Short Communications

Residual Effects of Bifenthrin on the Mortality of *Pardosa sumatrana* (Thorell 1890) (Araneae: Lycosidae)

Muhammad Khalid Mukhtar, Erum Choudhary and Hafiz Muhammad Tahir*
Department of Biological Sciences, University of Sargodha, Pakistan

Abstract.- In the present study, effects of Bifenthrin (pyrethroid insecticide) were evaluated on the common ground spider, *Pardosa sumatrana* (Thorell 1890). Spiders collected from the agricultural fields of District Sargodha, Punjab, Pakistan were exposed topically to different concentrations of Bifenthrin for 48 hours. Males were found to be more susceptible compared to the females. LT 50 values against Bifenthrin at field rate concentration were 17.60 and 21.21 hours for males and females, respectively. Similarly LD 50 values for males and females were 93.33 and 113.00 nL a.i./individual, respectively. When spiders were exposed to the insecticide treated soil high mortality was observed even after 15 days.

Keywords: Araneae, spiders, *Pardosa sumatrana*, citrus orchards, Bifenthrin, toxicity.

Spiders inhabit in all terrestrial ecosystems and are abundant in both natural habitats and agricultural fields (Dondale, 1970; Turnbull, 1973; Nyffeler and Benz, 1987; Tahir *et al.*, 2011). Many spider species effectively limit certain prey populations in the agricultural fields because of their specialization to feed on specific items (Maloney *et al.*, 2003). It has been a concern of many ecological researchers that current agricultural practices have become a threat to the invertebrate biodiversity including spiders (Bianchi *et al.*, 2006). Insecticides do not only cause mortality of spiders in the agricultural fields, but also have negative effects on their body structures (Rezac *et al.*, 2010). Amalin *et al.* (2009) documented that impact

of insecticides on the invertebrates cannot be observed instantly. They also reported higher densities of invertebrate predators in the non sprayed field than on the field treated with broad-spectrum herbicides and insecticides. A decrease in spider populations as a result of pesticide use can result in an outbreak of pest populations (Brown *et al.*, 1983; Yardim and Edwards 1998; Marc *et al.*, 1999; Holland *et al.*, 2000; Tanaka *et al.*, 2000). Some insecticides such as deltamethrin, dimethoate and phosalone do not cause mortality of spiders but affect their predatory potential over a considerable period (Cocquempot *et al.*, 1991). Fenvalerate and Lambda-Cyhalothrin inhibit emergence of spiderling from cocoons and delay web building activity (Dinter and Poehling, 1995).

The present study was designed to evaluate the effect of insecticide (Bifenthrin) on *Pardosa sumatrana* (Thorell, 1890) (Araneae: Lycosidae). *P. sumatrana* was selected for the study because of its dominance in the citrus fields in the study area. Bifenthrin is a pyrethroid insecticide and being used commonly in the agricultural fields of Punjab, Pakistan. Objectives of the study were: (i). to investigate the susceptibility of *P. sumatrana* to Bifenthrin (ii). to compare the effect on the rate of mortality in males and females (iii). to record the residual effect of Bifenthrin on the mortality of *P. sumatrana*.

Materials and methods
   Test organism
   Study was conducted from December, 2009 to April, 2010 in the Department of Biological Sciences, University of Sargodha. Specimens of *Pardosa sumatrana*, which was the dominant ground spider in the study area, were captured with a suction apparatus (Seimen VK 20C01) from unsprayed citrus fields of Sargodha, Punjab, Pakistan. Only the adult males and females were used in the experiments. The collected spiders were transferred into the sample bottles (10 cm long and 5.5 cm wide) as one spider per bottle with the help of camel hair brush. The mouth of each bottle was covered with cotton cloth. Humidity was maintained by adding half inch thick layer of moistened sand at the bottom of each bottle and by placing a piece of soaked piece of cotton over the cloth used to cover the mouth of bottles. The spiders

were fed with *Drosophila* larvae till satiation to standardize the level of hunger before using in the experiment.

Topical exposure

Four concentrations of Bifenthrin were tested *viz*. 120 (half field rate), 240 (recommended field rate), 360 (intermediate between field rate and double field rate) and 480 (double field rate) nL *a.i.* per individuals against *Pardosa samatrana*. Before the application of insecticide, spiders were anesthetized by exposing them with CO_2 gas for approximately two minutes. The insecticide was applied (0.5µl) topically on the dorsum of each spider using a micropipette. Twenty spiders (10 males, 10 females) were exposed to each concentration of the insecticide. Control specimens were exposed to water. Each experiment was replicated thrice. After the application of insecticide, each spider was placed singly in a glass bottle (10 cm long and 5.5 cm wide). Every day each spider was provided with food at satiation level. The mortality was recorded till 48 hours after the exposure. Mortality was defined as no movement in spider observed after stimulation.

Residual toxicity

For residual toxicity, experimental pots (10" diameter x 5" high) were prepared by adding a layer of half inch thick soil and were placed in sun light before spraying of insecticide. Only the field rate concentration of insecticide was prepared and sprayed on the pots by using Knapsack hand sprayer (10 pots for insecticide). Control pots (n= 10) were sprayed with water. Twenty spiders (10 males and 10 females) were released in each treated pot after 1, 3, 5, 7, 10, 15, 20 and 30 days of spray and mortality was observed after 2, 4, 8, 16, 24, 48 hours after the release of spiders in the experimental pots. During the experiment spiders were supplied with larvae of *Drosophila* as food.

Statistical analysis

Probit analysis was used to determine the LT50 and LT95 at the recommended field rate of insecticide. LD50 and LD95 were also calculated. To compare the mortality of spiders at different doses or at different time intervals (with insecticide) one-way ANOVA was used. All statistical analyses were performed using Minitab package (Version15) and SPSS package (Version 13).

Results

Topical bioassay

Significant difference in mortality was recorded at different doses of Bifenthrin (df 3, 36; F = 4.75; P = 0.012 for males and df 3, 36; F = 6.971; P = 0.002 for females). Results of Tukey's test are given in Table I. The mortality in females (18.33±9.45) was lower compared to the males (30.00±9.6) at the field rate concentration (240 nL). Mortality rate of spiders increased as the dose of insecticide increased.   LT 50 for males (17.60 hours) was lower compared to the females (21.21hous) (Table II). Similarly, values LT95 for male and females were 35.78 nL and 37.63 nL a.i./individuals respectively. The values of LD50 and LD 95 of both sexes are given in the Table III.

