs du coefficient de corrélation r calculées pour chaque modèle de croissance linéaire varient entre 0.10 (NS) et 0.22 ($p<0.05$) alors que durant le repos sexuel les valeurs de r augmentent et sont comprises entre 0.64 ($p<0.05$) et 0.82 ($p<0.05$).

Quelle que soit la période de l'année considérée, les relations allométriques reliant les différentes variables pondérales du bivalve à la longueur de la coquille (considérée comme taille de référence) se sont révélées significatives (Tableau 3). La séparation entre les individus en activité sexuelle et les individus au repos sexuel permet de comprendre l'effet du cycle sexuel sur les équations pondérales durant l'année de l'étude qui révèle des coefficients de corrélation r intermédiaires. Au repos sexuel, les valeurs de r sont maximales [0.65 ($p<0.05$) à 0.88 ($p<0.05$)] alors que durant l'activité sexuelle, ces valeurs diminuent notablement, de 0.15 ($p<0.05$) à 0.47 ($p<0.05$).

L'évolution saisonnière des acides gras totaux (% de la masse de la chair sèche) a montré des valeurs maximales enregistrées en automne (7.97±1.31) (Fig. 4). Le printemps est caractérisé par l'élévation du pourcentage des acides gras totaux dans le tissus sec de l'ensemble gonade-glande digestive (7.33±1.78) qui a diminué significativement ($p<0.05$) en été (4.87±1.60) (Fig. 4).

La dominance des acides gras polyinsaturés (AGPI, 57.93-65.22% des aides gras totaux) sur les acides gras saturés (AGS, 23.13-26.38% des aides gras totaux) et monoinsaturés (AGMI, 12.35-15.84% des aides gras totaux) a été mise en évidence durant l'année de l'étude (Fig. 5). Les variations des AGS ne se sont pas révélées significatives ($p<0.05$) durant la période d'étude. Les AGMI ont varié significativement ($p<0.05$) de l'été à l'automne et les AGPI du printemps à l'été ($p<0.05$).

Les acides gras polyinsaturés du type (n-3) constituent le groupe le plus important parmi les polyinsaturés dominés par l'acide eicosapentaenoïque (20:5 n-3, 10.68-12.15% des acides gras totaux) et l'acide docosapentaenoïque (22:6 n-3, 17.33-21.94% des acides gras totaux). Les acides gras saturés, essentiellement représentés par l'acide palmitique (16:0, 19.77-20.84% des acides gras totaux), constituent le deuxième groupe d'acides gras alors que l'ensemble des acides gras monoinsaturés, majoritairement représentés par 16:1(n-7), 18:1(n-7) et 20:1(n-7), n'ont pas représenté plus de 15.84% des acides gras (en automne).

Les valeurs maximales de l'acide palmitique sont enregistrées au printemps (20.84%±1.17 des acides gras totaux) et en automne (20.80%±3.95). La valeur maximale de C20:5 (n-3) est enregistrée en hiver (12.15±1.71% des acides gras totaux). Le pourcentage de l'acide docosahexaenoïque C22:6 (n-3) parmi les acides gras totaux a été maximale au printemps (21.94±1.73). Cet acide gras a diminué significativement ($p<0.05$) en été et a montré une augmentation significative ($p<0.05$) de nouveau en automne (21.22±1.10% des acides gras totaux) durant lequel les huîtres sont au repos sexuel. Les AGPI ont présenté les pourcentages maximums en hiver (65.22%±15.49 des acides gras totaux) et minimums en été (57.93%±13.69 des acides gras totaux). Le rapport AGPI (n-3)/AGPI (n-6) dans la chair de *C. gigas* ont présenté une valeur maximale en hiver (3.15) et minimale en été (2.45) (Tableau 4).

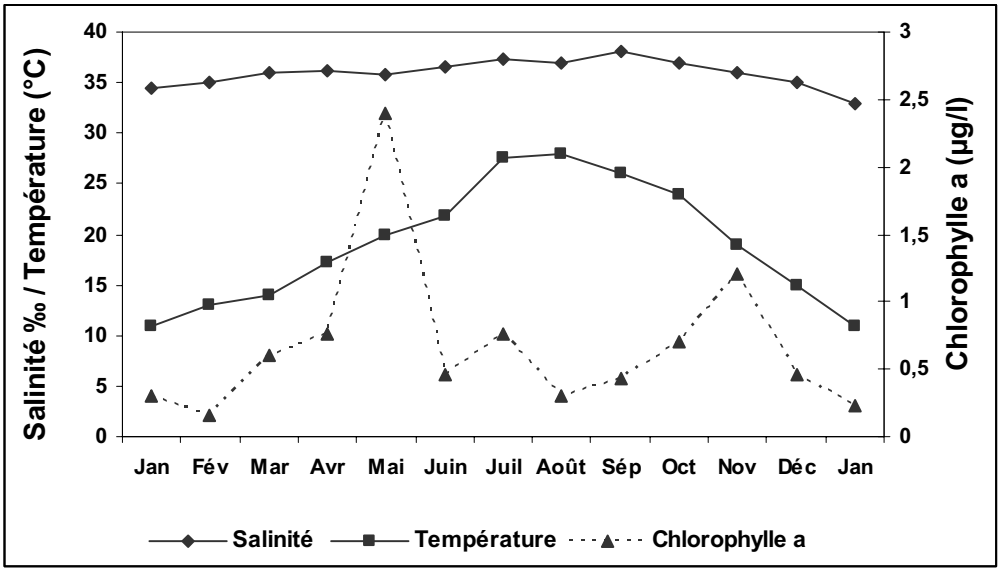
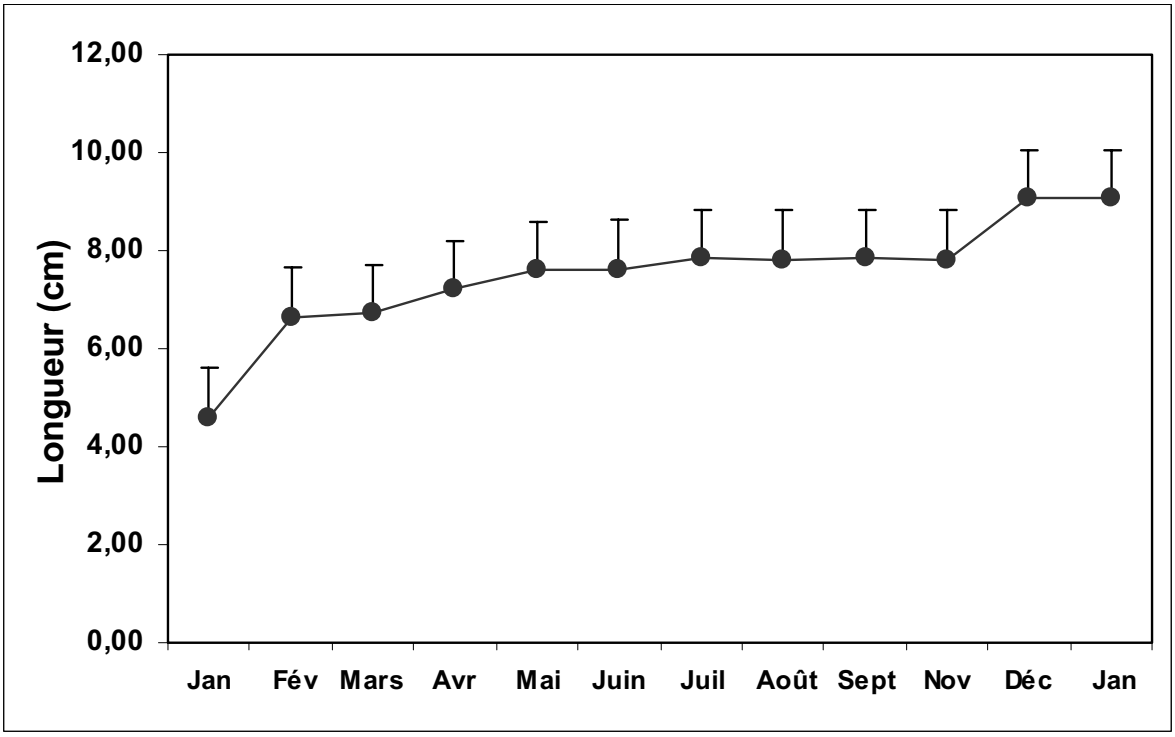
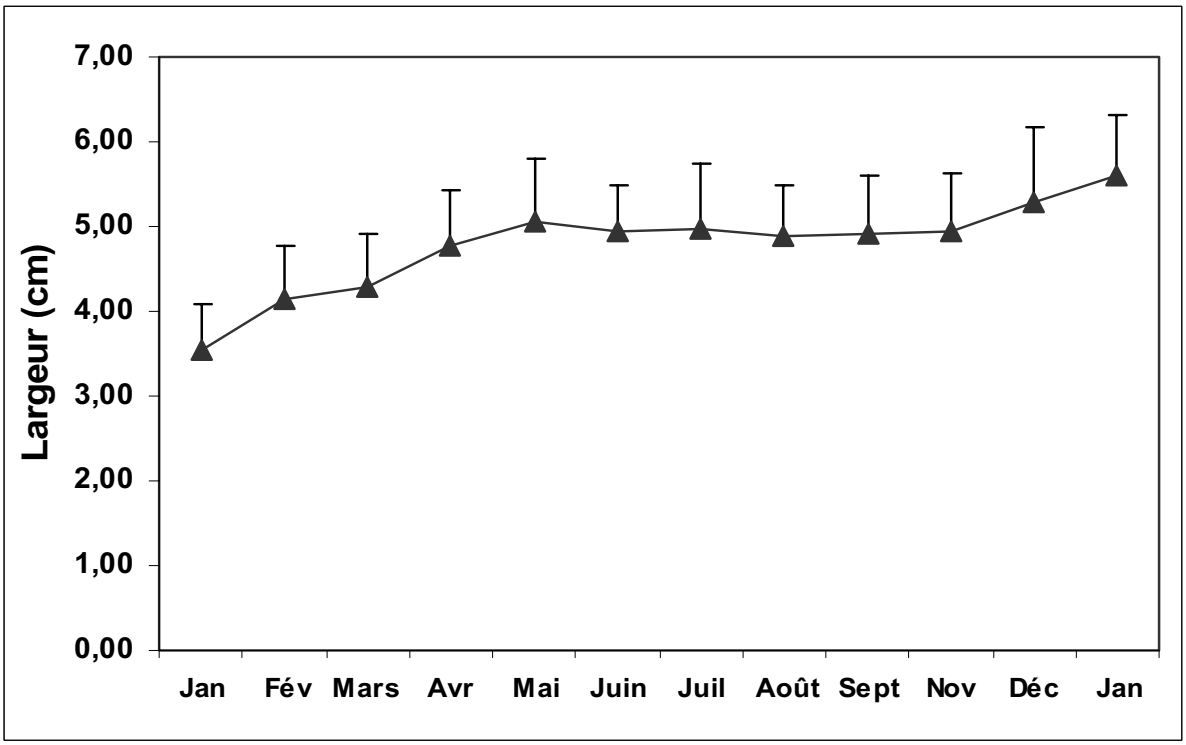


Fig. 2. – Variation mensuelle de la température (°C), salinité (‰) et chlorophylle a (µg/L) de janvier 2002 à janvier 2003.



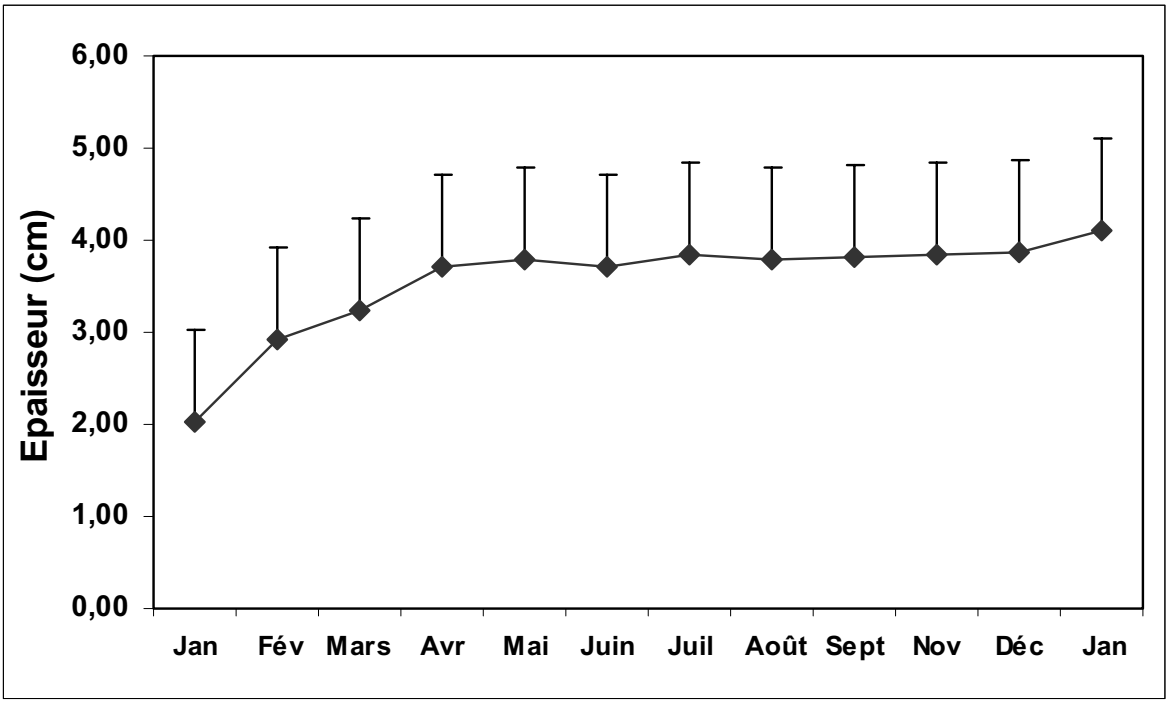
a

Fig. 3a. – Courbe de la croissance en longueur des huîtres de la station FMB de janvier 2002 à janvier 2003.



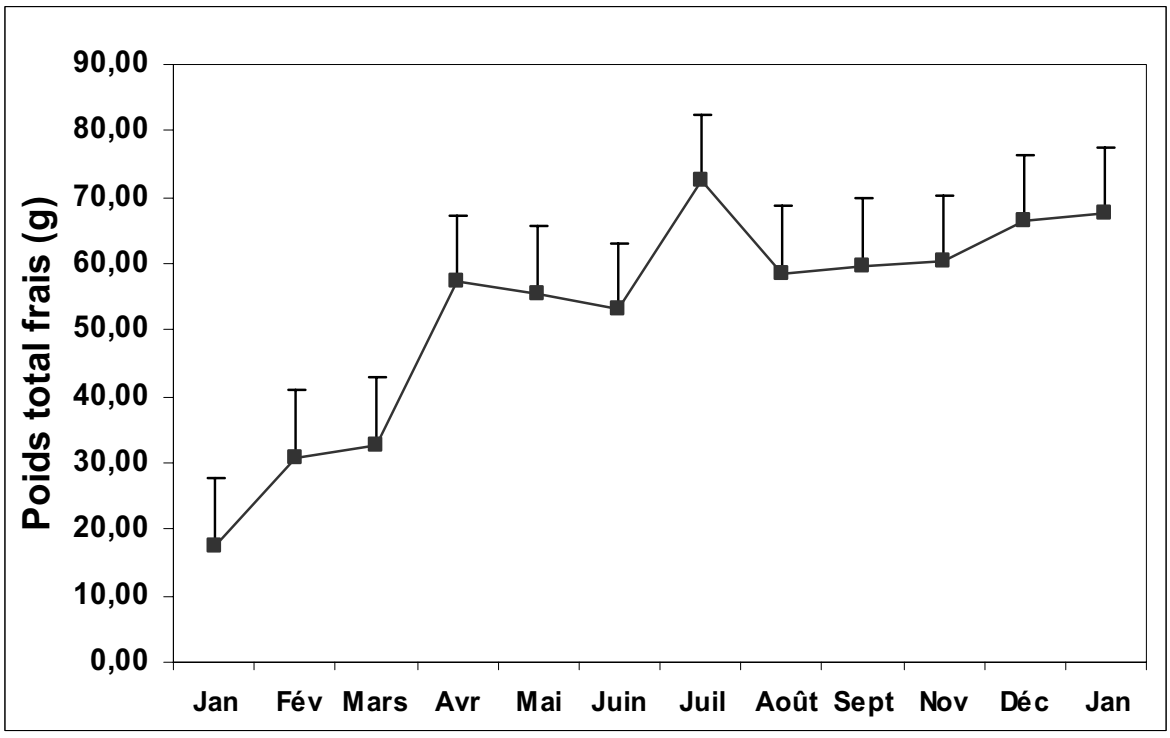
b

Fig. 3b. – Courbe de la croissance en largeur des huîtres de la station FMB de janvier 2002 à janvier 2003.



c

Fig. 3c. – Courbe de la croissance en épaisseur des huîtres de la station FMB de janvier 2002 à janvier 2003.



d

Fig. 3d. – Courbe de la croissance en poids total frais des huîtres de la station FMB de janvier 2002 à janvier 2003.

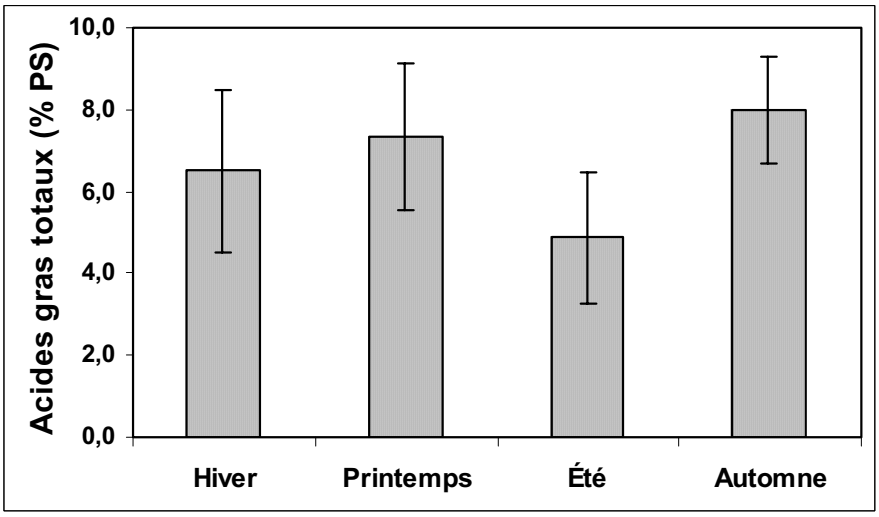


Fig. 4. – Variation saisonnière de la teneur en acides gras totaux (% du poids de chair sèche) chez *Crassostrea gigas* durant l'année de l'étude (2002) (moyenne ± DS).

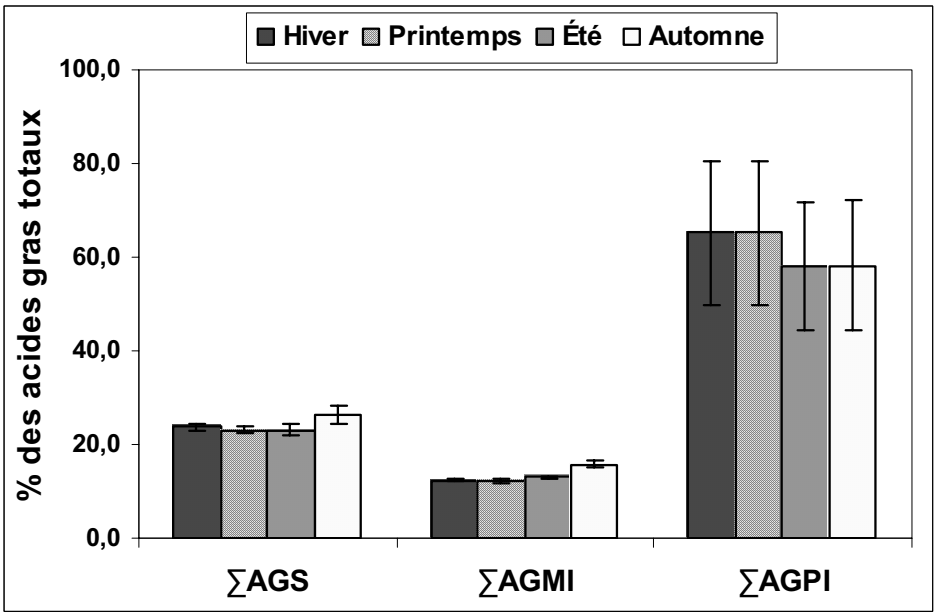


Fig. 5. – Variation saisonnière des acides gras saturés (AGS), acides gras monoinsaturés (AGMI) et acides gras polyinsaturés (AGPI) chez *Crassostrea gigas* durant l'année de l'étude (2002) (moyenne ± DS).

TABLEAU 1

Augmentations moyennes de tailles en longueur, largeur et épaisseur calculées selon les différentes phases du cycle sexuel de *Crassostrea gigas* dans la lagune de Bizerte.

	Janvier 02 – Février 02 (Repos sexuel)	**Mars 02 – Septembre 02 (Activité sexuelle)**	**Novembre 02 – Janvier 03 (Repos sexuel)**
Longueur	2.04±0.18	1.12±0.08	1.25±0.33
Largeur	0.58±0.08	0.64±0.08	0.65±0.11
Epaisseur	0.90±0.06	0.57±0.12	0.28±0.07

TABLEAU 2

Relations allométriques liant les paramètres de la croissance linéaire (L=Longueur, l=largueur, E=épaisseur) N: Nombre d'individus

Phases	Equations de régression	N	r	t
Cycle annuel	Log l=0.426+0.550 Log L	487	0.58	**
	Log E=-0.601+0.919 Log L	487	0.75	**
	Log E=0.075+0.744 Log l	487	0.57	**
Activité gamétogenique	Log l=0.888+0.326 Log L	282	0.22	**
	Log E=1.027+0.133 Log L	282	0.10	NS
	Log E=1.091+0.132 Log l	282	0.14	**
Repos sexuel	Log l=0.400+0.555 Log L	205	0.64	**
	Log E=-0.686+0.933 Log L	205	0.82	**
	Log E=-0.172+0.852 Log l	205	0.64	**

r: Coefficient de corrélation des équations de régression
**: Test Student significatif ($t>1.96$; $p<0.05$); NS=Non significatif

TABLEAU 3

Relations allométriques liant les paramètres de la croissance pondérale à la longueur de la coquille.

Phases	Equations de régression	N	R	t
Cycle annuel	Log Ptot=-0.154+2.025 Log L	487	0.83	**
	Log Pchf=-2.135+1.833 Log L	330	0.52	**
	Log Pchs=-3.190+1.661 Log L	330	0.44	**
	Log Pcoqs=0.268+1.584 Log L	330	0.56	**
Activité gamétogénique	Log Ptot=1.185+1.390 Log L	282	0.47	**
	Log Pchf=0.029+0.713 Log L	210	0.21	**
	Log Pchs=-1.078+0.575 Log L	210	0.15	**
	Log Pcoqs=1.140+1.166 Log L	210	0.42	**
Repos sexuel	Log Ptot=-0.128+1.967 Log L	205	0.88	**
	Log Pchf=-3.260+2.456 Log L	120	0.73	**
	Log Pchs=-4.384+2.306 Log L	120	0.65	**
	Log Pcoqs=-0.739+2.048 Log L	120	0.69	**

TABLEAU 4

Variation saisonnière de la composition en acides gras de l'ensemble gonade-glande digestive de *Crassostrea gigas* dans la lagune de Bizerte. Les résultats sont exprimés en pourcentages des acides gras totaux (moyenne±ET, n=6).