Table I.-   **Results of Tukey's test on topical application of Bifenthrin on male and female spiders of *Pardosa sumatrana* (Mean ±S.E).**

Concentration in *nL a.i.* per individuals	Male spider	Female spider
120	26.66±8.81[a]	16.33±9.92 [a]
240	30.00±9.6[a]	18.33±9.45 [a]
360	48.33±9.45[b]	38.33±9.09 [b]
480	78.33±11.15[c]	59.00±11.25 [c]

Note: Values in columns with different superscripts are significantly different**.**

Table II.-   **Estimated LT50 and LT95 of field rate of Bifenthrin (nL a.i./individuals) at 48th hours of both sexes of *Pardosa sumatrana***

Sex	LT %	Time in hours (confidence limits)
Male	50	17.60 (16.01-19.45)
	95	35.78 (32.17-40.72)
Female	50	21.21(19.53-23.31)
	95	37.63 (34.08-42.73)

Residual bioassay

Results of the residuals bioassay showed that in both sexes the mortality rate decreased

significantly with time (Table IV). Again in this experiment the mortality rate in males was higher than females. At 10th day mortality rate it was 26% in males while 22% in females. At 15th day there was 17% mortality in males while 12% in females. However at 30th day mortality rate in both sexes was negligible.

Table III.- Estimated LD50 and LD95 of field rate of Bifenthrin (nL a.i./individuals) at 24th hours of both sexes of *Pardosa sumatrana*

Sex	LD %	Dose (confidence limits)
Male	50	93.33 (87.31-107.11)
	95	129.32 (116.36-137.14)
Female	50	113 (105.12-121.42)
	95	149.26(134.12-163.72)

Table IV.- Residual toxicity of Bifenthrin on male and female spiders of *Pardosa sumatrana*

Days	Male spiders	Female spiders
1	46.00±6.35[d]	42.00±6.28[e]
2	45.00±6.87[d]	35.00±7.03[d]
3	26.00±6.69[c]	25.00±7.34[c]
5	27.00±7.00[c]	23.00±6.57[c]
10	26.00±5.61[c]	22.00±6.28[c]
15	17.00±4.95[bc]	12.00±3.88[b]
20	12.00±3.88A[b]	12.00±3.88[b]
30	5.00±1.66[a]	4.00±2.21[a]

Note: Values in columns with different superscripts are significantly different.

Discussion

Insecticides are widely used in the agro-ecosystems for the control of insect pests. Although many insecticides are giving better results in controlling the pests' populations, they are so toxic that they eliminate the useful arthropod predators from the fields. At different doses of Bifenthrin, the mortality rate was different in topical bioassays. Higher mortality in male spiders was recorded at all concentrations indicating that susceptibility of *Pardosa sumatrana* to insecticide is related with the weight of the body of spiders. Although males were more susceptible than females, high variability in susceptibility (in both sexes) was observed in all the replicate tests. A number of studies also reported

difference in susceptibility of both sexes (Dinter and Poehling, 1995). Higher mortality in males in the present study is in accordance to Peterson (2002) who also reported higher mortality in males. Tahir *et al.* (2011) studied the susceptibility of *Lycosa terrestris* against chlorpyrifos and also found higher mortality in males. Even though same dose was applied on both sexes, the male mortality rate was high due to its smaller size (Dinter, 1995).

The fate of the insecticides in the fields depends on the solubility in the water, absorbance in the soil, type of the soil and persistence (Wiltz, 2010). Results of residual bioassays showed that Bifenthrin caused higher mortality even after 15 days of exposure. It can be predicted that the absorbance of bifenthrin in the soil is high and it degrades slowly. Linde (1994) reported that Bifenthrin has a low solubility in water and a correspondingly strong tendency to bind to soil particles. Effects of the bifenthrin on spiders' numbers in the fields were not evaluated in the present study. Easterbrook (1997) reported that bifenthin reduced the number of spiders and other natural predators in the fields. Although the effects of insecticides on spiders in the fields may have less drastic effects compared to the laboratory studies due to availability of refugia to the spiders, which is helpful to escape from the spray residues but still insecticidal sprays are major threat to the spiders. It is recommended that insecticides in the agricultural fields should only be used when their application becomes inevitable and only those chemical sprays should be used that are highly effective against the target organism but least hazardous to non target animals.

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Genus *Aulacophora* Chevrolat, 1836 (Coleoptera: Chrysomelidae) From Pothohar, Punjab, Pakistan

Waqar Ahmad, Muhammad Naeem and Imran Bodlah*
Pir Mehr Ali Shah Arid Agriculture University, Rawalpindi, Pakistan

Abstract.- Two species of genus *Aulacophora* Chevrolat, 1836 have been recorded from Pothohar tract of Punjab province of Pakistan. One species, *Aulacophora lewisii* Baly, 1886 is reported for the first time from different localities of Pothohar tract of Punjab Province of Pakistan. New locality records for *Aulacophora foveicollis* have also been added from Thal tract of Punjab. The main identification characters along distribution range supported by micrographs have been given for future field and research identification. A short review on the distribution of species of genus *Aulacophora* in Pakistan has also been given.

Key words: Coleoptera, Chrysomelidae, *Aulacophora,* Pothohar.

Leaf-beetle subfamily Galerucinae is well distributed with over 5000 species in 520 genera and 6300 species (Gillespie *et al*., 2008; Scherz and Wagner, 2007). In the edispersed all over the world but mostly common in tropics (Aslan *et al*., 2000). The Galerucinae species are mostly known as plant pests which attack a wide variety of Mono and Dicotyledonous families. Adults mostly feed on foliage (parenchyma of the lower leaf surface) producing irregular holes and some species feed on pollen. Many species are severe pests of economic value which cause damage by feeding and transmit viruses (Aslan *et al.,* 2000).

Among the genera of Galerucinae, the genus *Aulacophora* Chevrolet, 1836 is mainly characterized by highest numbers of species (34) having triple modification of antennae (Mohamedsaid and Furth, 2011). This genus can be

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*   Corresponding author: imranbodlah@gmail.com

easily distinguished on the basis of the characters: eyes strongly convex, except the two basal antennal segments the remaining covered with delicate hairs, prothorax transverse, always narrower than the base of elytra and a median transversely impressed line; generally straight (Maulik, 1936).

This genus is represented by 17 species from Sulawesi (Indonesia), 14 from New Guinea (Papua Island), 48 from Sundaland (Indonesia) and 17 from Australia (Barroga and Mohamedsaid, 2002), 18 species from Thailand and Indochina (Kimoto, 1989), 4 species from New Caledenia (Beenen, 2008) and some 30 species from Indian subcontinent (Maulik, 1936; Anand and Cox, 1986).

The species of this genus are mostly linked with cucurbitaceous plants all over the world (Menon *et al.,* 1974). This genus is of great economic importance as a lot of work has been done on this genus all over the world: British India (Maulik, 1936), Sandaland (Barroga, 2001; Barroga and Mohamedsaid, 2002), Russia (Medvedev, 2001), Malaysia (Mohamedsaid, 1994), Indonesia (Laboissiere, 1934; Samoderzhenkov, 1992), India and Africa (Kim *et al*., 2003), Taiwan (Jolivet *et al*., 2009) but as for as Pakistan is concerned, no specific work on this genus has been done in Pothohar region of Punjab. So a systematic survey of various areas of Pothohar region (Punjab) was done during 2011 - 2012.