Acides gras	Hiver	Printemps	Eté	Automne
	Moy±ET	Moy±ET	Moy±ET	Moy±ET
14:0	2.55±1.87	1.15±1.28	1.22±0.64	2.46±0.27
15:0	0.42±0.21	0.28±0.14	0.51±0.30	0.72±0.11
16:0	19.77±1.41	20.84±1.17	20.05±3.29	20.80±3.95
18:0	0.91±0.13	0.85±0.05	1.33±0.71	2.32±1.18
ΣAGS	**23.66±0.86**	**23.14±0.65**	**23.13±1.38**	**26.38±1.78**
16:1(n-9)	0.21±0.19	0.33±0.30	0.45±0.33	0.49±0.15
16:1(n-7)	1.83±0.74	1.57±1.75	1.17±0.97	1.67±0.78
18:1(n-9)	1.78±0.89	1.57±0.36	2.23±0.84	2.26±1.81
18:1(n-7)	3.47±0.44	3.69±0.71	3.72±1.06	5.26±1.20
20:1(n-9)	1.91±0.12	1.94±0.76	1.88±0.56	2.66±0.54
20:1(n-7)	2.01±0.21	2.09±0.25	2.93±0.96	3.00±0.94
20:1(n-11)	1.19±0.17	1.14±0.12	0.61±0.51	0.48±0.31
ΣAGMI	**12.40±0.31**	**12.35±0.55**	**12.93±0.26**	**15.84±0.56**
18:2(n-6)	10.64±0.96	11.93±3.52	10.01±3.50	7.47±2.96
18:3(n-6)	0.17±0.06	0.16±0.07	0.14±0.09	1.14±0.55
20:2(n-6)	0.39±0.91	0.26±0.04	0.22±0.05	0.80±0.46
20:3(n-6)	0.18±0.18	0.13±0.12	0.20±0.12	0.12±0.12
20:4(n-6)	1.32±0.16	1.27±0.39	1.98±0.56	2.22±0.10
22:5(n-6)	0.51±0.04	0.47±0.15	0.53±0.30	0.82±0.10
22:4(n-6)	1.64±0.24	2.26±0.75	2.43±0.55	1.96±0.78
ΣAGPI(n-6)	**14.86±0.32**	**16.50±0.27**	**15.53±1.23**	**14.55±0.28**

TABLEAU 4

Variation saisonnière de la composition en acides gras de l'ensemble gonade-glande digestive de *Crassostrea gigas* dans la lagune de Bizerte. Les résultats sont exprimés en pourcentages des acides gras totaux (moyenne±ET, n=6).

Acides gras	Hiver	Printemps	Eté	Automne
	Moy±ET	Moy±ET	Moy±ET	Moy±ET
18:3(n-3)	4.38±0.95	4.00±0.82	3.39±0.89	3.12±1.11
18:4(n-3)	7.88±1.42	5.89±1.33	4.18±1.57	3.27±0.52
20:4(n-3)	1.04±0.25	0.73±0.06	0.62±0.22	0.73±0.24
20:5(n-3)	12.15±1.71	11.00±1.49	10.68±1.18	11.43±1.61
22:5(n-3)	0.74±0.18	0.81±0.26	1.19±0.23	0.77±0.25
22:6(n-3)	20.40±0.57	21.94±1.73	17.73±2.48	21.22±1.10
21:5(n-3)	0.32±0.28	0.24±0.26	0.33±0.29	0.69±0.43
ΣAGPI(n-3)	**46.94±0.61**	**44.63±0.67**	**38.15±0.84**	**41.25±0.52**
22:2i	0.15±0.13	0.67±0.33	0.80±0.14	0.05±0.05
22:2j	0.60±0.52	0.38±0.45	0.65±0.55	0.07±0.06
18:2(n-4)	2.66±0.33	2.95±1.08	2.78±0.98	2.16±0.16
ΣAGPI	**65.22±15.49**	**65.15±15.55**	**57.93±13.69**	**58.09±13.93**
(n-3)/(n-6)	3.15	2.70	2.45	2.83

DISCUSSION

L'étude de la croissance chez *C. gigas* permet de suivre l'évolution des caractères morphologiques des différentes parties du corps de l'animal au cours de son cycle biologique annuel. Il apparaît que le cycle sexuel influence clairement la régression linéaire entre la longueur et la largeur, la longueur et l'épaisseur et l'épaisseur et la largeur. Ces variables linéaires sont différemment liées entre elles suivant la phase d'activité sexuelle de *C. gigas*, en relation probablement avec les gains et les pertes de poids dus à la genèse ou l'émission des gamètes. Selon BERTHELIN et al. (2000), chez les huîtres comme chez les autres espèces de bivalves, le stockage de métabolites est intimement lié aux stades du cycle sexuel. Le tissu de réserve (cellules vésiculeuses) représente un important réservoir énergétique qui s'épuise au moment de l'activité sexuelle de l'animal.

Par contre, au repos sexuel, les valeurs de r augmentent, ce qui signifie que les différentes variables étudiées sont plus fortement liées entre elles. Cette période (automne) correspond également à une élévation de la concentration du milieu en chlorophylle a traduisant l'augmentation de la richesse nutritive du milieu. Celle-ci favorise, dans les tissus de l'huître, l'accumulation de réserves énergétiques, nécessaire à la croissance du tissu somatique et à la synthèse de $CaCO_3$ pour la croissance coquillière (SATO, 1994). Cette constatation corrobore celle de BROWN (1988) en montrant une corrélation positive entre la vitesse de croissance de *C. gigas* et, essentiellement, la chlorophylle a mais aussi la température.

Les valeurs de b calculées indiquent qu'il s'agit d'une allométrie majorante si b>1 ou minorante si b<1 (isométrie dans le cas où b=1). Les valeurs de b calculées pour la population de *C. gigas* de la lagune de Bizerte (<1) montrent qu'il s'agit d'une croissance plus rapide en longueur et en largeur plus qu'en épaisseur. Cela explique la forme allongée de la coquille selon l'axe antéro-postérieur.

Les relations qui relient les différentes variables pondérales du bivalve à la longueur de la coquille (considérée comme taille de référence) indiquent, qu'au cours de la phase d'activité sexuelle, les variations interindividuelles sont importantes. Elles sont probablement générées par des changements d'ordre physiologique en rapport avec la gamétogenèse et l'émission des gamètes. Cette situation engendre la réduction de la corrélation entre les deux variables étudiées démontrées par les faibles valeurs de l'indice de corrélation r. Par ailleurs, le coefficient d'allométrie b montre une valeur inférieure à 3 pour tous les modèles liant la longueur aux différentes variables pondérales. Il s'agit donc d'une allométrie minorante dans sa tendance générale. Nous supposons que la vitesse de croissance en longueur est plus rapide que la vitesse de la croissance pondérale.

Plusieurs facteurs influencent la vitesse de croissance linéaire et pondérale chez les bivalves tel que l'état physique et nutritionnel du milieu (ASKEW, 1972; UTTING, 1986) en plus des paramètres physiologiques (BAYNE et al., 1999) et génétiques des bivalves (NEWKIRK, 1980; GAFFNEY, 1988; HEDGECOCK et al.,1996). URRUTIA et al., 1999, ont montré qu'en présence d'un milieu trophique riche, le surplus d'énergie peut être partagé à la fois entre la croissance somatique des tissus et le développement gonadique du bivalve. LUBET (1991) a précisé que la croissance pondérale est influencée, entre autres facteurs, par la température qui a une action directe sur la cinétique de la gamétogenèse dont la résultante est la compétition entre les compartiments somatiques et germinaux induisant ainsi une croissance pondérale importante. Cet auteur a aussi montré que la température agit sur la croissance indirectement en agissant sur la productivité primaire du milieu et la disponibilité de la nourriture et, par conséquent, sur la nutrition du bivalve. Selon TOCHER & HARVIE (1988); WHYTE et al. (1990), la croissance pondérale résulte de l'accumulation des lipides en quantité absolue dans les tissus des bivalves, engendrée par le processus de gamétogenèse, essentiellement chez les femelles. Ceci nous a incités à étudier la composition en acides gras chez

C. gigas et à relier cette composition à la croissance pondérale des huîtres.

L'évolution saisonnière des acides gras totaux (% de la masse de chair sèche) montre des valeurs maximales enregistrées en automne (8±1.3) qui correspondent d'une part au repos sexuel du bivalve (Dridi et al., 2006) et d'autre part à l'élévation photosynthétique primaire (1.2µg/L) du mois de novembre dans la lagune de Bizerte. Selon Jarzebski et al. (1986), il existe une corrélation positive entre la disponibilité des aliments dans le milieu et la composition en acides gras chez le bivalve *Macoma balthica.* En hiver, la moyenne des teneurs en acides gras totaux (% de la masse de chair sèche) montre une diminution non significative qui peut être due au déclenchement de la gamétogenèse (Dridi et al., 2006) et à la diminution de la concentration en chlorophylle a dans le milieu d'élevage des huîtres. Le printemps est caractérisé par l'élévation du pourcentage des acides gras totaux dans les tissus secs de l'ensemble gonade-glande digestive (7.3±1.8) qui diminue significativement ($p<0.05$) en été (4.9±1.6). En effet, le printemps correspond à la maturité sexuelle des huîtres de la lagune de Bizerte (Dridi et al., 2006) et à l'élévation de la concentration du milieu en chlorophylle a (2.4µg/L). L'été est marqué par l'émission primaire des gamètes (juin) et le début de l'émission totale des gamètes du mois d'août (Dridi et al., 2006). Cette émission des gamètes représente une perte de matière organique qui induit la diminution de la concentration des acides gras totaux dans le poids sec de l'ensemble gonade-glande digestive. Dans le présent travail un suivi journalier ou même hebdomadaire de la concentration du milieu en chlorophylle a, aurait permis une évaluation plus précise de la richesse nutritionnelle du milieu d'élevage des huîtres dans la lagune de Bizerte.

La dominance des acides gras polyinsaturés (AGPI, 58-65.2% des aides gras totaux) sur les acides gras saturés (AGS, 23-26.3%) et monoinsaturés (AGMI, 12.3-16%) a été mise en évidence durant l'année d'étude. La prédominance des AGPI a également été observée chez des huîtres, des moules et des seiches de différents sites de la Méditerranée (Orban et al., 2002; 2004; Ozyurt et al., 2006). Nous n'avons pas observé de variations significatives des AGS durant la période d'étude, quant aux AGMI ils varient significativement ($p<0.05$) de l'été à l'automne et les AGPI varient significativement ($p<0.05$) du printemps à l'été.

Les acides gras polyinsaturés du type (n-3) constituent le groupe le plus important parmi les polyinsaturés (dominé par l'acide eicosapentaenoïque et l'acide docosapentaenoïque), suivi des acides gras saturés (essentiellement représenté par l'acide palmitique) et en dernier lieu les acides gras monoinsaturés.

Les valeurs maximales de C16:0 sont enregistrées au printemps (21%±1.2 des acides gras totaux) et en automne (21%±4) qui correspondent aux productions primaires maximales printanière et automnale. Selon Thompson et al. (1996), la présence de C16:0 dans les aliments ingérés par les huîtres leur assure une croissance plus rapide et diminue le taux de mortalité, essentiellement chez les juvéniles. Cela rappelle l'élévation des valeurs de C16:0 en automne notée dans la présente étude simultanément avec la croissance de la coquille en longueur (1.1±0.3cm), largeur (0.7±0.1cm) et épaisseur (0.3±0.1cm) de novembre 02 à janvier 03 (Fig. 3). Selon Von Elert (2004), l'élévation des pourcentages de 18:3 (n-3) et 18:4 (n-3) indique une meilleure assimilation de ces acides gras par *Daphnia galeata* se traduisant par une augmentation de la vitesse de croissance chez ce crustacé. Dans la présente étude, ces deux acides gras atteignent en hiver leurs valeurs maximales qui sont respectivement de 4.38%±0.95 et 7.88%±1.42 des acides gras totaux, sachant que cette saison de l'année correspond au repos sexuel du bivalve dans la lagune de Bizerte durant lequel nous avons observé une croissance meilleure en longueur, largeur et épaisseur de novembre 02 à janvier 03.

D'après Webb & Chu (1983); Whyte et al., 1990; Careas et al., 2002, les acides gras à 20 et 22 atomes de carbones sont essentiels pour la survie et la croissance des mollusques. Dans le même contexte, Langdon & Waldock (1981) et Enright et al. (1986) associent l'abondance de C20:5 et C22:6 dans les nutriments à l'élévation de la vitesse de croissance chez les naissains de *Crassostrea gigas* et *Ostrea edulis*.

Les résultats de ce travail, sont en accord avec ces dernières constatations. En effet, la valeur maximale de C20:5 (n-3) est enregistrée en hiver (12.1±2% des acides gras totaux) qui coïncide avec le repos sexuel et une croissance meilleure des coquilles des huîtres en longueur, largeur et épaisseur de janvier 02 à février 02 et de novembre 02 à janvier 03. Mais ce résultat est contradictoire avec les constatations de Thompson et al. (1996) qui ont montré une corrélation négative entre la teneur de C20:5 (n-3) dans l'aliment ingéré par les huîtres et l'accélération de la vitesse de croissance chez les larves de *C. gigas*. Le pourcentage d'acide docosahexaenoïque C22:6 (n-3) parmi les acides gras totaux est maximal au printemps (22±1.7) et correspond à la concentration maximale de chlorophylle a dans le milieu d'élevage des huîtres. Cet acide gras diminue significativement $p<0.05$ en été et montre une augmentation significative $p<0.05$ de nouveau en automne (21±1% des acides gras totaux) période durant laquelle les huîtres sont au repos sexuel, ce qui se traduit par l'accélération de la croissance du bivalve (exprimée par l'augmentation des moyennes de longueurs, largeurs et épaisseurs des coquilles des huîtres). Selon Bell et al. (1986), la diminution de la température induit généralement à l'augmentation des teneurs en AGPI dans les tissus des bivalves permettant de maintenir la souplesse des membranes, ce qui expliquerait dans notre cas les valeurs élevées des AGPI en hiver (65%±15.5 des acides gras totaux) et leur diminution en été (58%±13.7des acides gras totaux). La qualité et la quantité des aliments ingérés par les bivalves contribuent considérablement au changement du profil des acides gras dans la chair (Benninger & Stephan, 1985; Napolitano & Akman, 1993). La température et la nourriture pourraient agir en synergie sur la composition en acides gras dans la mesure où l'élévation de la concentration du milieu en chlorophylle a coïncide avec l'élévation de la température de l'eau. Ainsi les valeurs maximales de l'acide docosapentaenoïque au printemps pourraient être dues à la richesse nutritive du milieu.

Le calcul du rapport AGPI (n-3)/AGPI (n-6) dans la chair de *C. gigas* confirme davantage le rôle important

que joue la composition en acide gras dans le processus de croissance. Ce rapport est maximal en hiver (3.15) qui correspond au repos sexuel de l'huître et à l'élévation des moyennes de longueurs, largeurs et épaisseurs des coquilles de *C. gigas*. Selon SARGENT et al. (1993), la nécessité de maintenir un rapport élevé AGPI (n-3)/AGPI (n-6) dans la chair des organismes aquatiques est essentielle pour leur survie et leur croissance.

CONCLUSION

Selon BÉJAOUI (1998), BEN NAKHLA (2002) et DRIDI et al. (2006), la lagune de Bizerte est un milieu favorable à la reproduction et à la croissance des mollusques bivalves grâce à son faible hydrodynamisme, ses eaux tempérées et sa richesse phytoplanctonique. Ces auteurs ont tiré cette conclusion suites à leurs études respectives sur *Mytillus galloprovincialis*, *Flexopecten glaber* et *Crassostrea gigas*.

L'étude biométrique présentée dans ce travail, a permis de décrire la croissance, linéaire et pondérale de *Crassostrea gigas*. Les moyennes des mesures de la longueur, largeur et épaisseur des coquilles et les coefficients de corrélation r calculés à partir des modèles de croissances, ont montré des variations saisonnières en rapport direct avec le cycle sexuel du bivalve déterminé selon DRIDI et al. (2006) et la composition de ses tissus en acides gras. La présente étude a montré que l'accumulation et la diminution des réserves lipidiques au niveau des chairs des huîtres influencent la cinétique de croissance des huîtres. En outre, la variation des teneurs en acides gras dans les tissus des huîtres, sont tributaires de leurs stades sexuels et de la richesse du milieu en chlorophylle a (qui représente pour *C. gigas* la source nutritionnelle majeure, à son tour influencée par la température de l'eau).

REMERCIEMENTS

Nous remercions Dr Slim Tritar gérant de la Ferme Marine de Bizerte (FMB) pour la fourniture des échantillons d'huîtres nécessaires à la réalisation de cette étude.

RÉFÉRENCES

AMINOT A & CHAUSSEPIED M (1983). Manuel des analyses chimiques en milieu marin. Centre national pour l'exploitation des océans. CENEXO: 395 pp.

ASKEW CG (1972). The growth of *Ostrea edulis* and *Crassostrea gigas* in Ensworth Harbour. Aquaculture, 1:237-259.

BAYNE BL (1976). Aspects of reproduction in bivalve molluscs. In: VIELEY ML (ed), Estuarine processes. Academic press, New York: 432-448.

BAYNE BL, SVENSSON S & NELL JA (1999). The physiological basis for faster growth in the Sydney rock oyster, *Saccostrea commercialis*. Biology Bulletin, 197(3): 377-387.

BÉJAOUI N (1998). Écobiologie de la population de moules *Mytilus galloprovincialis* (Lamarck, 1819) du lac de Bizerte. Thèse de Doctorat. Faculté des sciences de Tunis: 259 p.

BELL MV, HENDERSON RJ & SARGENT JR (1986). The role of polyunsatured fatty acids in fishes. Comparative Biochemistry and Physiology, 83B: 711-719.

BEN NAKHLA L (2002). Contribution à l'étude biologique et à la valorisation du pétoncle *Flexopecten glaber* (Linnaeus, 1758) dans la lagune de Bizerte. DEA. Institut National Agronomique de Tunis: 73 p.

BENNINGER PG & STEPHAN G (1985). Seasonal variations in the fatty acids of the triacylglycerols and phospholipids of two populations of adult clam (*Tapes decussatus* L. and *T. philippinarum*) reared in a common habitat. Comparative Biochemistry and Physiology, 81B: 591-601.

BERTHELIN C, KELLNER K & MATHIEU M (2000). Storage metabolism in the Pacific oyster (*Crassostrea gigas*) in relation to summer mortalities and reproductive cycle (West Coast of France). Comparative Biochemistry and Physiology, 125B: 359-369.

BLIGH EG & DYER WJ (1959). A rapid method of total lipid extraction and purification. Canadian Journal of Biochemistry, 37: 911-917.

BROWN JR (1988). Multivariate analysis of the role of environmental factors in seasonal and site-related growth variation in the Pacific oyster *Crassostrea gigas*. Marine Ecology Progress Series, 45: 225-236.

CAREAS M, UTTING SD, COUTTEAU P, MILLICAN PF & SORGELOOS P (2002). Impact of the supplementation of a docosahexanoic acid-rich emulsion on the reproductive output of oyster broodstock, *Crassostrea gigas*. Marine Biology, 140: 1157-1166.

DRIDI S, ROMDHANE MS & EL CAFSI M (2006). Evidence of *Crassostrea gigas* reproduction in the Bizert lagoon, Tunisia. Journal of Biological Research, 5: 35-45.

ENRIGHT CT, NEWKIRK GF, CRAIGIE JS & CASTELL JD (1986). Growth of juvenile *Ostrea edulis* L. fed *Chaetoceros gracilis* Schutt of varied chemical composition. Journal of Experimental Marine Biology and Ecology, 96: 15-26.

FOLCH J, LEES M & SLOANE STANLEY GH (1957). A simple method for the isolation and purification of total lipids from animal tissues. Journal of Biological Chemistry, 266: 497-509.

FOOD AND AGRICULTURE ORGANIZATION OF THE UNITED NATIONS (FAO). July, 2006. Available from<ftp://ftp.fao.org/fi/stat/summary/default.htm#capture>

FRISONI GF, GUELORQET O & PETHUISOT JP (1986). Evaluation des potentialités aquacoles du lac de Bizerte. Rapport FAO/MEDRAP, Tunis, 79 pp.

GABBOTT PA (1983). Developmental and seasonal metabolic activities in marine molluscs. In: HOCHACHKA PW (ed), The Mollusca Vol (2). Environmental Biochemistry and Physiology, Academic Press, New York: 165-217.

GAFFNEY PM (1988). Genetic improvement of cultured bivalve species. Journal of Shellfish Research, 7: 158-159.

GIESE AC (1969). A new approach to the biochemical composition of the mollusc body. Oceanography and Marine Biology, 7: 175-229.

HEDGECCOCK D, MCGOLDRICK DJ, MANAHAN DT, VAVZA J, APPELMANS N & BAYNE BL (1996). Quantitative and molecular genetic analyses of heterosis in bivalve molluscs. Journal of Experimental Marine Biology and Ecology, 203: 49-59.

JARZEBSKI A, POLAK L, WENNE R & FALKOWSKI L (1986). Microgeographic differentiation in the lipid composition of the bivalve *Macoma baltica* from the Gulf of Gdansk (southern Baltic). Marine Biology, 91: 27-31.

LANGDON CJ & WALDOCK MJ (1981). The effect of algal and artificial diets on the growth and fatty acid composition of *Crassostrea gigas* spat. Journal of the Marine Biological Association of the United Kingdom, 61: 431-448.

LUBET P (1959). Recherches sur le cycle sexuel et l'émission des gamètes chez les mytilidés et les pectinidés (mollusques bivalves). Revue des travaux de l'institut des pêches maritimes, 23: 387-542.

LUBET P (1991). Bases biologiques de la culture des mollusques. In: BARNABÉ G (ed), Bases biologiques et écologiques de l'aquaculture: 99-210.

MEDHIOUB MN (1993). Aperçu sur la conchyliculture en Tunisie point de situation et réflexions. Revue de l'Institut National Agronomique de Tunisie, 8(2): 19-26.

METCALFE D, SCHMITZ A & PELKA JR (1966). Rapid preparation of fatty acid esters from lipids for gas chromatographic analysis. Analytical Chemistry, 38: 524-535.

NAPOLITANO GE & AKMAN RG (1993). Fatty acid dynamics in sea scallop *Plactopecten magellanicus* (Gmelin, 1791) from Georges Bank, Nova Scotia. Journal of Shellfish Research, 121: 267-277.

NEWKIRK GF (1980). Review of the genetics and the potential for selective breeding of commercially important bivalve. Aquaculture, 19: 209-228.

OJEA J, PAZOS AJ, MARTINEZ D, NOVOA S, SANCHEZ JL & ABAD M (2004). Seasonal variation in weight and biochemical composition of the tissues of *Ruditapes decussatus* in relation to the gametogenic cycle. Aquaculture, 238: 451-468.

ORBAN E, DI LENA G, MASCI M, NEVIGATO T, CASINI I & CAPRONI R (2004). Growth, nutritional quality and safety of oysters (*Crassostrea gigas*) cultured in the lagoon of Venice (Italy). Journal of the Science of Food and Agriculture, 84: 1929-1938.

ORBAN E, DI LENA G, NEVIGATO T, CASINI I, MARZETTI A & CAPRONI R (2002). Seasonal changes in meat content, condition index and chemical composition of mussels (*Mytilus galloprovincialis*) cultured in two different Italian sites. Food Chemistry, 77: 57-65.

OZYURT G, DUYSAK O, AKAMCA E & TURELI C (2006). Seasonal changes of fatty acids of cuttlefish *Sepia officinalis* L. (Mollusca: Cephalopoda) in the north eastern Mediterranean sea. Food Chemistry, 95: 382-385.

PAZOS AJ, ROMAN G, ACOSTA CP, SANCHEZ JL & ABAD M (1997). Lipid classes and fatty acid composition in the female gonad of *Pecten maximus* in relation to reproductive cycle and environmental variables. Comparative Biochemistry and Physiology, 117B: 393-402.

RUIZ C, ABAD M, SENADO F, GARCIA-MARTIN LO & SANCHEZ LOPEZ JL (1992). Influence of seasonal environmental changes on the gamete production and biochemical composition of *Crassostrea gigas* (Thunberg) in suspended culture in EL Grove, Galicia, Spain. Journal of Experimental Marine Biology and Ecology, 155: 249-262.

SARGENT JR, BELL JG, BELL MV, HENDERSON RJ & TOCHER DR (1993). The metabolism phospholipids and polyunsatured fatty acids in fish. In: LAHLOU B & VITIELLO P (eds), Aquaculture: Fundamental and applied Research, Vol. 43. Washington DC: Coastal and Estuarine Studies American Geophysical Union: 103-24.

SASTRY AN (1979). Pelecypoda (excl. Ostreidae). In: GIESE AC et al. (eds), Reproduction of marine invertebrates, New York: Academic Press 5: 113-292.

SATO S (1994). Analysis of the relationship between growth and sexual maturation in *Phacosoma japonicum* (Bivalvia veneridae). Marine Biology, 118: 663-672.

SOKAL R & ROHLF J (1969). Biometry. FREEMAN WH and Company, San Francisco, CA, USA.