Materials and methods

Several field trips of various localities (Rawalpindi, Islamabad, Taxila and Attcok) were done during 2011 to 2012. Beetles were collected by sweeping the weeds and bushes with aerial net. In the laboratory, specimens were pinned, dried, labeled and kept in collection boxes. Identification of the leaf beetles was done using the keys of Maulik (1936), Choate (1999) and Aston (2009). The micrographs were taken using *LEICA* MS-5 stereomicroscope attached with Samsung digital camera. The identified specimens have been deposited in Biosystematics Laboratory, Department of Entomology, PMAS- Arid Agriculture University (Pakistan).

Results and discussion

Two species of genus *Aulacophora* were recorded from studied area. *A. lewisii* Baly, 1886 is a new species for Pothohar tract; Punjab Province (Pakistan). New locality records for *Aulacophora foveicollis* have also been added from Thal tract of Punjab.

Aulacophora foveicollis (Lucas, 1849)

Aulacophora foveicollis P.H. Lucas, 1849 (as Galleruca)
Aulacophora africana Weise, 1903
Aulacophora nigriventris Redtenbacher, 1850

Identification characters

Body elongate, orange with reddish touch. Eyes black. Antennal 1st segment is the enlarged and broadest, 2nd is the smallest while the 3rd is a bit larger than 4th one and the 5th -11th are almost equal in length. Scutellum is triangular; having no punctures and shares the same body colour. 1st tarsal segment is much longer than 2nd and 3rd combined. 3rd tarsal segment is slightly bilobed and bent beneath the 4th. Abdominal segments are 4-5 and black (except the last one). The last sternite is same like the body's general colour. In ♂ (male), it is divided into three lobes; middle and two lateral. The lateral lobes are shorter than the middle, gradually narrowed and rounded at the apex. While in the ♀ (female), the sternite is simple. The last visible sternite is lessened and totally emarginate at the apex and concave dorsally.

Distribution

France, Greece, Italy, Portugal, Spain. Egypt, Afghanistan, Cyprus, Oman, Pakistan, Saudi Arabia, Russia, Syria, Turkey, Yemen, Afrotropical region and Oriental region.

Material examined

Rawalpindi: 11 ♂, 17 ♀, 23.iv.2011, Waqar; Attock: 4 ♂, 1♀, 19.iv.2012, Waqar; Taxila: 12 ♂, 20♀, 11.ii.2011, Waqar.

Distribution in Thal tract

1 ♂, 7 ♀, 23.iv.2011; Layyah, Imran; 2 ♂, 9 ♀, 29.iv.2011, Imran; Bhakar; 14 ♂, 7 ♀, 19.iv.2011, Imran; 1 ♂, 7 ♀, 27.iv.2011, Khushab, Imran.

Remarks

    The specimens collected from Pakistan were compared with the published description of *Aulacophora foveicollis* by Maulik (1936), the species varies in length of antennomere as 3rd is larger than 4th one.

<div align="center">

Aulacophora lewisii Baly, 1886

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Aulacophora lewissi Baly, 1886a
Aulacophora cattigarensis Weise, 1892 (Orthaulaca)
Aulacophora intermedia Jacoby, 1892

Identification characters

    Body oblong and elongate. Generally bicolour (head and thorax is reddish brown; elytra is black). Eyes golden. Antennal 1st segment is larger and wider than the 2nd one. 3rd to 9th is flattened. Scutellum is triangular in shape and reddish brown in colour. Abdomen is light brown in colour with 4-5 segments. In male, the last visible sternite is trilobed while in the female it is same like *A. foveicollis*. Pydigium is not exposed.

Habitat

    Specimens of this species were collected from wild vegetation *i.e. Spinacia oleracea*.

Distribution

    Afghanistan, Anhui, Arunnachal Pradesh, Bhutan, Fujian, Guandong, Guizhou, Guanxi, Hainan, Hongkong, Hubei, Hunan, Japan, Jiangxi, Liaoning, Nepal, Pakistan, Sichuan, Sikkim, Darjeeling, Shaanxi, Taiwan, Uttar Pradesh, Tibet,

    Peninsular Malaysia, Sabah, Sarawak, Kalimantan, Sri Lanka, Myanmar, Bhutan, Thailand, Cambodia, Laos, Vietnam, Zhejiang and Oriental region (Aston, 2009; Mohamedsaid, 2004)

Material examined

    Rawalpindi: 1 ♂, 23.iv.2011, Waqar; Taxila: 3♀, 19.iv.2011, Waqar; 2 ♂, 29.iv.2011, Rawalpindi, Imran; 4 ♂, 20.iv.2011, Islamabad, Imran.

Remarks

    This is recorded as a new species from this region. *A. lewisii* mainly differs from *A. foveicollis* due to the colour of its elytra and abdomen. The specimens collected from Punjab were compared with the published description of *Aulacophora lewisii* given by Aston (2009), this species is quite different with respect to the colour of eyes as they are golden in colour.

Distributional range of Genus Aulacophora *in Pakistan*

    In Pakistan this genus has been recorded from South to Northward direction and also from Azad Kashmir (Rizvi *et al*., 2012).

Aulacophora bicolor Weber: Northern area and Gilgit
Aulacophora excavata Baly: Murree, Azad Kashmir and Rawla Kot
Aulacophora foveicollis Lucas: Karachi
Aulacophora intermedia Jacoby: Murree
Aulacophora viridus Maulik: Azad Kashmir and Rawlakot

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Identification of a Gram Negative *Rahnella aquatilis* Strain from *Rana temporaria chensinensis* David in China

Yuan Xue[1], Duanling Zhang[1], Jianfei Chen[2], Yang Meng[1] and Yanlong Zhang[1] *

[1]College of Wildlife Resource of Northeast Forest University, Harbin 150040, People's Republic of China
[2]Division of Swine Infectious Diseases, State Key Laboratory of Veterinary Biotechnology, Harbin Veterinary Research Institute, Chinese Academy of Agricultural Sciences, Harbin 150001, People's Republic of China

Abstract.- *Rahnella aquatilis* strain bf008 is a rare Gram negative bacterium isolated from one of the liver samples of *Rana temporaria chensinensis* David in China. Phenotypic characterizations of the isolate were done based on the colony morphology, microscopic observations and biochemical tests. The result of antimicrobial susceptibility test revealed that bf008 was resistant to fosfomycin and cefazolin, and intermediately susceptible to cefoperazone and cefoxitin. 16S rRNA of bf008 was sequenced and analyzed with those of *R. aquatilis* reference strains. The phylogenetic relationship based on 16S rRNA sequence showed bf008 has a close genetic relationship with 12 *R. aquatilis* reference strains. The present study will be helpful to further understand the significance of different microbial species of the *Rana temporaria chensinensis* David.

Keywords: *Rahnella aquatilis*, *Rana temporaria chensinensis* David, 16S rRNA, phylogenetic analysis.

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* Corresponding author: zhangyanlong2013@yeah.net

*R*ana *temporaria chensinensis* David is a kind of frog with high commercial value (Yin *et al.*, 2006). This type of frog is distributed in the northeastern part of China. It has a long history of being used in traditional medicine and has been reported to improve health and immunity and prevent diseases such as antioxidant, antifatigue, inflammation (Wang, 2002).

Rahnella aquatilis is a rarely isolated Gram negative rod and a representative of the Enterobacteriaceae recognized in 1976–1979 (Gavini *et al.*, 1976; Izard *et al.*, 1979). Strains of *R. aquatilis* are isolated from open water reservoirs and soil (mostly from the rhizosphere of cereals, including wheat, corn, rice, etc.) as well as from clinical material. They are opportunistic pathogens causing a wide spectrum of diseases of the gastrointestinal and urinary tracts, respiratory organs, cardiovascular system, and others (Zdorovenko *et al.*, 2004). An important role in pathogenesis of diseases caused by Gram-negative bacteria belongs to the lipopolysaccharide (Zdorovenko *et al.*, 2008). As a result, some scientists believe that *R. aquatilis* is phytotrophic microorganisms, whilst others point to their ability to cause diseases in mammals. We report here a *R. aquatilis* strain isolated from *Rana temporaria chensinensis David* in China. The present study will be helpful to further understand the significance of different microbial species of *Rana temporaria chensinensis* David.

Materials and methods

During 2011-2012, 40 *Rana temporaria chensinensis* David were collected from a Rana-raising farm. Samples of livers were collected under sterile condition and inoculated onto broth plates with 10% bovine serum (Gibico). The plates were incubated at 37°C for 18-24 h. Several off-white, smooth, neat edged and round colonies were obtained on the plate inoculated with samples.

Pure cultures of the bacteria were analyzed by the Vitek 2 Compact System (bioMérieux Vitek) (Fang *et al.*, 2012) for rapid biochemical identification in Department of Microbiology of the First Affliated Hospital of Harbin Medical University.

The antimicrobial sensitivity phenotypes of the bacteria were determined using a Kirby–Bauer disk diffusion assay according to the standard and interpretive criteria described by Clinical and Laboratory Standards Institute guidelines (CLSI, 2009). The following antimicrobials were tested: cefoperazone, cefazolin, fosfomycin, cefoxitin. All antibiotic disks were provided by Oxoid.

In order to further identify the bacteria, the genomic DNA was purified and used as template to amplify a 1.5-kb fragment of 16S ribosomal RNA (rRNA) gene, using the universal prokaryotic primers of 16S-S (5′-AGAGTTTGATCCTGGCTCAG-3′) and 16S-AS (5′-AGGAGGTGATCCAGCCGCA-3′) (Baruque-ramos *et al.*, 2006). Briefly, DNA template was added to a reaction mixture (50 μL) containing 20 mM of each primer, 40 mM of dNTPs, 2.5 units of Platinum Taq polymerase (Transgene) in 10 x PCR buffer. For PCR, the samples was preheated at 94°C for 4 min, and followed by 30 cycles of amplification by using the following conditions: denaturation at 94°C for 1min, annealing at 56°C for 30s, and elongation at 72°C for 2 min, and then elongated at 72°C for 10 min. The PCR products were purified using a quick PCR purification kit (Omega), and then cloned into pMD18-T vector (Takara). The nucleotide sequence was edited, analyzed with EditSeq software (version 7.1.0, DNASTAR Inc., USA) and the program NCBI-BLAST (www.ncbi.nlm.nih.gov). The phylogenetic tree was constructed based on the neighbor-joining method using the MEGA 5.05 software.

Results and discussion

Several Gram negative rods were detected only in the slides prepared with the samples by microscope observation. The biochemical characteristics of the isolate (bf008) are given in Table I. Antimicrobial susceptibility testing with the disk diffusion method according to the CLSI guidelines (CLSI, 2009) revealed that bf008 was resistant to fosfomycin and cefazolin, and intermediately susceptible to cefoperazone and cefoxitin (Table II).

The 16S rRNA sequence of bf008 was submitted to GenBank with an accession number of KC480178. The 16S RNA sequence of bf008 had

Table I.- **Biochemical profile of *Rahnella aquatilis* isolate.**

No.	Reaction	Our isolate
2	Alanine - Phenylalanine - Proline aromatic aminotransferase	-
3	Side marigold alcohol	-
4	Pyrrolidinyl aromatic aminotransferase	+
5	n-Arabitol	-
7	D-cellobiose	+
9	β-galactosidase	+
10	Hydrogen sulfide	-
11	β-N-acetyl glucosidase	-
12	Glutamyl aryl amine enzyme PNA	-
13	D-glucose, acid	+
14	Y-glutamyl transferase	-
15	Fermentation / glucose	+
17	β-glucosidase	+
18	Maltose	+
19	D-mannitol	+
20	D-Mannose	+
21	beta-xylosidase	-
22	β-alanine arylamine enzyme PNA	-
23	L-proline arylamine enzyme	+
26	Lipase	-
27	Ancient sugar	+
29	Tyrosine aromatic amines enzyme	+
31	Urease	+
32	D-sorbitol	+
33	Sucrose	+
34	D-tagatose	-
35	Trehalose	+
36	Citrate	+
37	Malonate utilization	+
39	5 –ketone - glucosidase	+
40	L-lactic acid Alcaligenes	+
41	α-glucose	-
42	Succinate Alcaligenes	+
43	N-acetyl-β-galactosidase	-
44	α-galactosidase	+
45	Phosphatase	+
46	Amino acid aromatic amines enzyme	-
47	Ornithine decarboxylase	-
48	Lysine decarboxylase	-
53	Histidine assimilation	-
56	Courmarate	-
57	β-glucuronidase	-
58	O/129 tolerated	+
59	Glutamic acid - glycine - arginine aromatic aminotransferase	-
61	L-malate assimilation	-
62	Ellman	-
64	L-lactate assimilation	-

+, indicates positive; -, indicates negative.

99% identity with those of *R. aquatilis* reference strains deposited in GenBank by BLAST analysis. The phylogenetic relationship based on 16S rRNA sequence showed bf008 has a close genetic relationship with 12 *R. aquatilis* reference strains (Fig. 1).

The genus *Rahnella* is a group of facultative anaerobic, Gram negative, rod-shaped, non-pigmented bacteria that belongs to the Gammaproteobacteria class. Based on the biochemical profile (Table I) and 16S rRNA analysis, the bf008 isolated from one liver sample of *Rana temporaria chensinensis* David was identified as *Rahnella aquatilis* strain.