TOCHER DR & HARVIE DG (1988). Fatty acid composition of the major phosphoglycerides from fish natural tissues; (n-3) and (n-6) polyunsatured fatty acids in rainbow trout (*Salmo gairdneri*) and cod (*Gadus morhua*) brains and retinas. Fish Physiology and Biochemistry, 5: 229-39.

TOMPSON PA, GUO MX & HARRISON PJ (1996). Nutritiona value of diets that vary in fatty acid composition for larval Pacific oysters (*Crassostrea gigas*). Aquaculture, 143: 379-391.

URRUTIA MB, IBARROLA I, EGLISIAS JIP & NAVARRO E (1999). Energetics of growth and reproduction in a high–tidal population of the clam *Rudutapes decussatus* from Urdabai Estuary (Basque Country, N. Spain). Journal of Sea Research, 42: 35-48.

UTTING SD (1986). A preliminary study on growth of *Crassostrea gigas* larvae and spat in relation to dietary protein. Aquaculture, 56: 123-138.

VON ELERT E (2004). Food quality constraints in *Daphnia*: interspecific differences in the response to the absence of a long chain polyunsaturated fatty acid in the food source. Hydrobiologia, 526: 187-196.

WEBB KL & CHU FL (1983). Phytoplankton as a food source for bivalve larvae. In: PRUDER G D, LANGDON C & CONKLIN D (eds), Proceedings of the second International Conference on Aquaculture Nutrition Biochemical and Physiological Approaches to Shellfish Nutrition. Louisiana State University Press, Baton Rouge, LA: 272-291.

WHYTE JNC, BOURNE N & HODGSON CA (1990). Nutritional condition of rock scallop, *Crassadoma gigantean* (Gray), Larvae fed mixed algal diets. Aquaculture, 86: 25-40.

Received: June 20, 2007
Accepted: April 16, 2008

Belg. J. Zool., 138 (2) : 170-176 July 2008

Food quality affects diet preference of rabbits: experimental evidence

Nele Somers[1]*, Binke D'Haese[1], Beatrijs Bossuyt[1], Luc Lens[1] & Maurice Hoffmann[1,2]

[1] **Terrestrial Ecology Unit, Ghent University, K.L. Ledeganckstraat 35, B-9000 Ghent, Belgium.**
[2] **Research Institute for Nature and Forest, Kliniekstraat 25, B-1070 Brussels, Belgium.**

Corresponding author : * Nele Somers, Ghent University, Terrestrial Ecology Unit, K.L. Ledeganckstraat 35, B-9000 Ghent, Belgium. e-mail: Nele.Somers@UGent.be.

ABSTRACT. When foraging, small mammalian herbivores do not show a preference for the forage with the highest biomass, which can be explained by several hypotheses (e.g. antipredator considerations, more difficult handling of tall swards and/or the higher nutritional quality of shorter grasses). We tested the ability of rabbits to discriminate between plants of different nutritional value and whether they prefer the most nutritious. A feeding trial in which rabbits (*Oryctolagus cuniculus* (Linnaeus, 1758)) were offered two different types of grasses (fertilised and unfertilised) was executed under experimental conditions. The rabbits preferred the grasses with the highest protein percentage, when conditions were controlled for sward height/plant biomass. This observation is equivalent to results obtained in geese and provides experimental evidence about the capability of rabbits to select for plants with the highest nutritional quality.

KEY WORDS : diet selection, feeding trial, grazing, herbivore, rabbit

INTRODUCTION

Predicting the impact of herbivores on their environment requires insight into the criteria by which grazers select food patches. The mechanism of functional response (reviewed in CRAWLEY, 1983) predicts herbivores to prefer patches with the highest biomass and plant size (LUNDBERG, 1988; GROSS et al., 1993), in order to obtain as much forage as possible in a given time period. However, some studies evidenced that small herbivores, such as rabbits (*Oryctolagus cuniculus* (Linnaeus, 1758)) and geese, prefer rather swards of intermediate height (OLFF et al., 1997; WILMSHURST et al., 2000; HASSALL et al., 2001; IASON et al., 2002; BAKKER et al., 2005). Several explanations for this phenomenon have been mentioned. IASON et al. (2002) and VAN DE KOPPEL et al. (1996) suggested that rabbits and hares, *Lepus europaeus* (Pallas, 1778), prefer vegetation with medium standing crop swards because predators are most likely to occur in the cover offering higher vegetation. Moreover, a tall sward is more difficult to handle for small animals. A dislike for tall swards by brent geese, *Branta bernicla bernicla* (Linnaeus, 1758), and barnacle geese, *Branta leucopsis* (Bechstein, 1803), was explained by the larger costs of handling, more difficult locomotion, and decreased predator detection in the taller vegetation (VAN DER GRAAF et al., 2002).

However, preference for shorter swards may also be related to their higher food quality: grazing creates fast-growing and nutritionally-rich vegetation (so called 'grazing lawns', MCNAUGHTON, 1984), due to plant compensatory mechanisms operating after defoliation (MATTSON, 1980; MCNAUGHTON, 1983). Large herbivores need a larger plant biomass, but can tolerate low plant quality, while smaller herbivores can persist on small quantities of food on the condition that the plants are of high nutritional quality (OLFF et al., 2002). Small hindgut fermenters (e.g. the rabbit) depend on highly digestible forage because they have high metabolic requirements and their digestive system is very small (DEMMENT & VAN SOEST, 1985). The creation of grazing lawns by large herbivores may hence result in 'feeding facilitation' (ARSENAULT & OWEN-SMITH, 2002) benefiting smaller grazers. Hunger may strengthen this preference for nutritionally-rich forage (CRAWLEY, 1983).

The wild rabbit is considered to have a preference for shorter swards. For example, MORENO & VILLAFUERTE (1995) noticed that rabbit grazing pressure was higher in fresh, re-growing vegetation (after burning). Being a central-place forager (SCHOENER, 1979), this herbivore concentrates foraging in the neighbourhood of the burrow (DEKKER, 2007), less than 20m from cover (MORENO & VILLAFUERTE, 1995). With increasing distance from the burrow, grazing pressure of rabbits gradually decreases, causing a gradient pattern. Consequently, vegetation height increases and nitrogen content of forage decreases, due to repeated grazing of the rabbits, stimulating fresh regrowth (BAKKER et al., 2005). BAKKER et al. (2005) conclude that the grazing pattern has to be explained by food quality.

Univocal discrimination of factors determining feeding preferences is only possible in strictly controlled choice experiments (so-called 'cafeteria-trials'; CRAWLEY, 1983). Experimentally enhancing food quality in field experiments by fertilisation increases both biomass (BALL et al., 2000) and plant height. Moreover, feeding preferences are very difficult to measure in the field (CRAWLEY, 1983). For these reasons, we conducted a laboratory experiment to test the ability of rabbits to discriminate between grasses of low and high nutritional quality, while controlling for sward height or vegetation biomass. We predicted that rabbits would prefer grasses of the highest forage quality either when offered grass swards of comparable height or cut grasses of the same biomass.

MATERIALS AND METHODS

Study species

Twelve domestic rabbits (six males and six females, all between 1 and 3 years old) of the breed 'Steenkonijn' were used. The Steenkonijn is the oldest Belgian rabbit breed, and is most closely related to the wild rabbit (WERNER, 1980). Therefore, the behaviour of these animals is supposed to be comparable to the behaviour of their wild ancestor. The rabbits were housed in wire mesh pens (65cm x 110cm, height: 60cm) such that each individual could see a single other individual. This allowed social contact between the animals (DUNCAN et al., 2006). A vaccination against myxomatosis and viral haemorrhagic syndrome was administered. All the individuals received water *ad libitum*, and were fed with a mixture of commercial rabbit pellets and grains (Bonito 96, Aveve, Belgium). From the first day of the feeding trials, the pellet feeding was discontinued, so that the rabbits depended for their feeding on the experimental plants, supplemented with straw, which was provided in the pens.

We used *Festuca rubra* as forage in all trials. *Festuca rubra* plants were grown from seeds (Herbiseed, Twyford, England), sown on a mixture of 50% dune sand and 50% potting soil, in seed trays of 40cm by 45cm during July – October 2004. Immediately after sowing, half of the seed trays (selected at random) received 30g of organic fertiliser (8% nitrogen, 6% phosphorus, 7% potassium, 3% magnesium and 38% organic matter), further referred to as 'fertilised plants' (F^+). All trays received an inorganic fertiliser twice (once one month after sowing and once in March 2005). The 'fertilised plants' received 7.5g of inorganic fertiliser (20% nitrogen, 5% phosphorus and 8% potassium) at a time; the 'unfertilised plants' (F^-) received 2.5g at a time.

The trays were put inside the greenhouse immediately after sowing, and were watered every two days. From December 2004 until March 2005, the trays were put outside for better aeration. Fungicide (sulphur) was added twice to cope with a mildew infection, and an infection of aphids was treated by using a mix of piperonylbutoxide and pyrethrine.

Experimental design

The feeding trial took place in an experimental pen (Fig. 1) of 104cm depth, 91cm width and 73cm height, connected to a smaller pen (36cm by 26cm by 30cm) from which the rabbit was not able to see the surroundings. Two grass swards (trimmed just before the start of the trial to a height of 13cm (further called short swards ('S')) or of 33cm (tall swards ('T'))) or two dishes with clipped grass (100g per dish) were put in the larger pen, on the opposite side of the entrance from the small pen. A partition of 40cm high (in the middle between the two swards or dishes) divided the large pen into two halves.

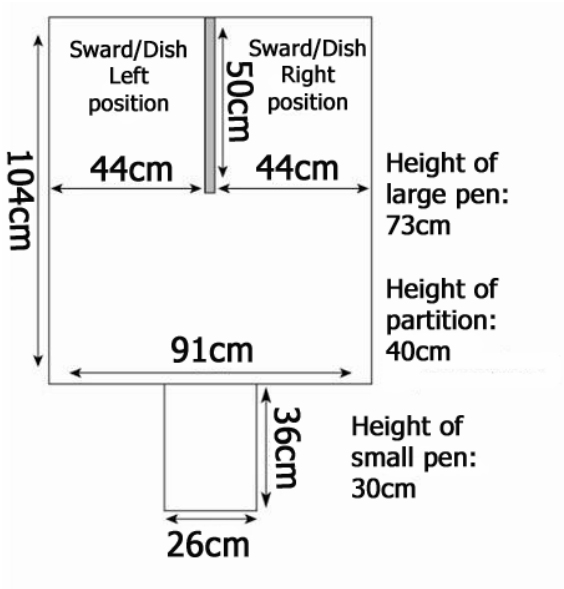


Fig. 1. – Design of the experimental pen.

In the sward trial (22-29 April 2005), there were four groups of three rabbits and two treatments (two combinations of swards): F^+T versus F^-T and F^+S versus F^-S. Each two groups of rabbits received the treatments in a different order. This total design was replicated once, but with a reverse of the left-hand and the right-hand sward. During the clipping trial (2-5 May 2005), the rabbits received dishes with clipped fertilised grass on one side of the pen, and unfertilised grass on the other side (F^+ versus F^-). In the replicate of the clipping trial, the position of F^+ and F^- was reversed. Since the design was randomized and fully balanced, possible effects of the order of treatments were minimized.

Before the start of the feeding trial, four learning days were organised: the rabbits were placed in the pen to habituate to the pen, the grass and the observer. Research carried out with ruminants shows that food preferences develop because of the experience of post-ingestive effects (satiety or malaise) and their interaction with the senses of mainly taste and smell (PROVENZA, 1995). Herbivores learn about grass quality through foraging consequences, which they link with pre-ingestive cues necessary to recognize the value of the forage (GINANE et al., 2005). Although this was only evidenced for ruminants, it is reasonable to assume that ruminants and non-ruminants do not differ in the non-cognitive aspects of how feedback is processed (PROVENZA, 1995). Non-ruminants have indeed been found to be able to discriminate between foods, even when the differences are relatively small (POST, 1993). This means that the rabbits would only be able to select the most nutritious food if they had the opportunity to experience the differences in post-ingestive effects between F^+ and F^-grasses. This condition was fulfilled through the learning days preceding the experiment.

At the start of each experimental session, the individual was weighed (to estimate its degree of hunger) and placed in the small pen. When the entrance to the experimental pen was opened and the rabbit approached the feeding

trays, we started an observation session of 20 minutes, from a central point which did not interfere with the experiment. A detailed description of the foraging behaviour of the rabbit was noted.

Forage quality analysis

After each session, plant material that had not been consumed was removed from the pen and dried at 60°C (WTB Binder with controller RD 2 EED/FED (Binder, Tuttlingen, Germany)), until no more mass loss was detected, after which the dried plants were ground. The percentage of crude protein (CP) and of cell wall constituents (cellulose, hemicellulose and lignin, which were derived from NDF (neutral detergent fibre), ADF (acid detergent fibre) and ADL (acid detergent lignin)) were obtained by Near Infrared Spectroscopy (NIRS – for more information see GIVENS et al. (1997)). A FOSS Feed and Forage Analyzer was used, combined with Winisi software (FOSS, Brussels, Belgium). The calibration for the NIRS was carried out by performing wet analyses for approximately 10% of the samples, following the method of Kjeldahl for CP and following the protocols of GOERING & VAN SOEST (1970) and VAN SOEST et al. (1991) for cell wall constituents.

The amount of digestible protein (DP) was calculated by multiplying CP by the mean digestibility coefficient of CP in grasses (value of 0.70; MAFF, 1986). DE (Digestible Energy) was calculated based on ADF, by multiplying GE (Gross Energy, value based on MAFF M (1986)) by GE_D (coefficient of digestibility of Gross Energy, in which GE_D is defined as follows: GE_D=0.867–0.0012 ADF (g/kg DM) (DE BLAS et al., 1992)). The DP/DE ratio was also calculated since this ratio has been proven to be very valuable in evaluating forage quality for rabbits (FRAGA, 1998).

Statistical analysis

All statistical analyses were performed using SAS 9.1 (SAS Institute Inc., Cary, North Carolina).

The feeding preference of rabbits for different types of grasses was tested by Mixed Linear Models, with "individual" as random effect and "relative foraging time" as dependent variable. This last variable was calculated by timing individual feeding bouts per sward or dish, starting from the moment when the experimental individual had tasted from either both swards or both dishes until the end of the session, divided by the total time left until the end of the session. As vegetation height or biomasses were controlled for, differences in handling time were not expected, so that the relative foraging time can be considered a good measure for intake rate. Furthermore, the level of satiety after eating (PROVENZA, 1995; GINANE et al., 2005) will have been higher for the more nutritious grasses. Since mammals learn to link the taste of the forage to the satiety level, directed foraging behaviour can only start from the moment they have tasted both grasses. Therefore, the relative foraging time, as defined above, is the most appropriate measure describing their preference.

Four independent categorical variables were included in the model: fertilisation (0/1), position of the sward or dish (left or right), day and sex. We started with a full model including all two-factor and higher-order interactions and applied a backward stepwise selection procedure.

The effect of fertilisation on forage quality was analysed by a General Linear Model. We tested the effect of fertilisation as a categorical variable separately on the percentages of CP, cellulose, hemicellulose, lignin, DP, DE and DP/DE. Mixed regression analysis modelling repeated measurements at individual level was used to examine shifts in weight during the feeding trials.

RESULTS

Table 1 summarizes the effect of fertilisation, day, sex and the position of the sward or dish (and all possible interaction terms) on the relative foraging time. Fertilised swards and dishes were preferred over unfertilised ones, as can be seen in Fig. 2: the mean of the relative foraging time was lower for F^-plants (sward trial: mean=20.02%±3.31 SE, N=48; clipping trial: mean=18.25±4.74%, N=24) than for F^+plants (sward trial: mean=31.26±3.31%, N=48; clipping trial: mean=37.13±4.74%, N=24).

The effects of fertilisation on forage quality measures are shown in Table 2, which highlights some significant differences between fertilised and unfertilised plants. CP and cellulose percentages were significantly higher in the F^+plants, as well in the sward trial as in the clipping trial. In the clipping trial, also the hemicellulose percentage was higher in the F^+plants, while the lignin percentage was lower in these plants. The value of digestible protein percentage was significantly higher in the F^+plants, compared to the F^-plants. This applied to the sward trial as well as to the clipping trial. The same result was obtained for the DP/DE-ratio. Also, the DE percentage differed significantly between F^+ and F^-plants, with a higher DE percentage in F^-plants, in the sward trial, but not in the clipping trial.

The effect of day on the relative foraging time was not significant, although the rabbits lost weight during the trials. These losses were statistically significant in both trials (sward trial: $F_{1,11}$=519.51, estimate for time effect=-22.74, P<0.001; clipping trial: $F_{1,11.3}$=7.11, estimate for time effect=-18.50, P=0.02).

DISCUSSION

The results of the experiment showed that only fertilisation had a significant influence on the preference of the animals: the relative foraging time was longer for the fertilised forage, both in the sward and in the clipping trial. The forage quality analysis revealed a higher percentage of both crude and digestible protein in F^+plants, compared to F^-plants. This was to be expected, since nitrogen is a principal component of the used fertilisers and its content is strongly related to protein content. We also observed a higher percentage of cellulose and hemicellulose, although the latter only in the F^+plants from the clipping trial, in which lignin decreased. As forage quality is believed to be enhanced by the protein level (LANGVATN

TABLE 1

Results of the Mixed Linear Model testing for the effect of the four main factors (fertilisation, position, day and sex) and interactions, on the relative foraging time, during a backward stepwise selection. The relative foraging time was calculated by timing individual feeding bouts per sward or dish, starting from the moment when the experimental individual had tasted from either both swards or both dishes until the end of the session, divided by the total time left until the end of the session. The p-values are those from the last step before the respective variable was removed. Num d.f.=numerator degrees of freedom, den d.f.=denominator degrees of freedom.

	Fixed effects	**num d.f.**	**den d.f.**	**F**	**P**
Sward trial	Fertilisation (F)	1	94	5.74	0.02
	Day (D)	7	87	1.21	0.3
	Position grass (P)	1	86	0.84	0.36
	Sex (S)	1	85	0.35	0.56
	F*P	1	84	0.85	0.36
	P*D	7	77	1.05	0.4
	D*S	7	70	0.9	0.51
	F*S	1	69	0.3	0.59
	P*S	1	68	0.02	0.89
	F*D	3	65	0.19	0.9
	F*P*D	3	62	1.72	0.17
	F*D*S	7	55	1.08	0.39
	P*D*S	3	52	0.29	0.83
	F*P*S	1	51	0.03	0.87
	F*P*D*S	3	48	0.23	0.87
	Random effect	*estimate*	*residual*		
	Individual	0	525.03		
Clipping trial	Fertilisation (F)	1	46	7.86	0.01
	Sex (S)	1	45	3.45	0.07
	Day (D)	1	44	0.08	0.78
	Position grass (P)	1	43	0	0.97
	F*S	1	42	2.97	0.09
	D*S	1	41	0.3	0.59
	P*D	1	40	0.26	0.61
	P*S	1	39	0.22	0.64
	F*D	1	38	0.2	0.66
	F*P	1	37	0.04	0.85
	F*D*S	1	36	0.98	0.33
	F*P*D	1	35	0.21	0.65
	P*D*S	1	34	0.1	0.76
	F*P*S	1	33	0.04	0.85
	F*P*D*S	1	32	0.13	0.72
	Random effect	*estimate*	*residual*		
	Individual	0	633.67		

& HANLEY, 1993) and to be diminished by the fibre content (OLFF et al., 1997), the question arises whether fertilisation effectively resulted in a higher food quality. Moreover, in the sward trial, the F^+plants even had a lower digestible energy content. However, it is reasonable to assume that the increase of digestible protein is the most important factor determining forage quality. Since some amino acids cannot be synthesized by the animal's body itself, organisms need amino acids, immediately available from the forage to maintain body conditions constant (FRAGA, 1998). The close agreement between the sum of individual amino acids levels in the body of the rabbit and the CP content (FRAGA, 1998), indicates that CP content provides a good estimate of forage quality. The DP/DE-proportion is mentioned to be an even better predictor (FRAGA, 1998). This ratio also proved to be significantly higher in the F^+plants, in both trials. We can hence conclude that the rabbits selected the forage with the highest nutritional quality.

This preference for high quality forage has been suggested for rabbits (KUIJPER et al., 2004; RÖDEL, 2005) and also for other relatively small mammal herbivores, e.g. small ruminants (WILMSHURST et al., 2000) and mountain hares (*Lepus timidus* (Linnaeus, 1758)) (LINDLÖF et al., 1974). However, studies eliminating the correlation between forage quality and sward height/biomass are scarce. Therefore, it is difficult to know whether the animals are really able to select for the higher nitrogen content, or whether this selection is just coincidental related to the selection of swards with medium standing crop. Some studies concerning geese (HASSALL et al., 2001; BOS et al., 2002; HASSALL & LANE, 2005) showed the capability of these birds to discriminate between high and low quality forage, by eliminating the relationship between forage quality and sward height. BAKKER et al. (2005) executed a field experiment which eliminated the relationship between distance from the rabbit burrow and forage quality, and showed that forage of a higher quality

TABLE 2

Results of the General Linear Model testing for the effect of fertilisation on forage quality measures of standing crop (sward trial) and clipped grass material (clipping trial). F^-plants=unfertilised plants, F^+plants=fertilised plants. CP=% Crude Protein, DP=% Digestible Protein, DE=% Digestible Energy. Num d.f.=numerator degrees of freedom, den d.f.=denominator degrees of freedom.

	num d.f.	den d.f.	F	P	mean F^-plants ± SE	mean F^+plants± SE
Sward trial						
CP (%)	1	94	60.95	<0.001	10.77±0.28	13.91±0.28
Cellulose (%)	1	94	19.99	<0.001	21.86±0.25	23.43±0.25
Hemicellulose (%)	1	94	0.21	0.65	22.62±0.25	22.79±0.25
Lignin (%)	1	94	0.36	0.55	3.17±0.07	3.23±0.07
DP (%)	1	94	60.95	<0.001	7.54±0.20	9.73±0.20
DE (%)	1	94	24.46	<0.001	10.54±0.05	10.17±0.05
DP/DE	1	94	86.67	<0.001	0.71±0.02	0.96±0.02
Clipping trial						
CP (%)	1	46	57.65	<0.001	10.17±0.30	13.44±0.30
Cellulose (%)	1	46	10.32	<0.001	21.55±0.22	22.56±0.22
Hemicellulose (%)	1	46	17.22	<0.001	21.54±0.19	22.65±0.19
Lignin (%)	1	46	19.5	<0.001	2.77±0.07	2.32±0.07
DP (%)	1	46	57.65	<0.001	7.12±0.21	9.41±0.21
DE (%)	1	46	2.69	0.11	10.70±0.05	10.57±0.05
DP/DE	1	46	49.18	<0.001	0.67±0.02	0.89±0.02

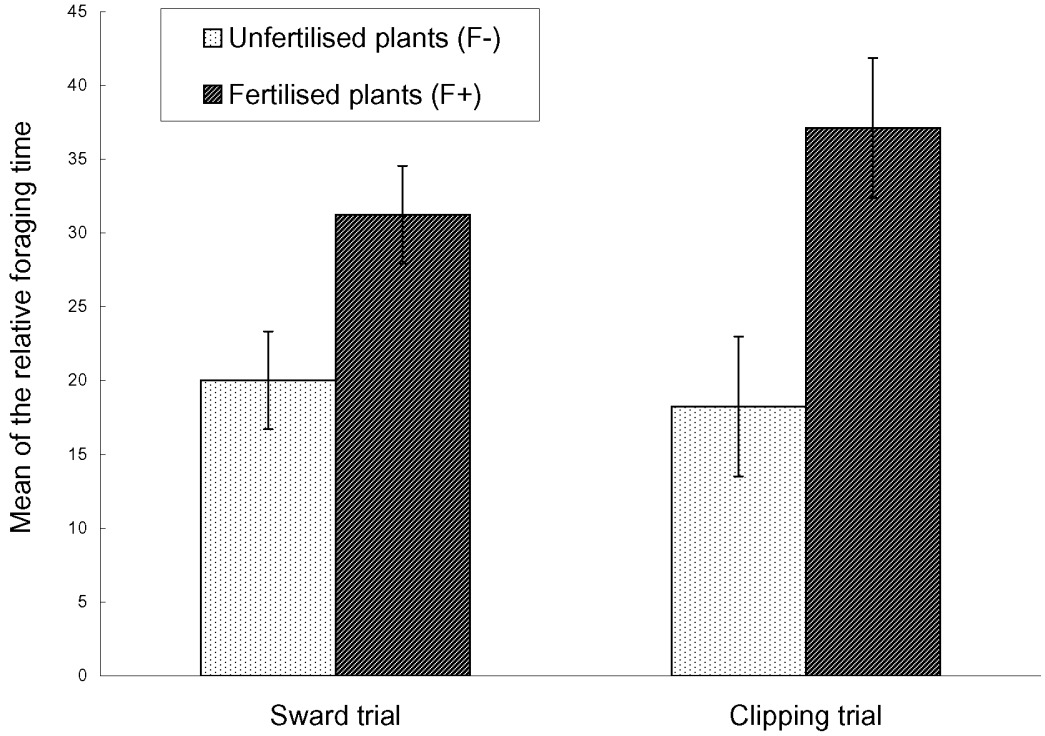


Fig. 2. – Mean and standard error of the relative foraging time of rabbits, when offered the choice between fertilised and unfertilised grasses, controlling for sward height (sward trial) or plant biomass (clipping trial). The relative foraging time was calculated by timing individual feeding bouts per sward or dish, starting from the moment when the experimental individual had tasted from either both swards or both dishes until the end of the session, divided by the total time left until the end of the session.

is preferred, even when farther from the burrow (and thus with a higher sward height). However, the highest (and farthest) swards in this trial were on average approximately as high as the short swards in our study. Therefore, it is possible that sward height in their trial did not show enough variation to really affect the rabbit behaviour. However, their results are confirmed by the results of the present study, controlling for plant height and biomass, which clearly indicate that plant quality, particularly nitrogen and related protein content, is a crucial factor for selecting foraging patches in rabbits.

The other factors included in the model (position of the sward or dish, experimental day and sex) did not significantly influence the food preference of the rabbits. The day of feeding was of no importance; although CRAWLEY (1983) mentions that a hungry animal will be more selective. Since the animals lost weight during the experiments, it could be expected that the animals would become more selective towards the end of the experiment, but this was not confirmed. Similar results were obtained by DUNCAN et al. (2006) who found no evidence that nutritional plane had an overall effect on the proportion of several plant species eaten during preference tests carried out with herbivores. The authors relate this to the more extreme forage deficits in the wild. Similarly, the scarcity of food during winter time in the temperate regions will cause stronger feelings of hunger than the rabbits in our experiment experienced.

Although we evidenced that food quality is important for determining preferences, we were not able to exclude the importance of anti-predation considerations and other sward height-related issues in diet selection: other factors, besides nutrient content, may also have played a role during the decision process of the rabbits. Other research indeed showed that rabbits also choose the swards with the lowest biomass, when there are no nutritional differences between the swards of different heights (IASON et al., 2002). However, the field experiment of BAKKER et al. (2005) demonstrates that the presence of predators causes a shift in the moment of feeding, but does not affect patch preferences. The presented results clearly showed that nutritional content plays an important role in the observed preference of small herbivores for swards of intermediate size, but further research is needed to unravel the relative importance of other potentially contributing factors.

ACKNOWLEDGEMENTS

We thank VLIZ and AWZ-WWK for the use of their infrastructure. We are grateful to INBO and ILVO for grinding and analyses of plant material. Invaluable technical support was provided by Frank Broucke (VLIZ), Ward Vercruysse (INBO), Wim De Belder (INBO) and Maarten Pevenage. Luc Maertens (ILVO) provided interesting information and literature on rabbit feeding trials and feed quality for rabbits. We also thank Chris Van Waes (ILVO) for the information on the plant analysis procedure. The first author is supported by the Research Foundation – Flanders (Aspirant of the FWO-Vlaanderen).

REFERENCES

ARSENAULT R & OWEN-SMITH N (2002). Facilitation versus competition in grazing herbivore assemblages. Oikos, 97: 313-318.

BAKKER ES, REIFFERS RC, OLFF H & GLEICHMAN JM (2005). Experimental manipulation of predation risk and food quality: effect on grazing behaviour in a central-place foraging herbivore. Oecologia, 146: 157-167.

BALL JP, DANELL K & SUNESSON P (2000). Response of a herbivore community to increased food quality and quantity: an experiment with nitrogen fertilizer in a boreal forest. Journal of Applied Ecology, 37: 247-255.

BOS D, DRENT R, RUBINIGG M & STAHL J (2002). The relative importance of food biomass and quality for patch and habitat choice in Brent Geese. In: BOS D (eds), Grazing in coastal grasslands, Brent Geese and facilitation by herbivory, PhD dissertation, Rijksuniversiteit Groningen, Groningen, the Netherlands: 23-41.

CRAWLEY MJ (1983). Herbivory. The dynamics of animal-plant-interactions. Blackwell Scientific Publications, Oxford, Great Britain.

DE BLAS C, WISEMAN J, FRAGA MJ & VILLAMIDE MJ (1992). Prediction of the digestible energy and digestibility of gross energy of feeds for rabbits. 2. Mixed diets. Animal Feed Science and Technology, 39: 39-59.

DEKKER JJA (2007). Rabbits, refuges and resources. How foraging of herbivores is affected by living in burrows. PhD dissertation, Wageningen Universiteit, Wageningen, the Netherlands.

DEMMENT MW & VAN SOEST PJ (1985). A nutritional explanation for body-size patterns of ruminant and nonruminant herbivores. The American Naturalist, 125: 641-672.

DUNCAN AJ, GINANE C, ELSTON DA, KUNAVER A & GORDON IJ (2006). How do herbivores trade-off the positive and negative consequences of diet selection decisions? Animal Behaviour, 71: 93-99.

FRAGA MJ (1998). Protein requirements. In: DE BLAS C & WISEMAN J (eds), The nutrition of the rabbit, CABI Publishing, Wallingford, UK: 133-143.

GINANE C, DUNCAN AJ, YOUNG SA, ELSTON DA & GORDON IJ (2005). Herbivore diet selection in response to simulated variation in nutrient rewards and plant secondary compounds. Animal Behaviour, 69: 541-550.

GIVENS DI, DE BOEVER JL & DEAVILLE ER (1997). The principles, practices and some future applications of near infrared spectroscopy for predicting the nutritive value of foods for animals and humans. Nutrition Research Reviews, 10: 83-114.

GOERING HK & VAN SOEST PJ (1970). Forage Fibre Analysis (Apparatus, Reagents, Procedures, and Some Applications). Agricultural Handbook No. 379, Agricultural Research Service, United States Department of Agriculture.

GROSS JE, SHIPLEY LA, HOBBS NT, SPALINGER DE & WUNDER BA (1993). Functional response of herbivores in food-concentrated patches: tests of a mechanistic model. Ecology, 74: 778-791.

HASSALL M & LANE SJ (2005). Partial feeding preferences and the profitability of winter-feeding sites for brent geese. Basic and Applied Ecology, 6: 559-570.

HASSALL M, RIDDINGTON R & HELDEN A (2001). Foraging behaviour of brent geese, *Branta b. bernicla*, on grasslands: effects of sward length and nitrogen content. Oecologia, 127: 97-104.

IASON GR, MANSO T, SIM DA & HARTLEY FG (2002). The functional response does not predict the local distribution of European Rabbits (*Oryctolagus cuniculus*) on grass swards: experimental evidence. Functional Ecology, 16: 394-402.

KUIJPER DPJ, VAN WIEREN SE & BAKKER JP (2004). Digestive strategies in two sympatrically occurring lagomorphs. Journal of Zoology, 264: 171-178.

LANGVATN R & HANLEY TA (1993). Feeding-patch choice by red deer in relation to foraging efficiency. An experiment. Oecologia, 95: 164-170.

LINDLÖF B, LINDSTRÖM E & PEHRSON (1974). Nutrient content in relation to food preferred by mountain hare. Journal of Wildlife Management, 38: 875-879.

LUNDBERG P (1988). Functional response of a small mammalian herbivore: the disc equation revisited. Journal of Animal Ecology, 57: 999-1006.

MAFF (1986). Feed Composition - UK Tables of Feed Composition and Nutritive Value of Ruminants. Chalcombe Publications, Marlow, Bucks, UK.

MATTSON WJ (1980). Herbivory in relation to plant nitrogen content. Annual Review of Ecology and Systematics, 11: 119-161.

MCNAUGHTON SJ (1983). Compensatory plant growth as a response to herbivory. Oikos, 40: 329-336.

MCNAUGHTON SJ (1984). Grazing lawns: animals in herds, plant form and co-evolution. The American Naturalist, 124: 863-886.

MORENO S & VILLAFUERTE R (1995). Traditional management of scrubland for the conservation of rabbits *Oryctolagus cuniculus* and their predators in Doñana National Park, Spain. Biological Conservation, 73: 81-85.

OLFF H, DE LEEUW J, BAKKER JP, PLATERINK RJ, VAN WIJNEN HJ & DE MUNCK W (1997). Vegetation succession and herbivory in a salt marsh: changes induced by sea level rise and silt deposition along an elevational gradient. Journal of Ecology, 85: 799-814.

OLFF H, RITCHIE ME & PRINS HHT (2002). Global environmental controls of diversity in large herbivores. Nature, 415: 901-904.

POST DM (1993). Detection of differences in nutrient concentrations by eastern woodrats (*Neotoma floridana*). Journal of Mammalogy, 74: 493-497.

PROVENZA FD (1995). Postingestive feedback as an elementary determinant of food preference and intake in ruminants. Journal of Range Management, 48: 2-17.

RÖDEL HG (2005). Winter feeding behaviour of European rabbits in a temperate zone habitat. Mammalian Biology/ Zeitschrift Für Säugetierkunde, 70: 300-306.

SCHOENER TW (1979). Generality of the size-distance relation in models of optimal foraging. American Naturalist, 114: 902-914.

VAN DE KOPPEL J, HUISMAN J, VAN DER WAL R & OLFF H (1996). Patterns of herbivory along a productivity gradient: an empirical and theoretical investigation. Ecology, 77: 736-745.

VAN DER GRAAF AJ, BOS D, LOONEN MJJE, ENGELMOER M & DRENT RH (2002). Short and long-term facilitation of goose grazing by livestock. In: BOS D (eds), Grazing in coastal grasslands, Brent Geese and facilitation by herbivory, PhD dissertation, Rijksuniversiteit Groningen, Groningen, the Netherlands: 63-83.

VAN SOEST PJ, ROBERTSON JB & LEWIS BA (1991). Methods for Dietary Fibre, Neutral Detergent Fibre, and Nonstarch Polysaccharides in Relation to Animal Nutrition. Journal of Dairy Science, 74: 3583-3597.

WERNER BJ (1980). Verzorgingsgids Konijnen. Grote-, midden-, kleine- en dwergrassen. Zuidgroep B.V. Uitgevers, Best.

WILMSHURST JF, FRYXELL JM & BERGMAN CM (2000). The allometry of patch selection in ruminants. Proceedings of the Royal Society of London, Series B, 267: 345-349.

Received: August 31, 2007
Accepted: December 12, 2007

Activation of the GFRa1/NCAM pathway stimulates Sertoli cell proliferation in vitro

Qingzhong Wang[1*], Huilian Liu[1], Yuqiang Shi[1], Zhifang Pan[2] & Jiangang Wang[1]

[1] **College of bio-engineering, Weifang University, NO. 5147, Dong feng dong street, Weifang, Shandong province, 261061, PR China**

[2] **Teaching and research section of cytobiology, Weifang medical college, NO. 288, Shen Li Dong street, Weifang, Shandong province, 261042, PR China**

Corresponding author : * Qingzhong Wang, College of bio-engineering, Weifang University, NO. 5147, Dong feng dong street, Weifang, Shandong province, 261061, PR China; E-mail: waqizh@163.com; Tel.: +86-0536-8785288. Fax: +86-0536-8785288

ABSTRACT. The proliferation and final density of Sertoli cells in the testis are regulated by hormones and local growth factors. Glial cell line-derived neurotrophic factor (GDNF), a distantly related member of the transforming growth factor-β (TGF-â) superfamily, and its receptor components: GFRα1, Ret and neural cell adhesion molecule (NCAM) have been recently reported to be expressed in the testis and to be involved in the proliferation regulation of immature Sertoli cells. However, the number of the reports is very low, and the signalling pathways have not been well characterized. In the present study, we used purified Sertoli cell cultures from 4- to 5-day-old mouse testis to detect the expression of GDNF, to examine the effect of GDNF on Sertoli cell proliferation and possible signalling pathways mediating such effect. GDNF expression in mouse Sertoli cells was detected at both RNA and protein levels by RT-PCR and immunocytochemistry methods. The expression was up-regulated by FSH. GDNF stimulated the proliferation of Sertoli cells and synergized with FSH to promote the proliferation to a higher level. GFRα1/Ret and GFRα1/NCAM are two receptor complexes for GDNF. As GFRα1 and NCAM not Ret were expressed in cultured Sertoli cells, we proposed that the stimulatory effect of GDNF on Sertoli cell proliferation was through pathways initiating from the GFRα1/NCAM receptor. This proposition was supported by the fact that the stimulatory effect of GDNF was significantly reduced by anti-NCAM antibody.

KEY WORDS : Sertoli cells; proliferation; glial cell line-derived neurotrophic factor; neural cell adhesion molecule

INTRODUCTION

GDNF signals through two independent pathways of GFRα1/Ret and GFRα1/NCAM to support several types of neurons in central and peripheral nervous systems (ARENAS et al., 1995; LIN et al., 1993; PARATCHA et al., 2003; ROUSSA & KRIEGLSTEIN, 2004; SARIOLA & SAARMA, 2003), and plays different roles in other mammalian tissue development such as the development of kidney (FUKUDA et al., 2003; SHAKYA et al., 2005), and the self-renewal and differentiation of spermatogonial stem cells (SSCs) in the testis (KANATSU-SHINOHARA et al., 2005; KUBOTA et al., 2004; OGAWA et al., 2004; RYU et al., 2005). Previous studies showed that GDNF was secreted by Leydig cells, Sertoli cells, some spermatocytes, round spermatids and smooth muscle cells in both human and mouse testis, and that GFRα1 was expressed in Sertoli and Leydig cells (DAVIDOFF et al., 2001; GOLDEN et al., 1999; MASURE et al., 1998). It has been reported that NCAM was expressed in fetal or immature Sertoli cells (LASLETT et al., 2000), and downregulated in the rat during maturation of Sertoli cells (ORTH et al., 2000; ORTH & JESTER, 1995). Moreover, it has been found that GDNF exerted a proliferation-promoting effect on Sertoli cells during mouse embryonic development and in the early postnatal period of rat testis (HU et al., 1999; WU et al., 2005). However, the mechanism and roles of GDNF in immature mouse Sertoli cells were not clearly understood. The aims of the present study were just to clarify the roles of GDNF on the proliferation of immature mouse Sertoli cells by using serum-free medium and purified Sertoli cell cultures, and to examine which signalling pathway mediated such effects.

MATERIALS AND METHODS

Reagents and experimental animals

Recombinant rat GDNF was obtained from R&D Systems. Ovine FSH, testosterone and β-estradiol were purchased from Sigma-Aldrich. One mouse line of ICR was used and obtained from Beijing Weitong River Laboratory Animal Inc., China. Animals were housed under 16h light, 8h dark schedule with food and water *ad libitum*, and treated in accordance with the NIH Guide for the Care and Use of Laboratory Animals. All the protocols were approved by the Animal Care and Use Committee of the Institute of Zoology, Chinese Academy of Sciences.

Preparation and culture of Sertoli cells

Sertoli cells of 4~5 days old mice were prepared by following the two-step enzymatic digestion protocol (OGAWA et al., 2004) with some modifications. Briefly, decapsulated testis tissue was treated with 10 volumes of Digestion solution I (2mg/mL collagenase type IV and 200mg/mL DNAse (Sigma-Aldrich) in Ca^{2+}- and Mg^{2+}-free PBS) at room temperature for 3 to 5 minutes with gentle agitation followed by 3 washes in 10 volumes of PBS. Collected specimens were then treated with 5 volumes of Digestion solution II (2mg/mL collagenase type

IV, 200mg/mL DNAse and 2mg/mL hyaluronidase (Calbiochem) in serum-free Dulbecco's Modified Eagle Medium (DMEM) at room temperature for 2 to 5 minutes with vigorous agitation until tubular clumps were invisible, followed by 3 washes in PBS. The dissociated cell suspension was filtered through a nylon mesh with 60mm pore size. Cells were washed twice by centrifugation at 200g for 5 minutes in DMEM medium containing 10% FBS. The pellet was resuspended in DMEM/F12 (Sigma-Aldrich) culture medium supplemented with 10% FBS, 2mM L-glutamine (Sigma-Aldrich), 100unit/mL penicillin and 100mg/mL streptomycin (Invitrogen), plated on 0.2% (w/v) gelatin (Sigma-Aldrich) -coated tissue culture flasks with a density of $2x10^5$cells/cm^2. Cells were cultured at 37°C in an atmosphere of 5% carbon dioxide in air for 1 hour. After gentle agitation, floating cells were removed as the medium was changed, and the cells attaching to the bottom were again incubated overnight. The cells were detached with trypsin-EDTA solution (0.25% (w/v) trypsin-0.04% (w/v) EDTA in Ca^{2+}- and Mg^{2+}-free PBS) (Sigma-Aldrich). After counting, the cells were seeded out again on 13mm round plastic cover slips pre-coated with 0.2% (w/v) gelatin in 24-well plates with 95,000 cells per well in a volume of 1mL medium. Following incubation at 37°C for another 24 hours in serum-free medium, the cells were washed and incubated with test material for 48 hours. In some experiments such as immunocytochemistry and RT-PCR analysis of gene expression, Sertoli cells of a higher concentration were used (125,000 cells per well) in order to get confluent cells in culture.

Cell viability assessment by MTT supravital staining

Cell viability was assessed with the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) calorimetric assay. The principle of the assay is that MTT, a nontoxic pale yellow substrate, is taken up by living cells but not dead cells to yield a dark blue formazan product that can be quantified spectrophotometrically at 570nm absorbance. The absorbance is directly proportional to the number of viable cells. The assay was performed as reported by Mosmann (Mosmann, 1983). Briefly, MTT (Sigma-Aldrich) was added to cultures in 24-well plates with an amount of 0.2mg/well 44 hours after the test reagents were added. Four hours later, cells in each well were lysed with 360mL 10% SDS in 0.01M HCl and incubated at 37°C for 4 hours. The absorbance at 570nm was measured in a microtiter plate reader. Triplicate cultures were used and each experiment was repeated 3 times.

Cell proliferation monitor by BrdU labelling

Cells cultured on cover slips were pulsed with 5-bromo-2'-deoxyuridine (BrdU) (BD Biosciences) at a final concentration of 10μM in culture medium for 4 hours. Cells were then fixed in ice-cold acetone–ethanol for 10 minutes at -20°C. Cell staining was performed by using BrdU in-situ detection kit (BD Biosciences) according to the manufacturer's instructions. Briefly, fixed cells on slides were washed 2 times in PBS for 5 minutes each time, and permeabilized by incubation with dilution buffer for 30 minutes. After two washes, slides were incubated with 0.3% H_2O_2 in PBS for 10 minutes to block endogenous peroxidase activity, followed by washes. The slides were placed in a jar containing the working solution of BD Retrievagen A and heated to 89°C for 10 minutes, and then allowed to slowly cool down to room temperature. Following 3 washes, the slides were incubated for 1 hour at room temperature with 100mL/slide of biotinylated anti-BrdU antibody diluted in the dilution buffer (1:10). After another wash, the slides were incubated with 100mL/slide of Streptavidin-HRP for 30 minutes at room temperature followed by 3 washes in PBS. Colour of BrdU-positive cells was developed by diaminobenzidine (DAB), and counterstaining was performed with hematoxylin. BrdU-positive cells were counted within at least three randomly chosen areas on each cover slip, with about 200 total cells in each area, and the experiment was repeated 3 times.

Immunocytochemistry

The primary antibodies used included rabbit anti-human GDNF polyclonal antibody, rabbit anti-human GFRα1 polyclonal antibody (Santa Cruz biotechnology), mouse anti-human CD56 (NCAM) monoclonal antibody (BD Biosciences), mouse anti-human Ret polyclonal antibody (R & D systems), goat anti-human vimentin polyclonal antibody (chemicon) and mouse anti-á smooth muscle actin monoclonal antibody (Sigma-Aldrich). The secondary antibodies included FITC-conjugated goat anti-rabbit IgG, FITC-conjugated goat anti-mouse IgG, FITC-conjugated mouse anti-goat IgG (Santa Cruz biotechnology), TRITC-conjugated goat anti-mouse IgG and TRITC-conjugated goat anti-rabbit IgG (Sigma-Aldrich).

For immunofluorescent staining, the cultured cells on slides were stained according to the standard procedure. Briefly, the cells were washed 2 times in PBS and fixed in 4% paraformaldehyde for 20 minutes at room temperature. Following two washes in PBS, cells were blocked with PBS containing 1% BSA, 0.1% Triton X-100 and 2% normal goat serum at room temperature for 45 minutes. The samples were then incubated overnight at 4°C with primary antibody diluted as described by manufacturers. The slides were washed 3 times with PBS containing 1% BSA, incubated with diluted secondary antibody for 1 hour at room temperature in the dark followed by another 3 washes. Nuclei of the cells were stained with Hoechst 33258 or Propidium iodide (Sigma-Aldrich). Colour of the slides was visualized and captured under Leica confocal microscope. For negative controls, the cells were incubated with non-immune rabbit serum.

RT-PCR analysis of gene expression

Total RNA was extracted from 8×10^6Sertoli cells by using Trizol reagent (Invitrogen) according to the manufacturer's instructions and was reverse transcribed into cDNA with SuperScript™ II reverse transcriptase (Invitrogen) and oligo (dT) primer. Then the cDNAs were amplified by PCR with Recombinant Taq DNA Polymerase (Takara) according to the manufacture's instruction. For semi-quantitative RT-PCR, equal amounts of RNA extracts

were used to generate first-strand cDNAs. Primer pairs specific for mouse GDNF, GFRα1, Ret, sulfated glycoprotein 2 (SGP2, a Sertoli cell marder) (COLLARD & GRISWOLD, 1987) and luteinizing hormone receptor (LH-R, a Leydig cell marker) cDNAs are listed in Table 1. The housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (G3PDH) was used as normalization control. To perform PCR amplification, reaction mixtures were first denaturalized at 94°C for 3 minutes, 30 cycles with the following conditions were then carried out: 1 minute of denaturalization at 94°C, 40 seconds of annealing at 58°C (65°C for Ret), 40 seconds of extension at 72°C. Subsequently, the reaction was incubated at 72°C for 7 minutes. The PCR products were verified by agarose gel electrophoresis.

TABLE 1

Primer sequences and PCR product size

Genes	Forward primer	Reverse primer	Product size
GDNF	5'-TCACTGACTTGGGTTTGGGCTAT-3'	5'-TCAGACGGCTGTTCTCACTCCTA-3'	477 bp
GFRα1	5'-ACTCCTGGATTTGCTGATGTCGG-3'	5'-CGCTGCGGCACTCATCCTT-3'	193 bp
Ret	5'-CTGCCGCTGCTAGGAGAAGCCCCAC-3'	5'-CTTCACACTGATGTTGGGACAAAGGAA-3'	555 bp
SGP2	5'-GACAATGAGCTCCA(G/A)GAA(A/C)TG-3'	5'-CAGGCATCCTGTGGAGTT(G/A)TG-3'	806 bp
LH-R	5'-AATCCCATCACAAGCTTTCAG-3'	5'-TGCCTGTGTTACAGATGC-3'	214 bp
G3PDH	5'-ACCACAGTCCATGCCCATCAC-3'	5'-TCCACCACCCTGTTGCTGTA-3'	450 bp

Statistical analyses

All the experiments were repeated at least three times if not otherwise stated. Differences between groups were analyzed for statistical significance by using one-way ANOVA and Tukey's post hoc test on raw data. $P<0.05$ was considered as significant.