Table II.- **Antimicrobial susceptibility patterns of** *Rahnella aquatilis* **isolate.**

Antimicrobial agent	Strains isolated
Cefoperazone	S
Cefazolin	R
Fosfomycin	R
Cefoxitin	S

S, susceptible; I, intermediate; R, resistant.
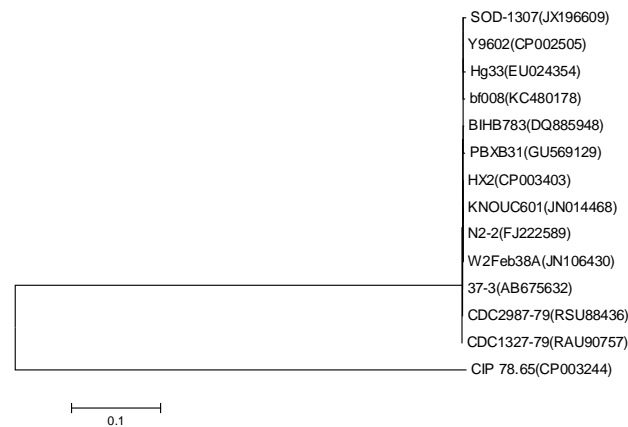
Fig. 1. Phylogenetic analyses of 16S rRNA sequences of *Rahnella aquatilis* strain bf008 and reference strains. The tree was constructed based on the neighbor-joining method using the MEGA 5.05 software. The scale bar indicates the branch lengths for 10% nucleotide differences. The accession numbers are given within brackets.

This is the first report of *R. aquatilis* from *Rana temporaria chensinensis* David from China. Using conventional microbiological methods alone might bias the results of microflora studies due to inaccurate information. Phenotypic characterization of the isolate was done based on the colony morphology, microscopic observations, and biochemical tests. We are not aware of any systematic antimicrobial susceptibility testing of *R. aquatilis* isolates. This is most likely a result of the limited number of strains available.

In 1985, the Enteric Bacteriology Section of the Centers for Disease Control reported the receipt of 15 strains of *R. aquatilis* (Farmer *et al.*, 1985). Fourteen of these strains were isolated from water (Izard *et al.*, 1979), but only one came from a clinical source (a burn wound). The first case of recovery of *R. aquatilis* from an intravenous drug abuser was reported in 1995, and that was the third published case of bacteremia due to this unusual Gram-negative bacterium (Funke and Rosner, 1995). The microbiological characteristics of the two *R. aquatilis* strains isolated in the faeces of two patients with acute gastroenteritis, one of whom was an AIDS patient was reported in 1996 (Reina and Lopez, 1996). *R. aquatilis* strain HX2 was isolated from vineyard soil in Beijing, China. This strain has the ability to solubilize mineral phosphate, fix nitrogen, and produce pyrroloquinoline quinine, indole-3-acetic acid and antibacterial substances (Chen *et al.*, 2007), and is a plant growth-promoting, disease-suppressive rhizobacterium (Guo *et al.*, 2012).

In conclusion, a *R. aquatilis* strain from *Rana temporaria chensinensis* David was identified by bacterial culture, biochemical identification and 16S rRNA sequence.

Conflict of interest declaration

There is no conflict of interest for all authors.

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Costs of Resistance to Insecticides in the Maize Weevil, *Sitophilus zeamais* (Coleoptera: Curculionidae)

Nasreen Memon[1] * and Francis Gilbert[2]
[1]*Department of Zoology, University of Sindh, Jamshoro, Sindh, Pakistan*
[2]*School of Biology, University of Nottingham, Nottingham NG7 2RD, UK*

Abstract. *Sitophilous zeamais,* the maize weevil is a main cause of infestation in stored maize throughout the tropical regions of the world. The attacked seeds are damaged badly, reduced their weight, nutritional values and market value. For the protection of maize crop,

farmers usually use pyrethroid and organophosphate insecticides. The indiscriminate use of these insecticides resulted in the development of resistance in Maize Weevil and affected their growth rate. Therefore for the determination of effect of insecticides on life history of *Sitophilous zeamais*, two pyrethroid-resistant strains from fields of the counties Juiz de Fora (JF) and Jacarezinho (JA) from State of Parana were compared with one strain susceptible to both insecticides from the county of Sete Lagoas (SL), which were reared in an insectary, under the controlled condition. We tested whether individual-based life histories of two strains of the maize weevil *S. zeamais* resistant to pyrethroid insecticides showed fitness costs relative to a susceptible strain. None were detected, despite such costs having been shown in a mass-rearing experiment using the same strains.

Key words: Insecticide resistance, maize weevils.

* Corresponding author: nasreen_kousarbks@hotmail.com

A major cause of losses to stored maize throughout the tropical and temperate regions around the world is the maize weevil *Sitophilous zeamais* Motschulsky 1855 (Coleoptera: Curculionidae). Damaged grains have reduced nutritional values, low percent germination and reduced weight and market value. Worldwide seed losses ranging from 20-90 % have been reported for untreated maize due (Giga and Mazarura, 1991). Pyrethroid and organophosphate insecticides are heavily used for maize weevil control (Fragoso *et al.,* 2003; Guedes *et al.,* 1995), and development of resistance is a serious worldwide challenge for pest management of such stored–product pests. Over-reliance on insecticides for controlling the maize weevil and other stored grain pests in tropical areas has made insecticide resistance a frequent problem (Champ and Dyte, 1976)

Insecticide resistance affects weevil physiology (Fragoso *et al.,* 2007) and components of the life history that map into population growth rate (Fragoso *et al*., 2005). The paradigm of such effects involves the idea that there is a cost to resistance, but in environments with insecticides the benefits outweigh the costs and resistance evolves (Baucom and Maurizio, 2004). The same paradigm is used in

interpreting insect resistance to plant secondary defensive chemicals (Zavala *et al.,* 2004), such as the furanocoumarins of umbellifers (Strauss and Zangerl, 2002). In the absence of herbivores, plants producing such chemicals are at a selective disadvantage. Thus insecticide resistance should disappear when insecticides are withdrawn, since then the costs outweigh the benefits, which are not expressed in an insecticide-free environment (Baucom and Maurizio, 2004). This is exactly what happens with the resistant weevil strains used here (Guedes *et al*., 1995; Ribeiro *et al.,* 2003; Fragoso *et al*., 2003, 2007).

Fragoso *et al.* (2005) studied the population growth of *S. zeamais,* assessing two resistant and two strains susceptible to pyrethroid pesticides. Using mass culturing methods, they found that both susceptible (from Bragança Paulista and Sete Lagoas) and one resistant strain (from Jacarezinho) were very similar to one another, while a further resistant strain (from Juiz de Fora) showed reduced developmental rate, delayed emergence, higher mortality and reduced growth rate. The main object of this study was to evaluate differences among three of these strains using individual-based data, to dissect further the relevant life-history differences.