RESULTS

Purity and morphology of immature mouse Sertoli cell cultures

In order to study the proliferation of immature mouse Sertoli cells, we set up an isolation and culture procedure as described in the Materials and Methods section by which highly purified Sertoli cell cultures could be established from 4~5 days old mice. The Sertoli cells in culture were frequently spindle-shaped, or occasionally elliptic (Fig. 1a). It is known that vimentin is specifically expressed in Sertoli cells, and smooth muscle á-actin in peritubular myoid cells in testis (PARANKO et al., 1986; TUNG & FRITZ, 1990). We, therefore, used immunocytochemical assay to assess the purity of our cultures. After immunocytochemical staining, the numbers of vimentin-labelled cells and propidium iodide-stained nuclei were counted respectively in each of three distinct areas per slide, with approximately 400-500 total cells per area. The purity of Sertoli cell cultures was 98%±1.2% as indicated by the percentage of vimentin positive cells to the propidium iodide-stained nuclei (i.e. total cells) (Fig. 1b). The 2% contaminated cells were the peritubular myoid cells as indicated by using antibody to α-actin, which were elongated and spindle-shaped (data not shown).

GDNF expression and hormonal regulation in cultured mouse Sertoli cells

As expected, our results indicated that basal level expression of GDNF mRNA and protein in cultured Sertoli cells was detected by RT-PCR and immunocytochemistry (Fig. 2). In order to determine whether the GDNF expression was regulated by FSH, we performed time-dependent and dose-dependent experiments. In the time-dependent experiments, the analysis was performed every 1 hour before 3 hours (data not shown) and then every 3 hours after treatment. In the dose-dependent experiments, 10ng/mL, 20ng/mL, 30ng/mL, 40ng/mL, 50ng/mL, 100ng/mL, 150ng/mL and 200ng/mL FSH were used respectively. As indicated in Fig.2, the GDNF expression was up-regulated by FSH in a time- and dose-dependent manner. The maximum mRNA expression was detected 3 hours after FSH treatment and dropped significantly starting from 6 hours (Fig. 2A-a), and the GDNF mRNA expression started to level off when the dose of FSH reached 50ng/mL and thereafter (Fig. 2A-b) (data for FSH treatment of less than 50ng/mL are not shown). A similar pattern was also observed in the immunocytochemical assays (Fig. 2B). Estrogen and androgen had no effects on the expression of GDNF (Fig. 2A-c and 2A-d).

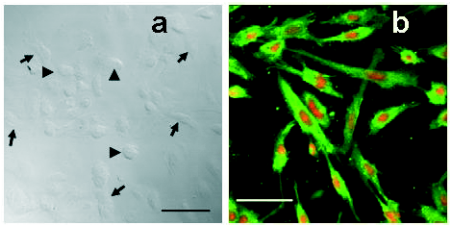


Fig. 1. – Morphological and immunocytochemical analysis of cultured Sertoli cells. a) Phase contrast microscopic image of cultured Sertoli cells, noting that the Sertoli cells were spindle-shaped (arrow) or elliptic (arrowhead); b) Immunofluorescent staining of cultured Sertoli cells. The cytoplasm of Sertoli cells was stained by using the goat anti-human vimentin polyclonal antibody and the FITC-conjugated mouse anti-goat IgG. The nuclei were stained by using propidium iodide. Images were acquired under a Leica confocal microscope. Scale bar: 50mm.

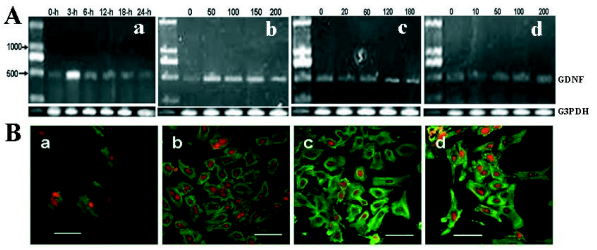


Fig. 2. – Semi-quantitative RT-PCR and immunocytochemical analysis of GDNF expression in cultured Sertoli cells. A: Semi-quantitative RT-PCR analysis of GDNF expression with FSH, estrogen and androgen treatment. a) The time-dependent expression of GDNF stimulated by 50ng/mL FSH. The expression gradually increased before 3 hours (data not shown) and was maximum 3 hours after treatment. b) The dose-dependent expression of GDNF detected 3 hours after FSH treatment. Expression increased gradually with the increase of FSH concentrations (data for FSH treatment of less than 50ng/mL were not shown) and levelled off with doses starting from 50ng/mL c) GDNF expression 4 hours after the addition of different concentrations of estrogen. d) GDNF expression 4 hours after the addition of different concentrations of androgen. B: Immunocytochemical analysis of GDNF expression. Sertoli cells were cultured for 20 hours and either untreated (a) or treated with 10ng/mL (b), 50ng/mL (c), or 100ng/mL FSH (d), and then fixed and stained with rabbit anti-human GDNF polyclonal antibody followed by FITC-conjugated goat anti-rabbit IgG for GDNF expression (green) and propidium iodide for nuclei (red). The staining was performed with the same protocol and same antibody dilution at the same day. The pictures were taken with identical Leica confocal microscope settings. The unit of numbers above figure: ng/ml; Scale bar: 50mm.

Sertoli cell viability and proliferation stimulation by GDNF

The viability of Sertoli cells was first monitored by MTT supravital staining. As shown in Fig. 3-A, GDNF at doses of both 10ng/mL and 20ng/mL stimulated the viability of Sertoli cells conspicuously compared with the cells without any treatment, although the effects of these two doses were not different significantly. As a positive control, 50ng/mL FSH also stimulated Sertoli cell viability significantly. Moreover, 10ng/mL GDNF in combination with 50ng/mL FSH increased the absorbance unit to a higher level, indicating a synergistic effect of these two factors or other mechanisms such as the up-regulation of GDNF expression by FSH. The stimulatory effect of GDNF and FSH on Sertoli cell proliferation was confirmed by BrdU-labelling assay (Fig. 3-B). Statistical analysis showed that significant differences could be identified between 10ng/mL GDNF treatment group and control group (DMEM/F12), and between group of 10ng/mL GDNF treatment and that of the combination of 10ng/mL GDNF with 50ng/mL FSH treatment. Although no statistical differences could be seen between 10ng/mL GDNF treatment group and 20ng/mL GDNF treatment group, and between group of 50ng/mL FSH and that of this dose in combination with 10ng/mL GDNF treatment, the proliferation-promoting effects of GDNF and FSH were obviously identified from Fig. 3-B. Also, in the presence of both GDNF and FSH, the number of BrdU-positive cells in total cells was increased compared with those under other treatments—clumps of BrdU-positive cells were frequently seen, probably resulting from a higher proliferation rate of the cells (Fig. 4).

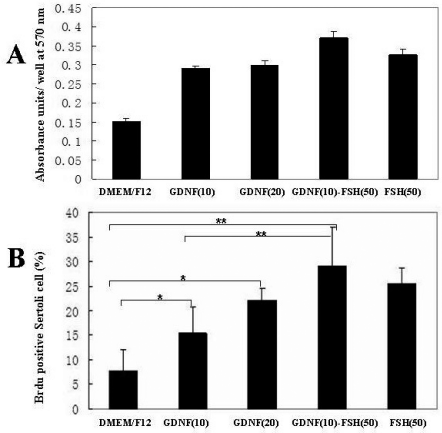


Fig. 3. – Enumeration of viable and proliferating Sertoli cells by supravital staining and BrdU labelling. A: Estimation of living Sertoli cells by MTT staining. The Sertoli cells were first plated and incubated in 24-well plates overnight, and again cultured and treated with GDNF, FSH separately or in their combination for 44 hours, then stained with MTT for 4 hours. Each bar represent 570nm absorbance units (AU) per well (mean±S.E.M. n=3). B: Enumeration of Sertoli cells in S-phase by BrdU labelling. For each experiment at least 3 areas under microscope (approximately 200 total cells) were chosen to count BrdU-positive cells. Values were represented as % Brdu-positive cells over total number of cells from three independent experiments (mean±S.E.M.; n=3). Statistical difference was analyzed by using Tukey's post hoc test. *, $p<0.05$; **, $p<0.01$. The unit of numbers in parentheses: ng/mL.

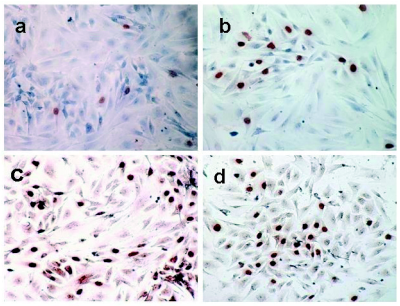


Fig. 4. – Light microscopic image of cultured Sertoli cells labelled by BrdU. a) no treatment; b) treated with 10ng/mL GDNF; c) treated by 10ng/mL GDNF plus 50ng/mL FSH; d) 50ng/mL FSH only.

Contribution of GFRα1/NCAM signalling to GDNF-induced Sertoli cell proliferation

We would like to know, as the first step to elucidating the molecular mechanism of the stimulatory effect of GDNF on mouse Sertoli cell proliferation, whether its receptor subunits were present on the membrane of mouse Sertoli cells. As shown in Fig. 5, the ligand binding subunit GFRα1 was detected both by RT-PCR and immunocytochemistry. Interestingly, Ret, which represented the first identified GDNF signalling pathway, was not expressed, while NCAM, which represented the alternative pathway, was expressed at both RNA and protein levels, suggesting that the stimulatory effect of GDNF on Sertoli cells was most likely mediated by signalling pathways starting from the GFRα1/NCAM receptor complex. This proposition was supported by the observation that anti-NCAM antibody (4mg/mL) reduced the stimulatory effect of GDNF significantly in serum-free culture (Fig. 6). The percentage of BrdU-positive cells in the total number of cells under GDNF treatment was decreased from 23.6% to 10.8% (P=0.028) by anti-NCAM antibody.

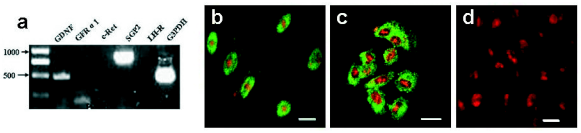


Fig. 5. – GDNF receptor subunit expression in immature mouse Sertoli cells. RT-PCR analysis (a) indicated that GFRα1 but not Ret was expressed in cultured immature Sertoli cells. SGP2 and G3PDH were included as positive controls while LH-R was used as a negative control. Immunocytochemical analysis of GFRα1 (b), NCAM (c) and Ret (d) on cultured Sertoli cells. Marker proteins were stained green while the nuclei were stained red by propidium iodide. Scale bar: 20mm.

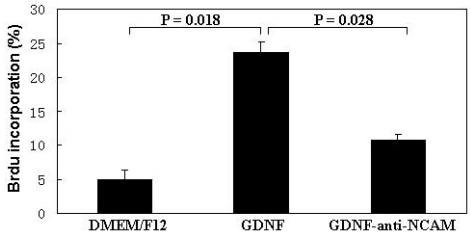


Fig. 6. – Reduction of stimulatory effect of GDNF on Sertoli cell proliferation by anti-NCAM antibody. Values were expressed as the percentages of BrdU-positive cells in total cells from results of three independent experiments (mean±S.E.M; n=3). Statistical differences were analyzed through Tukey's post hoc test. Concentrations of GDNF and anti-NCAM antibody were 10ng/mL and 4mg/mL respectively.

DISCUSSION

The proliferation of Sertoli cells has been reported to be stimulated by hormones, such as FSH (Griswold et al., 1977) and estrogen (Sharpe et al., 1998), and other paracrine growth factors including insulin-like growth factors I and II (IGF- I and IGF- II) (Borland et al., 1984), fibroblast growth factor (FGF) and transforming growth factor-α (TGF-α) (Petersen et al., 2001). Recently, studies reported that the stimulatory effect of GDNF on Sertoli cell proliferation was observed in cultured neonatal rat seminiferous tubules and in Sertoli cells from fetal mouse testis (Hu et al., 1999; Wu et al., 2005). In the present study, we used Sertoli cell cultures prepared from neonatal mice to examine whether and how GDNF stimulates the proliferation of Sertoli cells. To this end, highly purified Sertoli cell cultures were first established. The purity of our cultures was assessed by immunocytochemistry for cell markers. Vimentin and smooth muscle α-actin have been shown to be specific markers for Sertoli cells and peritubular myoid cells in the testis, respectively (Paranko et al., 1986; Tung & Fritz, 1990). The results of immunocytochemical assay verified that about 98% of the cultured cells were Sertoli cells with the remaining 2% being contaminating peritubular myoid cells, indicating that our Sertoli cell cultures, together with the serum-free medium, should be sufficient for elucidating the effects of GDNF on immature mouse Sertoli cells and signalling pathway mediating such effects.

A previous study indicated that FSH but not testosterone up-regulated GDNF mRNA expression (Tadokoro et al., 2002). This observation was confirmed in our present study by means of RT-PCR and immunohistochemistry. We also observed that estrogen had no effect on the expression of GDNF. Two studies indicated that GDNF stimulated the proliferation of immature Sertoli cells from newborn rats and fetal mice (Hu et al., 1999; Wu et al., 2005). Here we also confirmed the proliferation-promoting effects of GDNF on Sertoli cells in culture prepared with newborn mouse testes. However, the stimulatory effect of GDNF was only observed in the presence of FSH according to one study (Hu et al., 1999), a phenomenon not observed in another previous report (Wu et al., 2005) or in our study. The discrepancy was probably caused by the different cultures used – seminiferous tubule fragments and homogeneous cells were used respectively in the types of studies. Besides, the different animals (rat versus mouse) used in these studies should also be taken into account, and we suggested that the mechanisms of proliferation-promoting effects of GDNF on Sertoli cells in rat should slightly differ from those in mouse.

Our study also showed that GDNF in combination with FSH had more potent effects on Sertoli cell proliferation than GDNF alone, which was probably caused by accumulation of proliferation-promoting effects of the two factors on Sertoli cells. It has been known that FSH was a strong mitogenic hormone of Sertoli cells (Griswold et al., 1977; Meachem et al., 1996), and the expression of GDNF was up-regulated by FSH discussed as above, so the action of additional GDNF secreted by Sertoli cells should also be considered. Alternatively,

other possibilities might exist. For example, one factor could render the cells more sensitive to the action of the other one.

GDNF signals through the receptor GFRα1, which activates Ret (Durbec et al., 1996; Treanor et al., 1996). GDNF in complex with GFRα1 also signals through the NCAM independent of Ret (Paratcha, et al., 2003). However, the GDNF signalling in Sertoli cells had not yet been characterized. In the present study, we demonstrated for the first time that NCAM but not Ret was expressed in cultured mouse Sertoli cells at both RNA and protein levels. However, a previous study showed that the Ret mRNA was present in Sertoli cells from 20- and 55-day-old rats (Fouchecourt et al., 2006), which contradicts our results of this study. We suggest that this discrepancy was probably caused by the differences in experimental materials used: not 100% pure Sertoli cell fraction from 20- and 55-day-old rats in Fouchecourt's report, and purified Sertoli cell cultures from 4- to 5-day-old mice in our studies were used, indicating that the GDNF signalling might be divergent between rat and mouse. Therefore, we still propose that the stimulatory effect of GDNF on mouse Sertoli cell proliferation was mediated by pathway(s) starting from the NCAM, but not Ret subunit. This was supported by the fact that anti-NCAM antibody significantly reduced the proliferation-promoting effect of GDNF on Sertoli cells. The activation of downstream components of the NCAM pathway(s) should be addressed in future studies.

CONCLUSIONS

In conclusion, our data confirmed that GDNF, an autocrine growth factor, was produced by Sertoli cells and regulated by FSH, and played an important role in Sertoli cell proliferation via GDNF/NCAM pathway in the testis development of mouse.

ACKNOWLEDGEMENTS

This work was supported by the National Natural Science Foundation of China (Grant No. 30300182 and 30230190), and the National "973 Project" Fund (G199905590 and G1999055901).

REFERENCES

Arenas E, Trupp M, Akerud P & Ibanez CF (1995). GDNF prevents degeneration and promotes the phenotype of brain noradrenergic neurons in vivo. Neuron, 15(6): 1465-1473.

Borland K, Mita M, Oppenheimer CL, Blinderman LA, Massague J, Hall PF & Czech MP (1984). The actions of insulin-like growth factors I and II on cultured Sertoli cells. Endocrinology, 114(1): 240-246.

Collard MW & Griswold MD (1987). Biosynthesis and molecular cloning of sulfated glycoprotein 2 secreted by rat Sertoli cells. Biochemistry, 26(12): 3297-3303.

Davidoff MS, Middendorff R, Koeva Y, Pusch W, Jezek D & Muller D (2001). Glial cell line-derived neurotrophic factor (GDNF) and its receptors GFRalpha-1 and GFRalpha-2 in the human testis. Italian Journal of Anatomy and Embryology, 106 (2 Suppl 2): 173-180.

Durbec P, Marcos-Gutierrez CV, Kilkenny C, Grigotiou M, Wartiowaara K, Suvanto P, Smith D, Ponder B, Costantini F, Saarma M & et al. (1996). GDNF signalling through the Ret receptor tyrosine kinase. Nature, 381(6585): 789-793.

Fouchecourt S, Godet M, Sabido O & Durand P (2006). Glial cell-line-derived neurotropic factor and its receptors are expressed by germinal and somatic cells of the rat testis. The Journal of Endocrinology, 190: 59-71.

Fukuda N, Ichihara M, Morinaga T, Kawai K, Hayashi H, Murakumo Y, Matsuo S & Takahashi M (2003). Identification of a novel glial cell line-derived neurotrophic factor-inducible gene required for renal branching morphogenesis. Journal of Biological Chemistry, 278(50): 50386-50392.

Golden JP, DeMaro JA, Osborne PA, Milbrandt J & Johnson EM (1999). Expression of neurturin, GDNF, and GDNF family-receptor mRNA in the developing and mature mouse. Experimental Neurology, 158(2): 504-528.

Griswold MD, Solari A, Tung PS & Fritz IB (1977). Stimulation by follicle-stimulating hormone of DNA synthesis and of mitosis in cultured Sertoli cells prepared from testes of immature rats. Molecular and Cellular Endocrinology, 7(2): 151-165.

Hu J, Shima H & Nakagawa H (1999). Glial cell line-derived neurotropic factor stimulates sertoli cell proliferation in the early postnatal period of rat testis development. Endocrinology, 140(8): 3416-3421.

Kanatsu-Shinohara M, Miki H, Inoue K, Ogonuki N, Toyokuni S, Ogura A & Shinohara T (2005). Long-term culture of mouse male germline stem cells under serum-or feeder-free conditions. Biology of Reproduction, 72(4): 985-991.

Kubota H, Avarbock MR & Brinster RL (2004). Growth factors essential for self-renewal and expansion of mouse spermatogonial stem cells. Proceedings of the National Academy of Sciences of the United States of America, 101(47): 16489-16494.

Laslett AL, Li LH, Jester WF Jr & Orth JM (2000). Thyroid hormone down-regulates neural cell adhesion molecule expression and affects attachment of gonocytes in Sertoli cell-gonocyte cocultures. Endocrinology, 141(5): 1633-1641.

Lin LF, Doherty DH, Lile JD, Bektesh S & Collins F (1993). GDNF: a glial cell line-derived neurotrophic factor for midbrain dopaminergic neurons. Science, 260(5111): 1130-1132.

Masure S, Cik M, Pangalos MN, Bonaventure P, Verhasselt P, Lesage AS, Leysen JE & Gordon RD (1998). Molecular cloning, expression and tissue distribution of glial-cell-line-derived neurotrophic factor family receptor alpha-3 (GFRalpha-3). European Journal of Biochemistry, 251(3): 622-630.

Meachem SJ, Mclachlan RI, De Kretser DM, Robertson DM & Wreford NG (1996). Neonatal exposure of rats to recombinant follicle stimulating hormone increases adult Sertoli and spermatogenic cell numbers. Biology of Reproduction, 54(1): 36-44.

Mosmann T (1983). Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. Journal of Immunological Methods, 65(1-2): 55-63.

Ogawa T, Ohmura M, Tamura Y, Kita K, Ohbo K, Suda T & Kubota Y (2004). Derivation and morphological characterization of mouse spermatogonial stem cell lines. Archives of Histology and Cytology, 67(4): 297-306.

Orth JM, Jester WF, Li LH & Laslett AL (2000). Gonocyte-Sertoli cell interactions during development of the neonatal rodent testis. Current topics in Developmental Biology, 50: 103-124.

ORTH JM & JESTER WF (1995). NCAM mediates adhesion between gonocytes and Sertoli cells in cocultures from testes of neonatal rats. Journal of Andrology, 16(5): 389-399.

PARANKO J, KALLAJOKI M, PELLINIEMI LJ, LEHTO VP & VIRTANEN I (1986). Transient coexpression of cytokeratin and vimentin in differentiating rat Sertoli cells. Developmental Biology, 117: 35-44.

PARATCHA G, LEDDA F & IBANEZ CF (2003). The neural cell adhesion molecule NCAM is an alternative signaling receptor for GDNF family ligands. Cell, 113(7): 867-879.

PETERSON C, BOITANI C, FROYSA B & SODER O (2001). Transforming growth factor-alpha stimulates proliferation of rat Sertoli cells. Molecular and Cellular Endocrinology, 181(1-2): 221-227.

ROUSSA E & KRIEGLSTEIN K (2004). GDNF promotes neuronal differentiation and dopaminergic development of mouse mesencephalic neurospheres. Neuroscience Letters, 361(1-3): 52-55.

RYU BY, KUBOTA H, AVARBOCK MR & BRINSTER RL (2005). Conservation of spermatogonial stem cell self-renewal signaling between mouse and rat. Proceedings of the National Academy of Sciences of the United States of America, 102(40): 14302-14307.

SARIOLA H & SAARMA M (2003). Novel functions and signalling pathways for GDNF. Journal of Cell Science, 116(Pt 19): 3855-3862.

SHAKYA R, WATANABE T & COSTANTINI F (2005). The role of GDNF/Ret signaling in ureteric bud cell fate and branching morphogenesis. Developmental Cell, 8 (1): 65-74.

SHARPE RM, ATANASSOVA N, MCKINNELL C, PARTE P, TURNER KJ, FISHER JS, KERR JB, GROOME NP, MACPHERSON S, MILLAR MR & SAUNDERS PT (1998). Abnormalities in functional development of the Sertoli cells in rats treated neonatally with diethylstilbestrol: a possible role for estrogens in Sertoli cell development. Biology of Reproduction, 59(5): 1084-1094.

TADOKORO Y, YOMOGIDA K, OHTA H, TOHDA A & NISHIMUNE Y (2002). Homeostatic regulation of germinal stem cell proliferation by the GDNF/FSH pathway. Mechanisms of Development, 113(1): 29-39.

TREANOR JJ, GOODMAN L, DE SAUVAGE F, STONE DM, POULSEN KT, BECK CD, GRAY C, ARMANINI MP, POLLOCK RA, HEFTI F, PHILLIPS HS, GODDARD A, MOORE MW, BUJ-BELLO A, DAVIES AM, ASAI N, TAKAHASHI M, VANDLEN R, HENDERSON CE & ROSENTHAL A (1996). Characterization of a multicomponent receptor for GDNF. Nature, 382(6586): 80-83.

TUNG PS & FRITZ IB (1990). Characterization of rat testicular peritubular myoid cells in culture: alpha-smooth muscle isoactin is a specific differentiation marker. Biology of Reproduction, 42: 351-365.

WU Z, TEMPLEMAN JL, SMITH RA & MACKAY S (2005). Effects of glial cell line-derived neurotrophic factor on isolated developing mouse Sertoli cells in vitro. Journal of Anatomy, 206(2): 175-184.

Received: December 9, 2007
Accepted: April 16, 2008

Leptin promoted meiotic maturation of bovine oocytes and development of parthenogenetic activation and yak (*Bos grunniens*)-bovine interspecies cloned embryos

Zhilin Guo, Yongsheng Wang & Yong Zhang*

Institute for bioengineering, Northwest A & F University, Yangling, Shaanxi 712100, China.