Materials and methods

Three strains of *S. zeamais* were used in this study, originally collected from Brazil: two pyrethroid-resistant (>100-fold in bioassays) strains from fields of the counties Juiz de Fora (JF, from the State of Minais Gerais in 1999: Guedes *et al.,* 1995; Fragoso *et al.,* 2003) and Jacarezinho (JA, State of Parana in the late 1980s), and one strain susceptible to pyrethroids and organophosphates (Guedes *et al.,* 1994, 1995; Fragoso *et al.,* 2003; Ribeiro *et al.,* 2003; Araujo *et al.,* 2008) from the county of Sete Lagoas (SL, State of Minais Gerais, maintained for 20 years without exposure to insecticides in the National Research Centre of Maize and Sorghum of the Brazilian Agricultural Research Corporation, Embrapa Milho e Sorgho). All strains were kindly provided by Dr. Guedes and Rubia Araujo from the Ecotoxicology Laboratory of the Federal University of Viçosa, Minais Gerais, Brazil.

The deltamethrin-resistance of the JA strain is sex-linked (Guedes *et al.,* 1994, 1995; Ribeiro *et al.,* 2003). The JF population shows an overall fitness disadvantage in the absence of pyrethroids, unlike the JA population (Fragoso *et al.,* 2005; Oliveira *et al.,* 2007). All populations were maintained in mass-culture jars on whole maize grains (13% moisture content) at a temperature 25 ± 2ºC.

Weevils were reared at 27 ± 2ºC (in an insectary of School of Biology, University of Nottingham, Nottingham, UK) in male-female pairs in Petri dishes of 90 mm diameter with filter paper on the bottom, and provided with ten insecticide–free whole maize grains as feeding and oviposition substrates. The sexes were identified by the shape of their snout. Petri dishes were kept in boxes over salted water for maintaining humidity, in an insectary at 25-27ºC.

Ten newly emerged male-female pairs were selected at random from stock cultures of each strain. Every day for three months from 6th January 2010 to 7th April 2010, each pair was transferred to a new dish: the number of eggs laid in the maize grains of the old dish were counted, and the dish placed in a rearing box to await the emergence of the adults. Emergence was assessed every day. After the 7th April, pairs were simply maintained until death, without measuring egg production any more, or until the end of June 2010 when the experiment stopped.

Fewer eggs were counted than were laid because ovipositions were difficult to see. Females bite a small hole, lay an egg and coat it with a glycosidic substance that hardens rapidly into a solid egg plug that seals the hole (Woodbury, 2008). Often this is hardly any different from the maize surface, and in addition the hole is often drilled around or under imperfections in the surface, making some all but impossible to see. Thus the number of eggs counted does not match the number of emergences recorded, and very often the latter is greater than the former.

Statistical analysis was carried out using R (Zuur *et al*., 2009). All variable residuals were checked for normality, and non-parametric tests used where transformations (log or square-root) were unsuccessful in resolving the non-normality. KW indicates a Kruskal Wallis non-parametric test for one-way group differences, distributed as a x^2.

Results

About 50% more eggs were counted from females of the JA strain (1408) than either JF (961) or SL (936) females, mostly because fewer females failed to produce eggs rather than higher production per female (Fig. 1): the differences were not significant (KW=2.8, df=2, ns). The JA strain also produced more emerging adults (1690) than either JF (930) or SL (956), but again the values for each pair were very variable, and the means for the strains were not significantly different (KW = 4.2, df=2, ns). There were no differences in longevity among strains for males (KW = 2.0, df=2, ns) or females (KW = 2.5, df=2, ns).

To demonstrate the pattern of egg laying, we plotted the mean number laid per week for each strain (Fig. 2). Notice how the number rises rapidly to a peak, and then declines more or less linearly to reach zero or near zero by week 14. We therefore removed data from before day 10 and after day 90 to restrict them to the period within which the relationship between eggs laid and laying day is approximately linear. The number of eggs counted per day was then analysed by a generalised linear model with quasi-Poisson errors in a model consisting of pair-within-strain, strain, laying day (covariate) and an interaction between laying day and strain (*i.e.* a test for different slopes for the strains). Much of the deviance was accounted for by pairs-within-strains (Wald χ^2_{25} = 329.3, p<<0.001) and the overall regression with laying day (Wald χ^2_1 = 263.8, p<<0.001) with its negative slope. While there were no differences overall among strains (Wald χ^2_2 = 3.0, ns), there were significant differences among the strains in the slope of the relationship with laying day (Wald χ^2_2 = 14.5, p=0.001), all accounted for by the slope of strain JA (m = -0.015±0.0056) which was shallower than those of SL (-0.025 ± 0.0025) and JF (-0.023 ± 0.0058).

Egg-to-adult development times varied from 28 to 50 (Fig. 3), with a mean of 36.3 ± 1.3 days. In a mixed model of the effects of pair-within-strain, strain and laying day on development times for individual weevils, there was no evidence of any differences among strains ($F_{2,19.2}$ = 0.22, ns), but a strong negative effect of laying day ($F_{1,3533}$ = 63.5,

p<0.001; slope = -0.0246±0.0031) with no evidence for strain differences in slopes ($F_{2,3410}$ = 2.07, n.s.). The slope indicates that over the laying period of approximately 100 days, the egg-to-adult developmental period declined by about 2.5 days (Fig. 4).

The pattern of number of emergences from eggs laid on any one day was similar to that of the number of eggs. We similarly restricted the analysis to laying days after 10 and before 90. There were no strain differences overall (Wald χ^2=2.9, df=2, ns), but there was a strong negative effect of laying day (Wald χ^2=207.3, df=1, p<<0.001) with significant differences among strains in the slopes (Wald χ^2=22.5, df=2, p<0.001). Once again the JA strain had a shallower slope (-0.010) than the other two strains (SL, -0.021; JF -0.019).

Discussion

Maize weevil resistance to pyrethroid insecticide has been linked with reduced target-site sensitivity as the major mechanism, and increased detoxification by glutathione-S-transferases as a secondary mechanism (Fragoso *et al*., 2003, 2007; Guedes *et al*., 1995, 2006; Ribeiro *et al.,* 2003). The target site resistance was sex-linked (Guedes *et al*., 1994). Both resistant strains of the weevil were heavily subjected to pyretheroid insecticide application.

Fragoso *et al*. (2005) found that the Juiz de Fora (JF) population showed a fitness disadvantage in the absence of pyretheroids unlike that of Jacarezinho (JA). Based on mass data, they found that the JF population had reduced and delayed emergence relative to susceptible strains (Fragoso *et al.,* 2005; Oliveira *et al.,* 2007). Looking at individual-based data, we failed to detect any such reduced or delayed emergence in the JF strain. There were no differences of any kind between the resistant JF strain and the susceptible SL strain. The only possible differences among strains lay in the slightly more gently decline with female age in the number of eggs laid and the egg-to-adult development time of her offspring in the resistant JA strain relative to the other two. Although logistic reasons prevented us from using large numbers of pairs, and so statistically we are not sure, it is

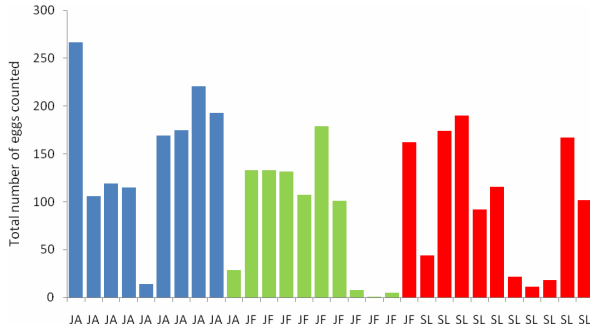certainly possible that JA females are less likely to fail to lay.