Corresponding author : * Dr. Yong Zhang; E-mail: gzl7511@yahoo.com.cn; Institute for bioengineering, Northwest A & F University, Yangling, Shaanxi 712100, China; Tel: +86 29 87080085; Fax: +86 29 87080085

ABSTRACT. Leptin has a central role in the regulation of oocyte and embryo development. The objective of the current study was to investigate the effect of leptin addition during oocyte maturation *in vitro* on meiotic maturation of bovine oocytes and development of parthenogenetic activation (PA) and yak-bovine interspecies cloned embryos. In addition, the effect of leptin supplementation in embryo culture medium on preimplantation development of yak-bovine interspecies cloned embryos was investigated. Leptin addition at 10 and 100ng/mL during oocyte maturation significantly increased the proportion of bovine oocytes that developed to metaphase II (MII) stage compared to the control (81.0±3.8 and 77.0±4.0 versus 62.2±1.8, $P<0.05$). Leptin at 10ng/mL significantly increased the blastocyst rate after PA compared to the control (31.6±4.3 versus 20.4±3.2, $P<0.05$). Leptin supplementation at 10ng/mL during oocyte maturation strikingly increased the blastocyst development of yak-bovine interspecies cloned embryos (33.2±4.0 versus 22.8±2.7, $P<0.05$) and the inner cell mass (ICM) and total cell number per blastocyst, and reduced the incidence of apoptotic cells per blastocyst compared to the control. When present in the embryo culture medium, leptin at 100ng/mL significantly increased the blastocyst development of yak-bovine interspecies cloned embryos compared to the control (34.2±4.9 versus 22.0±3.2, $P<0.05$). Meanwhile, leptin at 100 and 1000ng/mL significantly increased ICM and total cell number per blastocyst and reduced the proportion of apoptotic cells per blastocyst. In conclusion, leptin supplementation promoted the meiotic maturation of bovine oocytes and the developmental capacity of PA and yak-bovine interspecies cloned embryos.

KEY WORDS : yak, leptin, oocyte maturation, interspecies cloning

INTRODUCTION

Leptin, the 16-kDa product of the obese (ob) gene, has been implicated to play an important role in the regulation of food intake and energy expenditure (ZHANG et al., 1994). In addition, leptin is known to regulate diverse reproductive functions (HOLNESS et al., 1999). The ob/ob mice expressing a truncated form of leptin were obese and infertile (ZHANG et al., 1994). Exogenous leptin supplementation can restore normal weight and fertility in ob/ob mice, suggesting that leptin influences reproduction in a direct way (CHEHAB et al., 1996).

Recently, leptin has been detected to be expressed in mouse (ANTCZAK & VAN BLERKOM, 1997; KAWAMURA et al., 2002) and human oocytes (CIOFFI et al., 1997); mouse (KAWAMURA et al., 2002) and human (CIOFFI et al., 1997) follicular fluid; and human granulosa and cumulus cells (CIOFFI et al., 1997). The leptin receptor (LEPR) has high sequence homology to the class I cytokine receptor superfamily (CHEN et al., 1996), and different 3' terminal mRNA splicing produces six known isoforms (TARTAGLIA et al., 1995; CIOFFI et al., 1996; LEE et al., 1996). The long isoform containing a 302-residue-long intracellular domain was detected in the hypothalamus and also in some peripheral tissues (TARTAGLIA et al., 1995; BJORBAK et al., 1997), and this isoform regulates most leptin signalling (LEE et al., 1996; WHITE et al., 1997) by both pathways of the mitogen-activated protein kinase (MAPK) and the signal transducer and activator of transcription 3 (STAT3) (MATSUOKO et al., 1999). LEPR mRNA is expressed in mouse oocytes (MATSUOKO et al., 1999; KAWAMURA et al., 2002) and in bovine blastocysts (BOELHAUVE et al., 2005). Leptin, at physiological concentrations, causes tyrosine phosphorylation of STAT3 in mouse metaphase II (MII) oocytes (MATSUOKA et al., 1999). Thus, leptin is likely a regulator of oocytes and its surrounding cells.

The *in vitro* production (IVP) system in cattle, including somatic nuclear transfer (SCNT), is a crucial tool for basic research and for preservation of endangered mammals. Although there have been a few successes (WHITE et al., 1999; LANZA et al., 2000; LOI et al., 2001; LEE et al., 2003; SANSINENA et al., 2003), the efficiency of interspecies SCNT in birth of live offspring is still low. Incomplete cytoplasmic and nuclear maturation of the oocyte is a limiting step determining the ability of the oocyte to successfully support a somatic nucleus to undergo reprogramming, cleavage and embryo development. Growth factors and cytokines lead to proper nuclear and cytoplasmic maturation of the oocyte by a complex network (GREEWALD & ROY, 1994). Recently, there have been some reports that leptin supplementation promotes oocyte maturation and preimplantation development of embryos. It has been demonstrated that 10-100ng/mL leptin supplementation stimulates porcine oocyte maturation (CRAIG et al., 2004; ZHANG et al., 2007). Addition of leptin in embryo culture medium promotes preimplantation embryo development *in vitro* in a dose-dependent manner in the mouse (KAWAMURA et al., 2002). In bovines, the presence of leptin during oocyte maturation has been found to increase the proportion of oocytes developed to blastocyst stage after *in vitro* fertilization (IVF) and to

reduce the proportion of apoptotic cells per blastocyst (BOELHAUVE et al., 2005). However, the data about the effect of leptin on meiotic maturation of bovine oocytes and preimplantation development of interspecies cloned embryos when included in *in vitro* culture medium are insufficient. In addition, controversial results have been reported about the effect of leptin on preimplantation development of murine embryos (KAWAMURA et al., 2002; FEDORCSAK & STORENG, 2003; SWAIN et al., 2004). Thus, the role of leptin during *in vitro* development of mammalian oocytes and embryos needs to be further researched. Accordingly, in the present study, we investigated whether leptin addition to *in vitro* maturation (IVM) medium influences meiotic maturation of bovine oocytes and subsequent preimplantation development of parthenogenetic activation (PA) embryos. In addition, the effect of leptin supplementation in IVM medium of bovine oocytes on development of the embryo derived from yak-bovine interspecies nuclear transfer was assessed in this study. We also investigated the effect of leptin addition to embryo culture medium on preimplantation development of yak-bovine interspecies nuclear transfer embryo.

MATERIALS AND METHODS

1. Chemicals

Unless otherwise stated, chemicals were purchased from Sigma-Aldich Crop. (St. Louis, MO)

2. Donor cell preparation

Ear skin was biopsied from an adult black yak (*Bos grunniens*). The tissue was kept in PBS (Gibco, USA) at 4°C during transportation to the laboratory. After removal of cartilage, skin tissues were minced into about 1mm^3 pieces with a surgical blade, and seeded in the 25cm^2 tissue culture flask (Nunc, Roskilde, Denmark). The tissue was cultured in 5mL Dulbecco's modified Eagle's medium (DMEM, Gibco, USA) plus 20% (v/v) fetal bovine serum (FBS, Gibco, USA) under a humidified atmosphere of 5% CO_2 in air at 37°C. When primary cell layer reached 85% confluence after 8-10 days, the cells were harvested by 0.25% (w/v) trypsinization (Gibco, USA) and reseeded at a density of 100,000cells/mL in 25cm^2 tissue culture flasks. Fibroblast cells at passages 4-8 were harvested by 0.25% (w/v) trypsinization and frozen in DMEM supplemented with 20% (v/v) FBS and 10% (v/v) dimethylsulfoxide in liquid nitrogen at -192°C. Prior to SCNT, the cells were thawed, cultured for 3 days until 80% confluence and subjected to serum-starvation by being cultured for another 3-5 days in DMEM supplemented with 0.2% (v/v) FBS.

3. *In vitro* maturation of bovine oocytes

Bovine ovaries were collected from a local slaughter house and transported to the laboratory within 4h in saline solution at 30°C. Cumulus-oocyte complexes (COCs) from domestic cattle were aspirated from 2-8mm in diameter antral follicles using an 18 gauge needle and washed several times in IVM medium that contained TCM-199 (Gibco, USA), supplemented with 10% (v/v) FBS, 2mM $NaHCO_3$, 1.0% (v/v) penicillin-streptomycin (10,000IU/mL and 10,000μg/mL, respectively; pen-strep; Gibco), 10μg/mL LH and 1μg/mL estradiol-17β. Those oocytes that had uniform cytoplasmic appearance and were enclosed within three or more layers of viable compact granulosa cells were selected. Groups of 50 oocytes were matured in 500μl of IVM medium under mineral oil in four-well dishes at 39°C in a humidified atmosphere of 5% CO_2 in air.

4. Assessment of oocyte nuclear status

At the end of *in vitro* maturation culture, oocytes were denuded from cumulus cells by pipetting and vortexing in 0.5mg/mL hyaluronidase. Denuded oocytes were mounted on a glass slide and fixed with acetic acid: ethanol (1:3 v/v) for at least 30h at 4°C. Then, oocyte staining was performed in acetic acid-orcein (1% orcein in 45% acetic acid) and they were examined under a phase-contrast microscope. Oocytes were classed into intact nucleus (GV), the germinal vesicle break down (GVBD) and metaphase II (MII) stage. Oocytes with abnormal chromatin or no chromatin were selected as degenerated oocytes.

5. Somatic nuclear transfer and embryo culture

The zona-free method of Handmade Cloning (VAJTA et al., 2001; VAJTA et al., 2003; BHOJWANI et al., 2005) was used in this experiment. After *in vitro* maturation for 20-22h, cumulus cells' enclosed COCs were removed in 0.5mg/mL hyaluronidase dissolved in Hepes-buffered TCM-199 medium (T0) for 3min. The zona pellucida of nude oocytes was digested in 1.5mg/mL pronase dissolved in T0 at 39°C. Under stereomicroscopic control, manual bisections of oocytes were performed with Ultra Sharp Splitting Blades (AB Technology, Pullman, WA) in T0 supplemented with 20% (v/v) FBS, 5μg/mL cytochalasin B. Then, chromatin staining of all half-oocytes was performed with 10μg/mL Hoechst 33342 dissolved in T0. Half-oocytes without chromatin staining were registered under a fluorescence microscope. Registered half-oocytes were collected in TCM-199 supplemented with 20% (v/v) FBS (T20) as recipient cytoplasts. After serum-starvation, the donor cells were harvested by pipetting in 0.05% (w/v) trypsin dissolved in PBS and collected in T20 at room temperature.

For embryo construction, half of prepared cytoplasts were individually incubated in 200μg/mL phytohemagglutinin dissolved in T0 for 5sec. Cell-cytoplast pairing was achieved by gently pressing a prepared cytoplast over a single fibroblast cell. Then, the cell-cytoplast pairs were aligned using an alternating current of 15V AC in a platinum wire fusion chamber. Fusion medium contained 0.3M mannitol, 0.1mM $MgSO_4$ and 0.05mM $CaCl_2$. A second cytoplast was then aligned to the single cell-cytoplast pair in the fusion chamber. Fusion was performed with a single pulse of 3.7KV/cm for 4μsec using a BTX Electro Cell Manipulator 200 (Biotechnologies and Experimental Research Inc., San Diego, CA, USA). After fusion, reconstructed embryos were cultured in 1mL T20 containing 2μM Ca ionophore A23187 for 5min, then embryos were transferred into 5μl droplets of 2mM 6-dimethylaminopurine (6-DMAP) for 6h for further chemical activation. After chemical activation, each single

embryo was cultured in well of the wells (WOWs) (VAJTA et al., 2000) in 400µl modified synthetic oviductal fluid (mSOFaa) medium, as previously described (HOLM et al., 1999), covered with oil. Embryos were cultured at 39°C in 5% CO_2, 5%O_2 and 90%N_2 for 7 days.

6. Parthenogenetic activation

After bovine oocytes had matured for 20-22h, parthenogenetic activation was performed using the same method above. Oocytes were activated in 2µM Ca ionophore A23187 for 5min and then exposed in 2mM 6-DMAP for 6h. Activated oocytes were cultured in the mSOFaa at 39°C in 5% CO2, 5%O_2 and 90%N_2 for 7 days.

7. Embryo transfer

SCNT embryos of excellent quality at the blastocyst stage were transferred into Holstein recipients on Day 7±1 of the estrous cycle. Two or three embryos per recipient were nonsurgically introduced into the uterine horn ipsilateral to the ovary containing the corpus luteum. Pregnancy was diagnosed by rectal palpation at 60 and 120 days, respectively, after embryo transfer.

8. Different staining of inner cell mass (ICM) and trophectoderm (TE) cells

Day 7 blastocysts derived from yak-bovine interspecies nuclear transfer were randomly selected and subjected to different staining according to previously described methods (THOUAS et al., 2001). The zona pellucida of each blastocyst was removed by incubating in 0.5% pronase for 1min. Blastocysts were treated for 5-7sec with 0.2% (v/v) Triton X-100 and 0.3mg/mL propidium iodide dissolved in PBS and then transferred into 20µg/mL Hoechst-33342 dissolved in glycerol for 2-3h. Embryos were mounted on a glass slide in a drop of glycerol solution. Cell numbers were determined using a fluorescence microscope. Blue nuclei were considered as ICM cells and red or pink nuclei as trophoblast cells.

9. TUNEL assay for apoptosis detection

Apoptotic blastomeres were detected by TUNEL assay as previously described (GAVRIELI et al., 1992) using an In situ Apoptosis Detection Kit (Takara Bio Inc., Shigaken, Japan). Blastocysts were fixed in 4% (v/v) paraformaldehyde dissolved in PBS for 1h at room temperature. Blastocysts were permeabilized with 0.5% (v/v) Triton X-100 for 1h at room temperature. After blastocysts were washed three times with 1mg/mL BSA dissolved in PBS, they were incubated in a terminal deoxynucleatidyl transferase-labelling buffer for 3h at room temperature. Then, blastocysts were transferred into 25µl fluorescein isothiocyanate solution for 1.5h and incubated with 200µl 0.5µg/mL propidium iodide for 30min at room temperature. Blastocysts were treated with 0.2M diazabicyclo-octane in PBS supplemented with 50% (v/v) glycerol and then mounted on a glass slide. Each blastocyst was examined for total number of nuclei and number of TUNEL-labelled nuclei under a fluorescence microscope. The apoptotic cells appeared as yellow, but normal cells as red.

10. Experimental design

Experiment 1: This experiment was designed to investigate the effect of leptin addition in IVM medium on developmental capacity of bovine oocytes. COCs were cultured in IVM medium containing 0 (control), 1, 10 or 100ng/mL recombinant human leptin (Sigma Aldich Co.), designed according to BOELHAUVE et al. (2005). After *in vitro* maturation for 20-22h, some oocytes were selected to assess nuclear status, and others were subjected to PA or yak-bovine interspecies nuclear transfer.

Experiment 2: This experiment was designed to investigate the effect of leptin supplementation in IVM medium on developmental competence of PA embryos. After denuded oocytes derived from different dosage leptin treatment groups were performed to PA, the rates of cleavage and blastocysts formation were examined at Day 3 and Day 7 after activation, respectively.

Experiment 3: This experiment was designed to check the effect of leptin addition during oocyte maturation on development of yak-bovine interspecies cloned embryo. Accordingly, the results of the above two experiments, 10 or 100ng/mL leptin treatment were considered as optimum treatment groups and selected to be subjected to further SCNT experimentation. After maturation for 20-22h, oocytes were subjected to SCNT. The cleavage rate at Day 3 after reconstruction, and development to the blastocyst stage at Day 7 after reconstruction, were assessed. This experiment was replicated five times, using 278-313 oocytes per treatment. TE/ICM cell numbers per blastocyst derived from different dosage leptin treatment groups were examined at Day 7 after reconstruction. TUNEL analysis to blastocyst was performed at Day 7 after reconstruction.

Experiment 4: This experiment was designed to investigate the effect of leptin supplementation in embryo culture medium on developmental capacity of cloned embryos derived from yak-bovine interspecies nuclear transfer. Reconstructed embryos were randomly allocated and cultured in the mSOFaa supplemented with 0, 1, 10, 100 or 1000ng/mL leptin from Day 0 to Day 7. The concentrations of leptin were selected according to KAWAMURA et al. (2002). The cleavage rate at Day 3 and development of the blastocyst stage at Day 7 after reconstruction were assessed. This experiment was replicated five times, using 222-291 oocytes per treatment. ICM/TE cell number and TUNEL analysis were performed at Day 7 after reconstruction.

11. Statistical analysis

All data were analyzed using a statistical system (SPSS for windows 13.0) program. The differences in embryos development among groups were analyzed using one-way ANOVA. Post hoc analysis was performed according to LSD test. Meanwhile, ICM/TE cell number and apoptosis cells per blastocyst among experimental groups were analyzed using the same methods. The data are presented as mean±SEM. A P value less than 0.05 indicated significant differences among the groups.

RESULTS

1. Effect of leptin addition in IVM medium on developmental capacity of bovine oocytes

The effect of leptin addition in IVM medium on developmental capacity of bovine oocytes was evaluated in this experiment. There was no effect of leptin supplementation in the 1ng/mL leptin treatment group on the proportion of oocytes reaching MII stage, as compared to the control. However, treatment of oocytes with 10 or 100ng/mL leptin increased the proportion of oocytes developed to MII stage, as compared to the control (P<0.05) (Table 1).

TABLE 1

Effect of leptin addition in *in vitro* maturation medium on developmental capacity of bovine oocytes.

Group (leptin treatment)	No. of oocytes treated	No. of oocytes developed to a specific stage (%)			No. of degenerated oocytes
		GV	GVBD	MII	
0ng/mL	104	8 (7.8±1.8)	17 (16.6±2.4)	65 (62.2±1.8)^{c}	14 (13.4±2.6)
1ng/mL	157	16 (10.2±1.1)	23 (15.2±2.4)	107 (68.0±1.9)^{bc}	11 (6.8±1.3)
10ng/mL	178	10 (6.0±2.0)	13 (7.0±2.3)	143 (81.0±3.8)^{a}	12 (6.3±3.1)
100ng/mL	163	18 (10.6±3.2)	11 (6.0±3.4)	124 (77.0±4.0)^{ab}	10 (5.6±2.6)

Values with different letters (a, b and c) in the same column are significantly different (P<0.05).
Oocytes were classed into intact nucleus (germinal vesicle, GV), the germinal vesicle break down (GVBD) and metaphase II (MII) stage according to the specific stage of development. Oocytes with abnormal chromatin or no chromatin were selected as degenerated oocytes.

2. Effect of leptin supplementation in IVM medium on developmental competence of embryo derived from PA

The purpose of this experiment was to assess whether leptin increases development capacity of embryo derived from PA. There was no effect of leptin addition during IVM on cleavage rate of PA embryo at Day 3 after activation (71.2±3.5, 73.0±3.1, 73.6±4.6, and 69.2±5.9, cleavage rate for 0, 1, 10 and 100ng/mL leptin addition, respectively), but leptin treatment at 10ng/mL (31.6±4.3) increased the proportion of oocytes developed to the blastocyt stage at Day 7 after activation as compared to the control (20.4±3.2) (Fig. 1). In addition, leptin treatment at 100ng/mL (29.0±3.1) also increased the proportion of oocytes developed to the blastocyst stage, although there was no significant difference compared to the control.

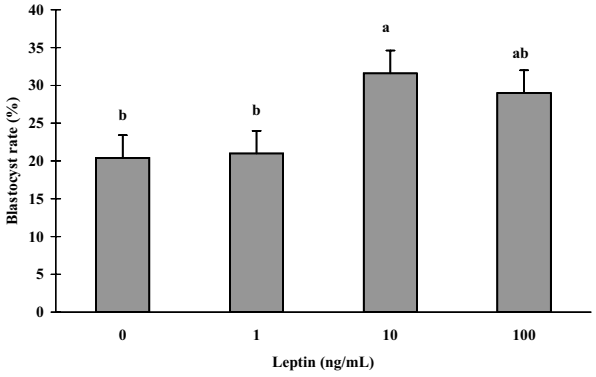


Fig. 1. – Effect of leptin addition in *in vitro* maturation medium on developmental capacity of parthenogenetic activation (PA) embryo in bovine. The proportion of oocytes developed to the blastocyst stage at Day 7 after activation was assessed. Results are shown as means±SEM of five replicates, using 132-185 oocytes per treatment. Different letters (a and b) denote significant differences (P<0.05).

3. Effect of leptin supplementation during *in vitro* maturation on preimplantation development of yak-bovine nuclear transfer embryos

In this experiment, the effect of leptin on preimplantation development of yak-bovine interspecies cloned embryos was determined. There was no effect of leptin addition during oocyte maturation on cleavage rate of yak-bovine cloned embryos at Day 3 after reconstruction (70.2±2.4, 72.8±2.3 and 69.6±4.8, cleavage rate for 0, 10 and 100ng/mL leptin treatment, respectively). However, as shown in Fig. 2A, leptin addition at 10ng/mL (33.2±4.0) increased the proportion of oocytes developed to the blastocyst stage as compared to the control (22.8±2.7) (P<0.05). Meanwhile, as shown in Fig. 2B and Fig. 2C, leptin at 10ng/mL (47.8±8.6, 111.0±17.1) increased significantly inner cell mass and total cell number per blastocyst as compared to the control (28.3±5.0, 75.4±10.4) (P<0.05), and leptin at 10 (4.7±0.4) and 100ng/mL (4.4±0.4) decreased the incidence of TUNEL-positive blastomeres per blastocyst as compared to the control (10.7±1.1) (P<0.01).

4. Effect of leptin supplementation in embryo culture medium on development of yak-bovine interspecies cloned embryos

As shown in Fig. 3A, the rate of blastocyst formation of yak-bovine interspecies cloned embryos significantly increased in the 100ng/mL leptin treatment group (34.2±4.9) compared to the control (22.0±3.2), but the cleavage rate did not differ significantly between the treatment groups (75.4±5.3, 70.0±6.1, 71.0±5.0, 69.4±6.9 and 69.2±5.9, cleavage rate for 0, 1, 10, 100 and 1000ng/mL leptin treatment, respectively). As shown in Fig. 3B, the ICM/Total cell number per blastocyst increased in 100 (49.8±8.9, 104.2±11.7) and 1000ng/mL (49.0±6.8, 107.1±9.3) leptin treatment group compared to the control (28.7±5.9, 74.3±6.7) (P<0.05). The proportion of apoptotic blastomeres per blastocyst in 100 (5.4±0.5) and 1000ng/mL (4.4±0.3) leptin treatment group decreased as compared to the control (7.3±0.5) (P<0.05), which is shown in Fig. 3C.

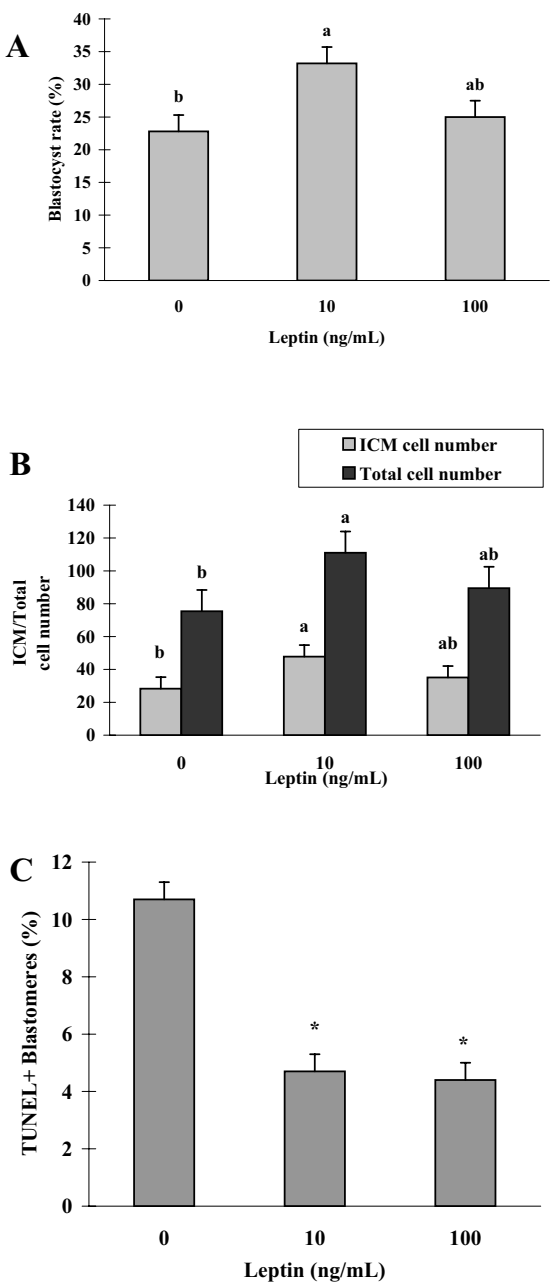


Fig. 2. – The effect of leptin at 0, 10 and 100ng/mL in *in vitro* maturation medium of bovine oocytes on preimplantation development, blastocyst cell number and blastomere apoptosis of yak-bovine interspecies cloned embryos. Blastocyst rate (A) and inner cell mass (ICM) /Total cell number per blastocyst (B) at Day 7 after reconstruction was assessed. The proportion of apoptotic blastomeres per blastocyst (C) are shown as mean±SEM of five replicates, using 20 blastocysts per treatment. Different letters (a and b) denote significant differences (P<0.05). Significant difference (P<0.01) from the control group is indicated by *.

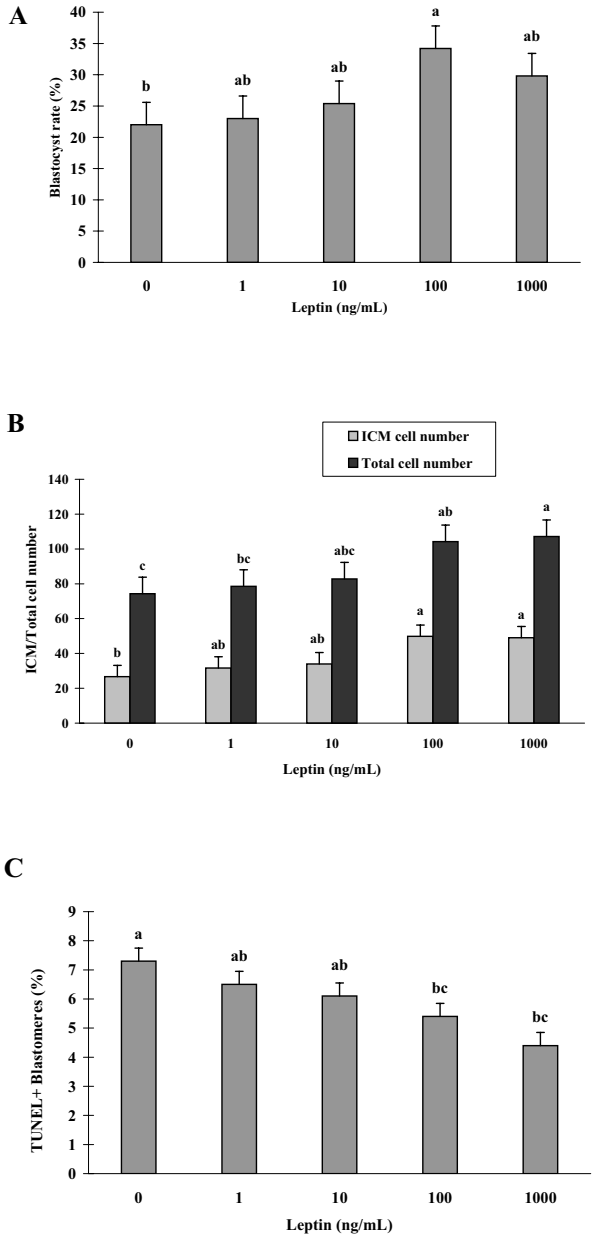


Fig. 3. – Influence of different concentrations of leptin supplementation in embryos culture medium on preimplantation development and cell apoptosis of yak-bovine interspecies cloned embryos. Rate of blastocyst development and inner cell mass (ICM) /Total cell number per blastocyst were evaluated at Day 7 after reconstruction. Number of apoptotic blastomeres per blastocyst was assessed at Day 7 after reconstruction, and shown as mean±SEM of five replicates, using 20 blastocysts per treatment. Different letters (a, b and c) denote significant difference (P<0.05).

5. Effect of leptin treatment during *in vitro* maturation of oocytes on pregnancy of yak-bovine interspecies cloned embryos in surrogate mothers

As shown in Table 2, blastocysts derived from 10ng/mL leptin treatment during oocyte maturation achieved pregnancies at pregnant Day 60 and 120 after embryo transfer, respectively. However, there was no pregnancy at Day 60 in the control group in the absence of leptin.

TABLE 2

Effect of leptin treatment in *in vitro* maturation medium of bovine oocyte on pregnancies of yak-bovine cloned embryo.

Leptin treatment	Embryos transferred	Recipients	Pregnant		Offspring
			Day 60	Day 120	
0ng/mL	45	15	0	0	0
10ng/mL	54	20	3	2	0

DISCUSSION

Previous reports have shown that leptin has a possible role in regulation of oocyte and preimplantation embryo development in the mouse, pig and bovine. Leptin addition at 100ng/mL, which is the most prominent treatment, enhanced the development of porcine oocytes reaching MII stage determined by extrusion of the first polar body (CRAIG et al., 2004). In contrast, ZHANG et al. (2007) reported that 10ng/mL leptin treatment in IVM medium is the most significant treatment to improve the rate of porcine oocytes reaching MII stage by acid-orcein staining after oocytes maturation. In the present study, 10 and 100ng/mL leptin treatment increased the proportion of bovine oocytes developed to MII stage compared to the control, and 10ng/mL leptin treatment was the most significant one. Leptin treatment reduced the rate of stained oocytes with abnormal chromatin configuration compared to the control. In pigs, 10 and 100ng/mL leptin treatment strikingly increased the rate of blastocyst formation of PA embryo, and 10ng/mL leptin treatment obviously increased the cleavage rate of PA embryo (CRAIG et al., 2004). In contrast, in another report, only 10ng/mL leptin significantly increased the rate of blastocyst formation of PA embryo (ZHANG et al., 2007). In bovine, leptin addition at 1 or 10ng/mL during oocyte maturation increased the rate of blastocyst formation and cell number per blastocyst derived from IVF embryo (BOELHAUVE et al., 2005). In the current study, 10ng/mL leptin addition obviously increased the rate of PA embryo developed to the blastocyst stage in bovine. To determine the influence of leptin on the developmental capacity of yak-bovine interspecies nuclear transfer embryo, we investigated the effect of 10 and 100ng/mL leptin treatment designed according to the results of PA experiment. In the present study, we proved for the first time that leptin treatment at 10ng/mL during oocyte maturation strikingly improved the development rate to the blastocyst stage of the yak-bovine interspecies cloned embryo. Moreover, leptin treatment at 10ng/mL produced pregnancies of yak-bovine interspecies cloned embryo at Day 120. Embryonic developmental capacity is an indicator of oocyte quality. So, our study suggests that leptin promoted the cytoplasmic maturation of bovine oocytes so that the efficiency of yak-bovine interspecies nuclear transfer was improved. Therefore, this study may indicate a possible way to overcome the inefficiency of interspecies nuclear transfer by promoting nuclear and cytoplasmic maturation of bovine oocytes.

Leptin receptor mRNA and protein are present in the mouse preimplantation embryo (KAWAMURA et al., 2002). The levels of leptin secreted by cultured human blastocysts are significantly higher than that of arrested embryos (GONZALEZ et al., 2000). Leptin and leptin receptor proteins are expressed in *in vitro* matured oocytes, and IVF or SCNT embryos in porcine (KIM et al., 2006). In pigs, CRAIG et al. (2005) demonstrated that 10ng/mL of leptin in the culture medium increased the rate of cleavage and blastocyst significantly in the IVF embryos. KIM et al. (2006) also reported that 1000ng/mL leptin addition in embryo culture medium increased the rate of blastocyst formation and the number of TE and total cells in SCNT embryos, but leptin treatment had no effect on that of IVF embryos. In mouse, KAWAMURA et al. (2002) reported that 10, 100 and 1000ng/mL leptin addition promoted the development of blastocyst, expanded blastocyst and hatched blastocysts. In the present study, leptin at 100ng/mL to the embryo culture medium increased the rate of blastocyst formation of yak-bovine interspecies cloned embryo. In addition, leptin at 100 and 1000ng/mL increased ICM/total cell number of SCNT embryo compared to the control.

It is well known that ICM/total cell number and apoptotic blastomeres in blastocysts may reflect embryo quality. In the present study, leptin at 10ng/mL in IVM medium increased ICM/total cell number of yak-bovine interspecies cloned embryo, and 10 or 100ng/mL leptin obviously reduced the incidence of apoptotic blastomeres in SCNT embryo. Therefore, our study has shown that leptin plays a positive role to improve the viability of SCNT embryo when it is present in IVM medium or embryo culture medium.

In conclusion, it was demonstrated that leptin supplementation during oocyte maturation promoted the meiotic maturation of bovine oocytes and developmental capacity of PA or SCNT embryo. Moreover, leptin addition in the embryo culture medium improved the development of yak-bovine interspecies cloned embryo.

ACKNOWLEDGEMENTS

We thank Dr. Yang Yongmei for help in manuscript writing, Mr. Zhang Xiao for help in ovary collection. This study was funded by National High Technology Research and Development Program of China (863 Program) (No.2001AA213081).

REFERENCES

ANTCZAK M & VAN BLERKOM J (1997). Oocyte influences on early development: the regulatory proteins leptin and STAT3 are polarized in mouse and human oocytes and differentially distributed within the cells of the preimplantation stage embryo. Molecular Human Reproduction, 3: 1067-1086.

Bhojwani S, Vajta G, Callesen H, Roschlau K, Kuwer A & Becker F (2005). Developmental competence of HMCTM derived bovine cloned embryos obtained from somatic cell nuclear transfer of adult fibroblasts and granulosa cells. Journal of Reproduction and Development, 51: 465-475.

Bjorbak C, Uotani S, Silva B & Flier JS (1997). Divergent signaling capacities of the long and short isoforms of the leptin receptor. Journal of Biological Chemistry, 272: 32686-32695.

Boelhauve M, Sinowatz F, Wolf & Paula-Lopes FF (2005). Maturation of bovine oocytes in the presence of leptin improves development and reduces apoptosis of *in vitro*-produced blastocysts. Biology of Reproduction, 73: 737-744.

Chehab FF, Lim ME & Lu R (1996). Correction of the sterility defect in homozygous obese female mice by treatment with the human recombinant leptin. Nature Genetics, 12: 318-320.

Chen H, Charlat O, Tartaglia LA, Woolf EA,Weng X, Ellis SJ, Lakey ND, Culpepper J, Moore KJ, Breitbart RE, Duyk GM, Tepper RI & Morgenstern JP (1996). Evidence that the diabetes gene encodes the leptin receptor: identification of a mutation in the leptin receptor gene in db/db mice. Cell, 84: 491-495.

Cioffi JA, Shafer AW, Zupancic TJ, Smith-Gbur J, Mikhail A, Platika D & Snodgrass HR (1996). Novel B219/OB receptor isoforms: possible role of leptin in hematopoiesis and reproduction. Nature Medicine, 2: 585-589.

Cioffi JA, Van Blerkom J, Antczak M, Shafer A, Wittmer S & Snodgrass HR (1997). The expression of leptin and its receptors in pre-ovulatory human follicles. Molecular Human Reproduction, 3: 467-472.

Craig J, Zhu H, Dyce PW, Petrik J & Li J (2004). Leptin enhances oocyte nuclear and cytoplasmic maturation via the mitogen-activated protein kinase pathway. Endocrinology, 145: 5355-5363.

Craig J, Zhu H, Dyce PW, Wen L & Li J (2005). Leptin enhances porcine preimplantation embryo development *in vitro*. Molecular and Cellular Endocrinology, 229: 141-147.

Fedorcsak P & Storeng R (2003). Effects of leptin and leukemia inhibitory factor on preimplantation development and STAT3 signaling of mouse embryos *in vitro*. Biology of Reproduction, 69: 1531-1538.

Gavrieli Y, Sherman Y & Bem-Sasson SA (1992). Identification of programmed cell death in situ via specific labelling of nuclear DNA fragmentation. The Journal of Cell Biology, 119: 493-501.

Gonzalez RR, Caballero-Campo P, Jasper M, Mercader A, Devoto L & Pellicer A (2000). Leptin and leptin receptor are expressed in the human endometrium and endometrial leptin secretion is regulated by the human blastocyst. Journal of Clinical Endocrinology Metabolism, 85: 4883-4888.

Greewald GS & Roy SK (1994). Follicular development and its control. In: Knobil E & Neill JD (eds), The Physiology of Reproduction, Raven Press, New York: 629-724.

Holm P, Booth PJ, Schmidt MH, Greve T & Callesen H (1999). High bovine blastocyst development in a static *in vitro* production system using SOFaa medium supplemented with sodium citrate and myo-inositol with or without serum-proteins. Theriogenology, 52: 683-700.

Holness MJ, Munns MJ & Sugden MC (1999). Current concepts concerning the role of leptin in reproductive function. Molecular and Cellular Endocrinology, 157: 11-20.

Kawamura K, Sato N, Fukuda J, Kodama H, Kumagai J, Tanikawa H, Nakamura A & Tanaka T (2002). Leptin promotes the development of mouse preimplantation embryos *in vitro*. Endocrinology, 143: 1922-1931.

Kim JH, Lee SH, Kim S, Jeong YW, Koo OJ, Hashem MD, Park SM, Lee EG, Hossein MS, Kang SK, Lee BC & Hwang WS (2006). Embryotrophic effects of ethylenediaminetetraacetic acid and hemoglobin on *in vitro* porcine embryos development. Theriogenology, 66: 449-455.

Lanza RP, Cibelli JB, Diaz F, Moraes CT, Farin PW & Farin CE (2000). Cloning of an endangered species (*Bos gaurus*) using interspecies nuclear transfer. Cloning, 2: 79-90.

Lee B, Wirtu GG, Damiani P, Pope E, Dresser BL & Hwang W (2003). Blastocyst development after intergeneric nuclear transfer of mountain bongo antilope somatic cells into bovine oocytes. Cloning Stem Cells, 5: 25-33.

Lee GH, Proenca R, Montez JM, Carroll KM, Darvishzadeh JG, Lee JI & Friedman JM (1996). Abnormal splicing of the leptin receptor in diabetic mice. Nature, 379: 632-635.

Loi P, Ptak G, Barboni B, Fulka J, Cappai P & Clinton M (2001). Genetic rescue of an endangered mammal by cross-species nuclear transfer using post-mortem somatic cells. Nature Biotechnology, 19: 962-964.

Matsuoka T, Tahara M, Yokoi T, Masumoto N, Takeda T, Yamaguchi M, Tasaka K, Kurachi H & Murata Y (1999). Tyrosine phosphorylation of STAT3 by leptin through leptin receptor in mouse metaphase 2 stage oocyte. Biochemical and Biophysical Research Communications, 256: 480-484.

Sansinena MJ, Taylor SA, Taylor PJ, Denniston RS & Godke RA (2003). Production of interspecies nuclear transfer embryos using male and female llama (*Lama glama*) cell lines. Theriogenology, 59: 287 (Abstract).

Swain JE, Dunn RL, McConnell D, Gonzalez-Martinez J & Smith GD (2004). Direct effects of leptin on mouse reproductive function: regulation of follicular, oocyte, and embryo development. Biology of Reproduction, 71: 1446-1452.

Tartaglia LA, Dembski M, Weng X, Deng N, Culpepper J, Devos R, Richards GJ, Campfield LA, Clark FT, Deeds J, Muir C, Sanker S, Moriarty A, Moore KJ, Smutko JS, Mays GG, Wool EA, Monroe CA & Tepper RI (1995). Identification and expression cloning of a leptin receptor, OB-R. Cell, 83: 1263-1271.

Thouas GA, Korfiatis NA, French AJ, Jones GM & Trounson AO (2001). Simplified technique for differential staining of inner cell mass and trophectoderm cells of mouse and bovine blastocysts. Reproductive BioMedicine online, 3: 25-29.

Vajta G, Lewis IM, Hyttel P, Thouas GA & Trounson AO (2001). Somatic cell cloning without micromanipulators. Cloning, 3: 89-95.

Vajta G, Lewis IM, Trounson AO, Purup S, Maddox-Hyttel P & Schmidt M (2003). Handmade somatic cell cloning in cattle: analysis of factors contributing to high efficiency *in vitro*. Biology of Reproduction, 68: 571-578.

Vajta G, Peura TT, Holm P, Paldi A, Greve T & Trounson AO (2000). New method for culture of zona-included or zona-free embryos: the well-of-the-wells (WOW) system. Molecular Reproduction and Development, 55: 256-264.

White KL, Bunch TD, Mitalipov S & Reed WA (1999). Establishment of pregnancy after the transfer of nuclear transfer embryos produced from the fusion of argali (*Ovis ammon*) nuclei into domestic sheep (*Ovis aries*) enucleated oocytes. Cloning, 1: 47-54.

White DW, Kuropatwinski KK, Devos R, Baumann H & Tartaglia LA (1997). Leptin receptor (OB-R) signaling: cytoplasmic domain mutational analysis and evidence for receptor homo-oligoomerization. Journal of Biological Chemistry, 272: 4065-4071.

Zhang K, Wang S, Ma Y, Lou Y, Wei H, Sun X, Zhao Y, Li Y, Dai Y, Zhang L & Li N (2007). Effects of leptin supplementation in *in vitro* maturation medium on meiotic maturation of oocytes and preimplantation development of parthenogenetic and cloned embryos in pigs. Animal Reproduction Science, 101: 85-96.

Zhang Y, Proenca R, Maffei M, Barone M, Leopold L & Friedman JM (1994). Positional cloning of the mouse obese gene and its human homologue. Nature, 372: 425-432.

Received: January 18, 2008
Accepted: April 16, 2008

Belg. J. Zool., 138 (2) : 191-195 July 2008

Discrimination between susceptible and non-susceptible *Biomphalaria alexandrina* snails – intermediate hosts of *Schistosoma mansoni* in Western Saudi Arabia – using random amplified polymorphic DNA analysis

Abdel Elah A. Banaja[1], Manal B. Jamjoom[2], Ismail M. Shalaby[1] & Youssuf A. Gherbawy[1]

[1] **Biological Sciences Dept., Faculty of Science, Taif University, Taif, Saudi Arabia.**
[2] **Faculty of Medicine, King Abdel Aziz University, Jeddah, Saudi Arabia.**
Corresponding author : Prof. Youssuf Gherbawy: Youssufgherbawy@yahoo.com

ABSTRACT. Eight samples from infected and susceptible and from non-susceptible snails (*Biomphalaria alexandrina*) to *Schistosoma mansoni* infection were used in this study. Snails samples were studied at the genotypic level by RAPD analysis with two arbitrary primers. The genetic distances between samples were measured by the percentage of unshared bands. The studied samples separated into two groups according to their DNA fingerprinting. Thus this study has shown that the RAPD markers method has proved useful in discrimination between susceptible and non-susceptible snails of *Biomphalaria alexandrina*.

KEY WORDS : Schistosomiasis, *Biomphalaria alexandrina*, susceptibility, genetic diversity, RAPD-PCR, primers.

INTRODUCTION

Schistosomiasis, is a disease caused by infection with *Schistosoma mansoni* and *S. haematobium*. *Biomphalaria alexandrina* and *Bulinus truncatus* are, respectively, intermediate hosts of these parasites. *S. mansoni* and *S. haematobium* are of great economic as well as medical importance. Some 200 million people are probably infected and 500-600 million more exposed to infection (WEBBE, 1981). From laboratory observation, it has been noticed that some snails of the genus *Biomphalaria* exposed to infection with miracidia of *S mansoni*, develop infection and produce cercariae, while the rest remain uninfected. Sometimes non-susceptible snails outbreed the susceptible ones and replace them (SHOZAWA et al., 1989; MKOJI et al., 1990). These observations lead to the inquiry: do non-susceptible strains of freshwater snails occur in Western Saudi Arabia? If so, it would be beneficial to take advantage of this fact to develop methods of control by introducing a resistant strain into water containing a susceptible strain, in order to have a chance of displacing them.

Susceptibility of snails to schistosome infection depends on the metabolic status of the snail itself. One of the metabolic activities depends upon the production of reactive oxygen species by hemocytes from the snail (BENDER et al., 2005). Phagocytes play a crucial rule in host defence against pathogens, and their arsenal includes the ability to initiate a respiratory burst (HAMPTON et al., 1998). The generation of reactive oxygen species is apparently essential for efficient killing of bacteria and fungi (ROOS & WINTERBOURN, 2002; REEVES et al., 2003).

The possibility that snail susceptibility or resistance to *S. mansoni* is a consequence of differences in the respiratory burst has been addressed in previous studies with schistosome-resistant and susceptible strains of *Biomphalaria glabrata* (CONNERS & YOSHINO, 1990). HAHN et al. (2000) and DIKKEBOOM et al. (1988a; b) did not detect strain differences.

The identification of molluscs is normally based on morphological characters of the shell, renal, and reproductive systems (PARAENSE, 1975). However, the identification of some species may be complicated by the similarity among these characters (PARAENSE, 1988). Recently, molecular tools based on polymerase chain reaction and restriction fragment length polymorphism (PCR-RFLP) of the ribosomal RNA intergenic spacer regions (ITS) have been used to overcome this problem (VIDIGAL et al., 1998; CALDEIRA et al., 1998; 2000). The detection *S. mansoni* infection in snails of the genus *Biomphalaria* is usually through cercariae shedding induced by artificial light exposure, or by squeezing snails between two glass slides. However, these methods cannot detect the parasite in dead snails or in the pre-patent period. In the latter, infection diagnosis is only possible after the parasite has completed its life cycle (3 to 4 weeks after infection), when cercarial release begins. Thus, molecular methods have been used to detect *S. mansoni* infection for those two situations (HANELT et al., 1997; JANNOTTI-PASSOS et al., 1997; HAMBURGER et al., 1998). Molecular techniques for *S. mansoni* detection in snails have been used as a complementary tool when the conventional techniques are not efficient. HAMBURGER et al. (1992) diagnosed *S. mansoni* in *Biomphalaria* sp., through a DNA probe marked with 32P directed to a repeated genome region of the parasite. HANELT et al. (1997) were able to detect the presence of *S. mansoni* in *B. glabrata* snails, during the pre-patent period, and distinguished *S. mansoni* from two other trematode species by amplifying its 18S region from rDNA through "nested" PCR.

The objective of this work was to determine if susceptible and non-susceptible populations of snails coexist in Western Saudi Arabia, and to characterize such strains on a molecular basis by comparing the DNA patterns of the population of snails. Thereafter, it would be beneficial to produce the non-susceptible snails in large numbers and introduce them into the field. Natural selection would fur-

ther act to increase the proportion of alleles for insusceptibility and eventually provide some measure of biological control of schistosomiasis in natural populations.

MATERIALS AND METHODS

Snail collection

One hundred snails were collected from different localities and habitats (irrigation canals and drains, current stream, ponds, dry canals etc ...) in the South-western region of Saudi Arabia, and examined individually for cercarial production. Snails were reared singly in either a 250ml or 400ml beaker with a Petri dish cover at an ambient temperature at 26ºC. Tap water, aerated for at least 24 hours, was used for humidification, and snails were fed oven-dried or fresh lettuce 2 or 3 times a week. Reproduction was by self-fertilization. Rooms in which snails were maintained and experiments conducted were kept at a temperature of about 26ºC. Eggs laid by adult snails of the first generation were reared. Each snail progeny to be isolated from rearing was numbered from "1" on, maturity being based on onset of egg laying. Each snail's progeny were maintained under standard laboratory conditions as described above to give the second and third generations of snails which were subjected to the same biological studies.

DNA extraction

Total DNA was extracted from the combined foot and eggs of selected snails. Briefly, the snail's foot and eggs were mechanically disrupted in 50mM Tris–HCl, pH 8.0 (100mM NaCl, 50mM EDTA, 0.5% SDS) and incubated overnight at 37°C with 50mg/ml proteinase K. as described by VIDIGAL et al. (1994). Following phenol/chloroform extraction and ethanol precipitation, DNA was resuspended in 10mM Tris–HCl, pH 8.0, 1mM EDTA, and DNA concentrations were estimated by comparison with known standards on 2% ethidium bromide stained agarose gels.

DNA amplification

The protocol used was that previously applied to the study of schistosome and *Trypanosome cruzi* (DIAS NETO et al., 1993; STEINDEL et al., 1993).

RAPD data analysis

Computer analyses of RAPD patterns were performed as described by HALMSCHLAGER et al. (1994). The analysis of data was based on the NEI & LI (1979) Coefficient. A dendrogram was constructed by the unweighted paired group method of arithmetic average (UPGMA) based on Jaccard's similarity coefficient by using Phoretix ID software (version 5.2).