Fig. 1. Total number of eggs counted for each pair of the three different strains. (JF, Juiz de For a (resistant); JA, Jacarezinho (resistant); SL, Sete Lagoas (susceptible).
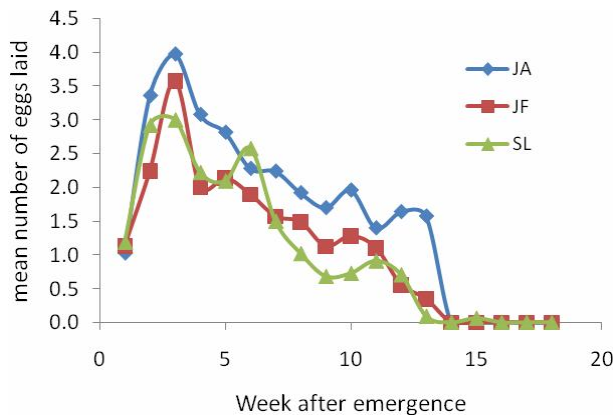

Fig. 2. The number of eggs laid per week by pairs of *Sitophilus zeamais* weevils of three different strains. For abbreviations see Figure 1.

Fragoso *et al.* (2005) did not find differences between the resistant JA strain and susceptible strains, suggesting that resistance might be fixed in this laboratory population, accounting for its relatively good demographic performance. They also pointed to the good conditions of laboratory culturing. It is certainly true that demographic trade-offs might only be evident under stressful conditions (Zera and Harshman, 2001), unlike those of the laboratory environment. Thus if we were to isolate weevils without food for periods of time, then such costs might be measurable.

Acknowledgements

We thank Dr RNC Guedes and Rubia Araujo from the Ecotoxicology Laboratory of the Federal
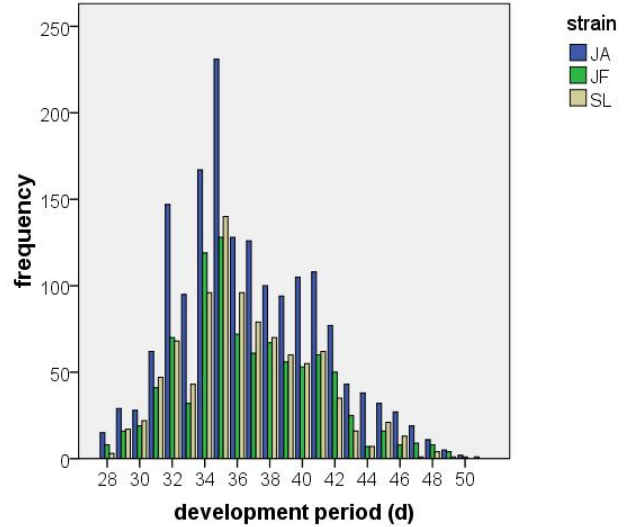
Fig. 3. Frequency distribution of the egg-to-adult developmental period for the three strains of *Sitophilus zeamais*. For abbreviations, see Figure 1.
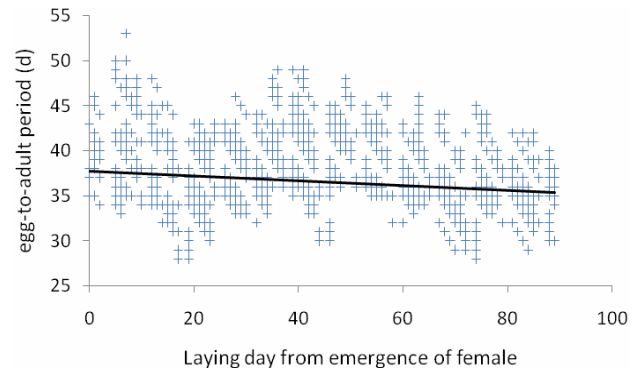

Fig. 4. Relationship between egg-to-adult developmental period (in days) and laying day (from emergence of female).

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Consequences of Spring Warming for the Black Redstart (*Phoenicurus ochruros*) in N.W. Croatia

Zdravko Dolenec,[1]* **Jelena Kralj,**[2] **Damir Sirovina,**[1] **and Darinka Kiš-Novak**[3]
[1]Department of Zoology, Faculty of Science, University of Zagreb, Rooseveltov trg 6, HR-10000 Zagreb, Croatia
[2]Institute of Ornithology, Croatian Academy of Sciences and Arts, HR-10000, Zagreb, Croatia
[3]Faculty of Teacher Education, University of Zagreb, Centre in Čakovec, Starčevićeva 55, HR-40000 Čakovec, Croatia

Abstract.- Average global temperatures in Earth have increased over the past 100 years and climate change impacts wildlife in countless ways. Numerous of the works demonstrated climatic impacts on bird phenology. We used 26 years of data from black redstart (*Phoenicurus ochruros*) in population from Mokrice village (northwestern Croatia), 1987-2012. Correlation between timing of arrivals and year was significant. Black redstart arrival dates have become 8.84 days earlier during the research period. The relationship between local mean spring temperatures and year was significant. The date trends in spring migration correspond with increasing mean spring air temperatures (March-April). This result suggests that black redstart respond to air spring temperatures by earlier arrival at breeding grounds. Our results are consistent with many other long-term studies of the timing of birds migration and suggest that air spring temperature change may be responsible for shifts in arrival dates.

Key words: Spring temperatures, black redstart, Croatia

Average global temperatures in Earth have increased over the past 100 years (IPCC, 2007), and climate change impacts wildlife in countless ways (Parmesan, 2006). For example, studies from throughout the world have demonstrated that tree

* Corresponding author: dolenec@zg.biol.pmf.hr

phenophases are becoming earlier in spring and are closely associated with rising temperatures (*e.g.* Wielgolaski, 1999) and red squirrels (*Tamiasciurus hudsonicus*) have shifted their breeding period towards earlier dates (Réale *et al*., 2003). Furthermore, in numerous bird populations in temperate areas, temperature has been demonstrated to effect of breeding (*e.g.*, Dunn and Winkler, 1999; Dolenec *et al*., 2011b), clutch size (Möller, 2002), population dynamics (*e.g.,* D'Alba *et al*., 2010), breeding range (*e.g.,* Thomas and Lennon, 1999), etc. Most studies look at phenology performance (*e.g.,* Wesolowski and Cholewa, 2009). Numerous of the works illustrated climatic impacts on bird migration timing concentrate on the last three or four decades of rapid increase in air temperatures. For example, the blackcap (*Sylvia atricapilla*) across northwestern Croatia have advanced arrival by as many as nine days from 1979 to 2007 (Dolenec and Dolenec, 2010b).