RESULTS AND DISCUSSION

All snails were subjected to miracidia to determine their susceptibility or resistance to infection. Eight snails of *Biomphalaria alexandrina* were then selected for DNA analysis: 4 infected with *Schistosoma mansoni* and 4 free from *Schistosoma mansoni* cercariae. The primers (5'-ACCTACCGTACTATGACG-3') and (5'-GTTCCAGCC-3') were used in this study, and generated a considerable number of amplification products for comparison. Different DNA banding patterns were present in almost every isolate. Comparison of each profile for each of the two primers was based on the presence (1) versus absence (0) of RAPD amplimers that migrated to the same position in the gel. Bands of the same size obtained by the same primer were scored as identical, but only bands repeatable in at least two experiments with the same primer at different times were evaluated. Individual amplifications of both susceptible and non-susceptible snails with the two primers are illustrated in Figs 1 & 2. There are very limited differences in the amplified bands of the individuals within each population. The only reproducible differences we have noticed are between the two types of snails (susceptible and non-susceptible). The total number of bands scored was 165, with the average number of bands scored per lane being 10.3 (Figs 1 & 2). According to DNA patterns, the primers we used revealed a very high degree of similarity among the samples of the non-susceptible group, and also, separately, among the samples of the susceptible group (Figs 1 & 2). On the other hand DNA patterns of the two primers showed big differences between the samples of non-susceptible group and those of the susceptible group (Figs 1 & 2). So, the results showed that there are intrapopulation differences in *Biomphalaria alexandrina* between susceptible and non-susceptible snails.

Using RAPD-PCR, ABDEL-HAMID et al. (1999) showed that there are intrapopulation differences within a species (*Biomphalaria tenagophila*) between susceptible and non-susceptible snails. Thus, when tissues from several individuals of the same population are pooled, one can obtain a pattern which is to some extent distinctive of the strain. Also, they reported that as there is relatively restricted genetic variation between susceptible and non-susceptible snails within the same species, it is indeed possible that the susceptibility of snails to parasitic infection is genetically controlled.

Our combined data from all samples using two different primers were analyzed to produce a dendrogram (Fig. 3). According to the dendrogram constructed from RAPD data, the samples of *Biomphalaria alexandrina* split into two RAPD groups corresponding to the infection status. Our results indicated that RAPD primers were useful for distinguishing snail populations with respect to susceptible and non-susceptible properties. ABDEL-HAMID et al. (1999) suggested that RAPDs should be highly useful for phylogenetic analysis among closely related individuals. This suggestion is in agreement with both BARRAL et al. (1991) and VIDIGAL et al. (1994) who indicated that RAPD markers are a highly resolving and helpful tool for investigation of variability. They provide a simple technology that can be used to rapidly distinguish species, strains and sexes in laboratory conditions. Also, SIMPSON et al. (1993) proved that RAPD is undoubtedly a powerful approach for analysis of genetic variation and the identification of genetic markers. So, RAPD is of particular value in the study of genetic variation of snails and would allow the design of specific primers for genome analysis. RAPD is finding increasing use because of its technical

simplicity. NEWTON (1962) considered that susceptibility or resistance of a snail to infection is an hereditary character. RICHARDS (1970), RICHARDS & MERRIT (1972) and NABIH & EL-ANSARY (1990) confirmed this contention by extensive studies on the genetics of *B. glabrata* and *B. alexandrina* snails. They suggested that the specificity relationship between the parasite and its intermediate host snail is genetic. Moreover, RICHARDS (1984) showed that resistance to infection in adult snails is governed by a single gene according to mendelian genetics. This suggestion has some support from the findings of KNIGHT et al. (1991) and LARSON et al. (1996) who described the occurrence of genetic polymorphisms between resistant and susceptible snails through the use of restriction fragment length polymorphism (RFLPs) in the rRNA gene as well as RAPD assays. The differences in the DNA patterns were expected, since it was previously reported by KNIGHT et al. (2000) that there were different genetic strains of a single species of snails from the same locality and they varied in their degrees of resistance, and even among susceptible stocks certain individuals were more resistant than others. Recently, ABDEL-HAMID et al. (2006) studied genetic variations between strains susceptible and resistant to *Schistosoma* infection within *B. alexandrina* snails using random amplified polymorphic DNA analysis technique. Their results showed that 39.8% of the examined field snails were resistant, while 60.2% of these snails showed high infection rates. ABDEL-HAMID et al. (1999) reported that the availability of isogenic snail lines has made it possible to use molecular tools to determine the degree of genetic variability between them.

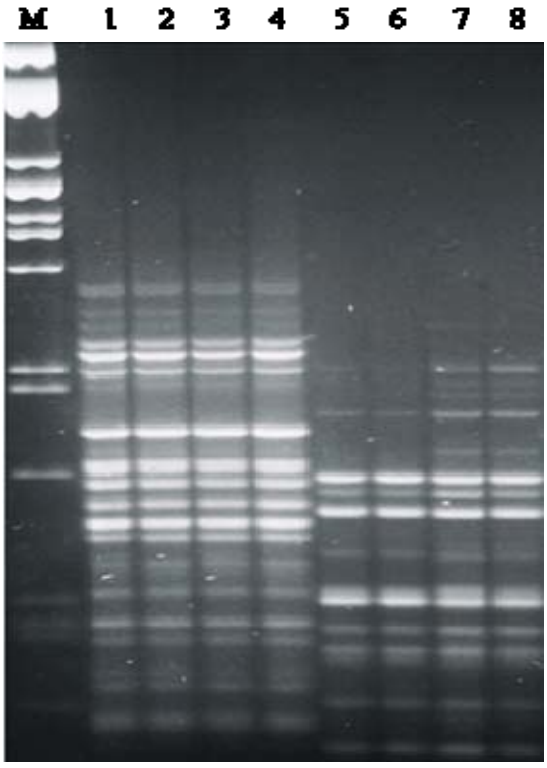


Fig. 1. – RAPD fragments generated by the primer (5'-ACCTACCGTACTATGACG-3') for eight samples of *Biomphalaria alexandrina*. Lanes: 1-4, non-susceptible samples and Lanes: 5-8, susceptible samples.

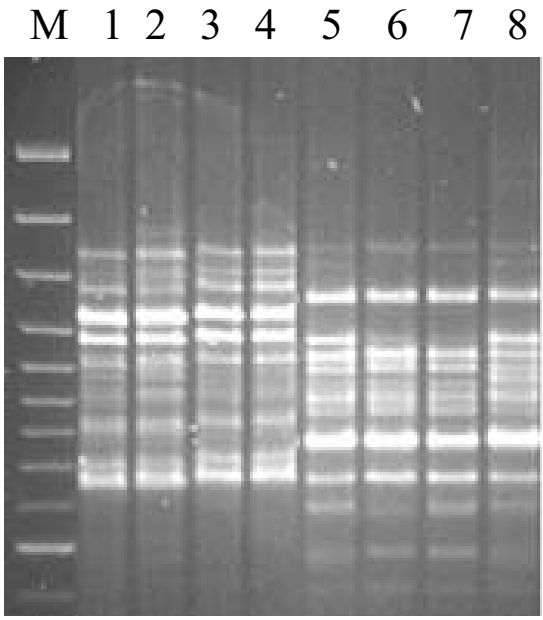


Fig. 2. – RAPD fragments generated by the primer (5'-GTTGCCAGCC-3') for eight samples of *Biomphalaria alexandrina*. Lanes: 1-4, non-susceptible samples and Lanes: 5-8, susceptible samples.

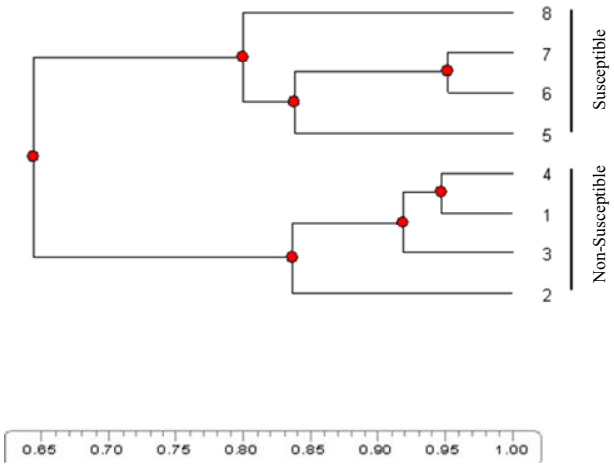


Fig. 3. – The dendrogram showing the relationships of different *Biomphalaria alexandrina* samples. Genetic distances were obtained by random amplified polymorphic DNA analysis using two different primers.

By comparing genomic DNA of susceptible and non-susceptible snails using two primers, we found a component that the susceptible ones produced that was lacking in the non-susceptible ones, that would make them non susceptible. In subsequent studies, it might be possible to insert (or delete) this component in susceptible snails by treatment with specific mutagens (ABDEL-HAMID et al., 1999).

So, we suggest that such genetically altered varieties in snails of *Biomphalaria alexandrina* could then be

released into areas endemic for schistosomes, following treatment of these areas to remove most or all of the genetically unaltered susceptible snails. This concept will lead to genetic control of schistosomiasis.

ACKNOWLEDGEMENTS

This work was supported by grant from Taif University, Saudi Arabia.

REFERENCES

ABDEL-HAMID AZ, MOLFETTA JB, FERNANDEZ V & RODRIGUES V (1999). Genetic variation between susceptible and non-susceptible snails to schistosoma infection using random amlified polymorphic DNA analysis (RAPDs). Revista do Instituto de Medicina Tropical de São Paulo, 41: 291-295.

ABDEL-HAMID AZ, RAWI SM & ARAFA AF (2006). Identification of a genetic marker associated with the resistance to *Schistosoma mansoni* infection using random amplified polymorphic DNA analysis. Memórias do Instituto Oswaldo Cruz, Rio de Janeiro, 101: 863-868.

BARRAL V, THIS P, IMBERT-ESTABLET D, COMBES C & DELSENY M (1991). Genetic variability and evaluation of *Schistosoma* genome analyzed by using random amplified polymorphic DNA markers. Molecular and Biochemical Parasitology, 59: 211-222.

BENDER RC, BRODERICK RJ, GOODALL CP & BAYNE CJ (2005). Respiratory burst of *Biomphalaria glabrata* hemocytes: *Schistosoma mansoni*-resistant snails produce more extracellular H_2O_2 than susceptible snails. The Journal of Parasitology, 91: 275-279.

CALDEIRA RL, VIDIGAL TA, PAULINELLI ST, SIMPSON AJG & CARVALHO OS (1998). Molecular identification of similar species of the genus *Biomphalaria* (Mollusca: Planorbidae) determined by PCR-RFLP. Memórias do Instituto Oswaldo Cruz, Rio de Janeiro, 93: 219-225.

CALDEIRA RL, VIDIGAL TA, MATINELLA L, SIMPSON AJG & CARVALHO OS (2000). Study of planorbids from Venezuela by polymerase chain reaction amplification and restriction fragment length polymorphism (PCR-RFLP). Memórias do Instituto Oswaldo Cruz, Rio de Janeiro, 95: 171-177.

CONNERS VA & YOSHINO TP (1990). *In vitro* effect of larval *Schistosoma mansoni* excretory-secretory products on phagocytosis-stimulated superoxide production in hemocytes from *Biomphalaria glabrata*. The Journal of Parasitology, 76: 895-902.

DIAS NETO E, SOUZA CP, ROLLINSON D, KATZ N, PENA SDJ & SIMPSON AJG (1993). The random amplification of polymorphic DNA allows the identification of strains and species of schistosome. Molecular and Biochemical Parasitology, 57: 83-88.

DIKKEBOOM R, BAYNE CJ, VEN DER KNAAP WP & TUNAGEL JM (1988a). Possible role of reactive forms of oxygen in *in vitro* killing of *Schistosoma mansoni* sporocysts by hemocytes of *Lymnaea stanalis*. Parasitology Research, 75: 48-154.

DIKKEBOOM R, VAN DER KNAAP WP, VAN DEN BOVENKAMP W, TUNAGEL JM & BAYNE CJ (1988b). The production of toxic oxygen metabolites by hemocytes of different snail species. Developmental and Comparative Immunology, 12: 509-520.

HAHN UK, BENDER RC & BAYNE CJ (2000). Production of reactive oxygen species by hemocytes of *Biomphalaria glabrata*: Carbohydrate-specific stimulation. Developmental and comparative immunology, 24: 531-541.

HALMSCHLAGER E, MESSNER T, KOWALSKI R & PRILLINGER H (1994). Differentiation of *Ophiostoma piceae* and *Ophiostoma quercus* by morphology and RAPDanalysis. Systematic and Applied Microbiology, 17: 554-562.

HAMBURGER J, WEIL M, OUMA JH, KOECH D & STURROCK RF (1992). Identification of schistosome-infected snails by detecting schistosomal antigens and DNA sequences. Memórias do Instituto Oswaldo Cruz, Rio de Janeiro, 87: 243-247.

HAMBURGER J, HE-NA, XIN XY, RAMZY RM, JOURDANE J & RUPPEL A (1998). A polymerase chain reaction assay for detecting snails infected with bilharzias parasites (*Schistosoma mansoni*) from very early prepatency. American Journal of Tropical Medicine and Hygiene, 59: 872-876.

HAMPTON MB, KETTLE AJ & WINTERBOURN CC (1998). Inside the neutrophil phagosome: oxidants, myeloperoxidase, and bacterial killing. Blood, 92: 3007-3017.

HANELT B, ADEMA CM, MANSOUR MH & LOKER ES (1997). Detection of *Schistosoma mansoni* in *Biomphalaria* using nested PCR. The Journal of Parasitology, 83: 387-394.

JANNOTTI-PASSOS LK, VIDIGAL TA, DIAS-NETO E, PENA SDJ, SIMPSON AJG, DUTRA WO, SOUZA CP & CARVALHO-PARRA JF (1997). PCR amplification of the mitochondrial DNA minisatellite region to detect *Schistosoma mansoni* infection in *Biomphalaria glabrata* snails. The Journal of Parasitology, 83: 395-399.

KNIGHT M, BRINDLEY PJ, RICHARDS CG & LEWIS EA (1991). *Schistosoma mansoni*: use of a cloned ribosomal RNA gene probe to detect restriction fragment length polymorphisms in the intermediate host *Biomphalaria glabrata*. Experimental Parasitology, 73: 285-294.

KNIGHT M, ONGELE E & LEWIS FA (2000). Molecular studies of *Biomphalaria glabrata*, an intermediate hosts of *Schistosoma mansoni*. International Journal of Parasitology, 30: 535-541.

LARSON SE, ANDERSON PL & MILIER AN (1996). Use of RAPD-PCR to differentiate genetically defined lines of an intermediate host of *S. mansoni, A. glabrata*. The Journal of Parasitology, 82: 237-244.

MKOJI GM, SMITH JM & PRICHARD RK (1990). Effect of oltipraz on the susceptibility of adult *Schistosoma mansoni* to killing by mouse peritoneal exudates cells. Parasitology Research, 76: 435-439.

NABIH I & EL-ANSARY A (1990). Genetic studies on freshwater snails "specific intermediate hosts for schistosomiasis". 11. Isolation and base composition determination of deoxyribonucleic acid. Cellular and Molecular Biology, Including Cyto-Enzymology, 26: 455-458.

NEWTON WE (1962). The comparative tissue reaction of two strains of *Australorbis glabratus* to infection with *Schistosoma mansoni*. The Journal of Parasitology, 38: 362-370.

NEI M & LI WH (1979). Mathematical model for studying genetic variation in terms of restriction endonuclease. Proceeding of the National Academy of Sciences of the United states of America, 76: 5269-5273.

PARAENSE WL (1975). Estado atual da sistemática dos planorbídeos brasileiros. Arquivos do Museu Nacional do Rio de Janeiro, 55: 105-128.

PARAENSE WL (1988). *Biomphalaria kuhniana* (Clessin, 1883), planorbid mollusk from South America. Memórias do Instituto Oswaldo Cruz, Rio de Janeiro, 83: 1-12.

REEVES EP, LU H, JACOBS HL, MESSINA CG, BOLSOVER S, GABELLA G, POTMA EO, WARELY A, ROES J & SEGAL AW (2003). Killing activity of neutrophils is mediated through activation of proteases by K+ flux. Nature, 416: 291-297.

RICHARDS CG (1970). Genetics of a molluscan vector of schistosomiasis. Nature (Lond.), 227: 806.

RICHARDS CG (1984). Influence of snail age on genetic variation in susceptibility of *Biomphalaria glabrata* for infection with *Schistosoma mansoni*. Malacologia, 25: 493-503.

RICHARDS CG & MERRITT JR (1972). Genetic factors in the susceptibility of juvenile *Biomphalaria glabrata* to *Schistosoma mansoni* infection. American Journal of Tropical Medicine and Hygiene, 21: 425-434.

ROOS D & WINTERBOURN CC (2002). Immunology. Lethal weapons. Science, 296: 669-671.

SHOZAWA A, SUTO C & KUMADA N (1989). Superoxide production by the hemocytes of the freshwater snail, *Biomphalaria glabrata*. Zoological Science, 6: 1019-1022.

SIMPSON AJG, NETO ED & STEINDEL M (1993). The use of RAPDs for the analysis of parasites. In: PENA SDJ, CHAKRABORTY R, EPPLEN LT & JEFFREYS J (eds), DNA fingerprinting: state of the sciences. Boston, Birkhauser, 1993. p. 331-337.

STEINDEL M, DIAS NETO E, MENEZES CLP, ROMANHA AJ & SIMPSON AJG (1993). Random amplified polymorphic DNA analysis *of Trypansoma cruzi* strains. Molecular and Biochemical Parasitology, 60: 71-80.

VIDIGAL TA, DIAS NETO E, CARVALHO OS & SIMPSON AJG (1994). *Biomphalaria glabrata:* Extensive genetic variation in Brazilian isolates revealed by random amplified polymorphic DNA analysis. Experimental Parasitology, 79: 187-194.

VIDIGAL TA, SPATZ L, NUNES DN, SIMPSON AJG, CARVALHO OS & DIAS NETO E (1998). *Biomphalaria* spp: identification of the intermediate snail hosts of *Schistosoma mansoni* by polymerase chain reaction amplification and restriction enzyme digestion of the ribosomal RNA gene intergenic spacer. Experimental Parasitology, 89: 180-187.

WEBBE G (1981). Schistosomiasis: some advances. British Medical Journal, 283: 1-8.

Received: June 17, 2007
Accepted: May 26, 2008

Belg. J. Zool., 138 (2) : 196-197 July 2008

SHORT NOTE

Paedomorphosis in the banded newt, *Triturus vittatus* (Jenyns, 1835)

Uğur Kaya, Ferah Sayım, Eyup Başkale & İbrahim Ethem Çevik

Ege University, Faculty of Science, Biology Section, Zoology Department, Bornova-İzmir, 35100, Turkey

Corresponding author : Uğur Kaya, e-mail: ugur.kaya@ege.edu.tr

KEY WORDS : Paedomorphosis, banded newt, *Triturus vittatus,* development.

Paedomorphosis, the phenomenon of gaining sexual maturity while retaining the external larval morphological features, has been well known in salamanders and newts since its first description in *Ambystoma mexicanum* by Dumerill in 1866 (1). Paedomorphosis has been noted in 57 species of newts and salamanders, distributed in nine families. Of the 12 species in the genus *Triturus* (2), the paedomorphic forms of nine (*T. alpestris*, *T. boscai*, *T. carnifex*, *T. cristatus*, *T. helveticus*, *T. italicus*, *T. marmoratus*, *T. montandoni* and *T. vulgaris*) have been described (3-11). However, paedomorphosis has not been previously documented in *T. vittatus*.

In this paper, we introduce new data regarding the occurrence of paedomorphosis in the genus *Triturus* by describing the paedomorphic females for the first time in *T. vittatus*.

The study site comprised two separate but nearby irrigation pools of approximately 513m^2 connected by large pipes, in the vicinity of Sakarya, Turkey (41º 02' N, 30º 37' E). We captured 47 specimens of *T. vittatus* on 23 March 2006, of which four were paedomorphic, and transported them alive to our amphibian laboratory. We placed each of them with a metamorphic male in separate 20 litre glass aquaria and provided some small-leaved aquatic plants.

Paedomorphs and metamorphs in captivity displayed courtship behaviour and then breeding activity. Three of the four paedomorphic females deposited 174 eggs in total (n: 35, 41 and 98 by each female) and 54 larvae developed from these eggs. Retention of external gills in the mature individuals was an external cue to their paedomorphosis (Fig. 1A). The females started to loose their external gills gradually in captivity after laying their eggs (Figs 1B-C). SVL of the paedomorphic females ranged from 3.6-4.5cm with a mean of 3.9±0.86cm. Their external pattern and colouration did not differ from the other adult forms of the same population. A large sized (SVL=41mm) larva of *T. vittatus* has also been noted by Kosswig (12) but it had not been demonstrated to be a paedomorphic form.

Until now, the banded newt, *Triturus vittatus* was not known to be paedomorphic; here we describe the occurrence of paedomorphosis in this species for the first time. However, because of the small sample size of the paedomorphic forms, we cannot yet point out the exact cause of this phenomenon.

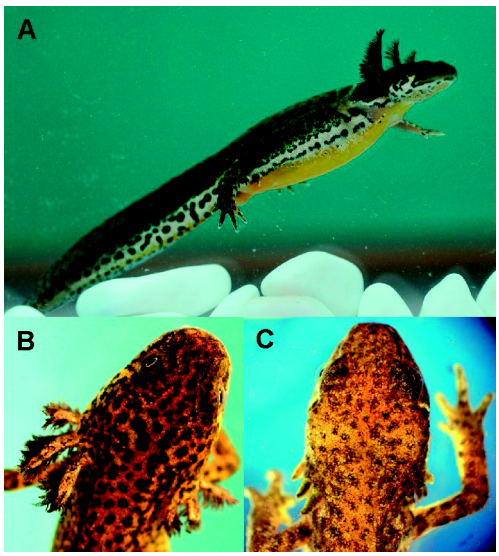


Fig. 1. – A paedomorphic female of *Triturus vittatus* (A); the external gills of paedomorphic female: retained (B), reduced (C).

ACKNOWLEDGEMENTS

We thank Prof. Dr. M. K. Atatür and Dr. M. Denoël for their valuable comments on the manuscript. The permissions for field work and handling of the newts were issued by the Animal Ethics Committee of Faculty of Medicine, Ege University.

REFERENCES

1. Denoël M, Joly P & Whiteman HH (2005). Evolutionary ecology of facultative paedomorphosis in newts and salamanders. Biological Reviews, 80: 663-671.
2. Zajc I & Arntzen JW (1999). Phylogenetic relationships of the European newts (genus *Triturus*) tested with mitochondrial DNA sequence data. Contributions to Zoology, 68: 73-81.
3. Radovanovic M (1961). Neue Fundorte neotenischer Bergmolche in Jugoslawien. Zoologischer Anzeiger, 166: 206-218.
4. Dely OG (1967). Neuere angaben zur kenntnis des neotenischen teichmolches (*Triturus vulgaris* L.). Acta Zoologica Academiae Scientiarum Hungaricae, 13: 253-270.

5. Roček Z (1974). Beitrag zur Erkennung der Neotenie des Alpenmolches *Triturus alpestris* (Laurenti, 1786). Vestnik Ceskoslovenske Spolecnosti Zoologicke, 38: 285-294.
6. Gabrion J, Sentein P & Gabrion C (1977). Les populations néoténiques de *Triturus helveticus* des causses et du Bas-Languedoc. I. Répartition et caractéristiques, Terre et Vie, 31: 489-506.
7. Arnold EN & Burton JA (1978). A field guide to the reptiles and amphibians of Britain and Europe. Collins, London.
8. Tiedemann F & Häupl M (1979). Ein neuer Fund neotener *Triturus v. vulgaris* (L.) in Österreich. Annalen des Naturhistorischen Museums in Wien, 82: 467-470.
9. Kalezic ML, Cvetkovi D, Djorovi A & Uki GD (1994). Paedomorphosis and differences in life-history traits of two neighbouring crested newt (*Triturus carnifex*) populations. The Herpetological Journal, 4(4): 151-159.
10. Çevik İE, Atatür MK, Arİkan H & Akyurtlaklİ N (1997). Occurrence of neotenic *Triturus vulgaris* (Urodela: Salamandridae) larvae in western Anatolia. Israel Journal of Zoology, 43: 301-304.
11. Denoël M, Duguet R, Dzukic G, Kalezic M & Mazzotti S (2001). Biogeography and ecology of paedomorphosis in *Triturus alpestris* (Amphibia, Caudata). Journal of Biogeography, 28: 1271-1280.
12. Kosswig C (1951). Herpetologisches aus der Turkei. Mitteilungen aus dem Museum für Kulturgeschichte und dem Naturwissenschaftlichen Arbeitskreis, Magdeburg, 3: 13-23.

Received: April 3, 2007
Accepted: November 19, 2007