The black redstart (*Phoenicurus ochruros*), is a small, insectivorous, socially monogamous short/medium-distance migrant bird species (Cramp, 1998) and common breeding species in north-western Croatia. The main aim of this work is to describe the potential change in spring migration of the black redstart and identify relationship between mean spring air temperatures and arrival date.

Material and methods

Data was collected between 1987 and 2012 for the black redstart in Mokrice village (mixed farming); 46.00 N 15.55 E; *ca.* 140 m above sea level; 6.4 km^2; northwestern Croatia). For detailed description of the study site see Dolenec *et al.* (2011a). Dates were converted to numerical values such that 1 March = 1, *etc.* Arrival date for each year was calculated as the mean of the first five birds arrivals recorded for that year (method previously used by Both *et al.*, 2005). Observations from 1987 to 2012 were recorded by Z. Dolenec. The data were collected daily (sometimes in the morning and sometimes in the afternoon) by author who lives in the village Mokrice. To investigate the impact of spring temperatures on the date of arrival, we used local climate (temperature) data for the

months of March and April (arrival period). Mean monthly Mokrice area air temperatures were provided by the Meteorological Office in Zagreb, (weather station at Maksimir, 20 km from the centre of the research area, 123 m a.s.l.)(March-April, mean = 9.4±1.26ºC, range = 6.5 to 11.3ºC; separately: March, mean = 7.1±2.08, range = 1.7 to 10.3ºC and April, mean = 11.7±1.44ºC, range = 8.2 to 14.5ºC). Local temperatures have been commonly used in different phenological studies (*e.g.,* Sokolov *et al.,* 1998; Biaduń *et al.*, 2011; Dolenec and Dolenec, 2011a,b).

Statistical analysis of long-term trends in the timing of spring arrival and spring mean air temperatures was performed by regressions analysis. All analyses were performed using SPSS 13.0 for Windows. Significance was set al P <0.05 for all statistical tests.
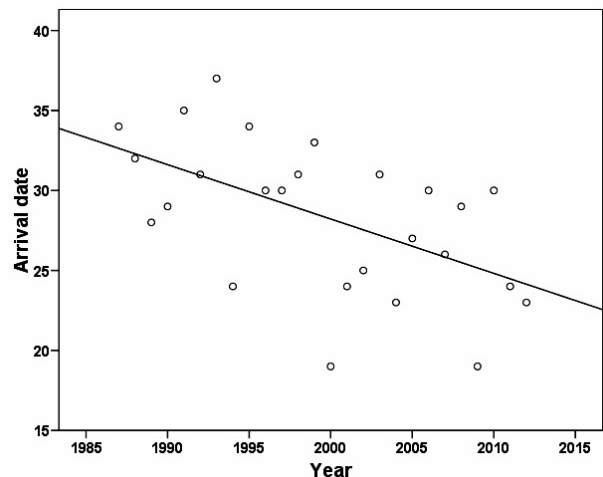

Fig. 1. Relationship between arrival date and year for black redstart, 1987-2012.

Results and discussion

The black redstart arrived between 19 March (2000) and 6 April (1993) (mean = 28 March, SD = 4.73). A regression analysis of the arrival dates for the researches period is illustrated in Figure 1; it indicates an overall increase in advances over the 26-year period, with a slope (linear regression) of -0.34 days per year (8.84 days during the research period), and with a 99, 6% probability of significance (Pearson's coefficient r = -0.55, p = 0.004, N = 26; slope = -0.34). During the study

period, local air spring temperatures (March-April) have significantly increased by approximately 1.82 °C in the arrival period (r = 0.43, p = 0.029, N = 32; slope = 0.07; Fig. 2). The relationship between spring temperatures and arrival date was also significant (r = 0.6, p = 0.001, N = 32; slope = 2.26; Fig. 3).
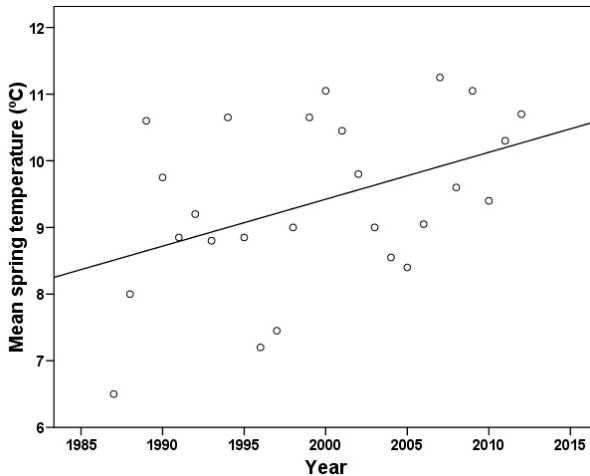

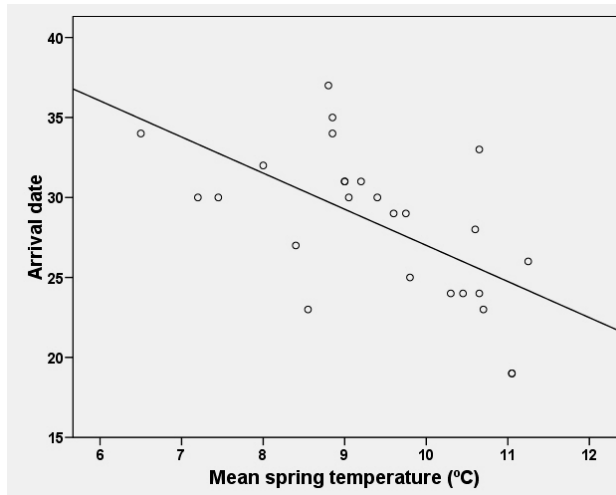Fig. 2. Relationship between local air spring temperatures (March-April) and year, 1987-2012.

Fig. 3. Relationship between arrival date and local air spring temperatures (March-April) in black redstart, 1987-2012.

This result suggests that black redstart respond to air spring temperatures by earlier arrival at breeding grounds. This change in migration

behavior has coincided with an increase in spring air temperature. This results agree with those of Tryjanowski *et al.* (2002) that air temperatures are important determinant of arrival date in the black redstart (western Poland, 1970–1996). A study of 34 years (1971-2004) of data for 40 species of bird migrants (16 short/medium- and 24 long-distance migrants) in Lithuania (Zalakevicius *et al.*, 2006) showed that 28 species have altered their arrival dates significantly, with most arriving earlier, in conjunction with climate change. In the case of the black redstart, he reported advancement in arrival dates. Furthermore, in Germany (Peintinger and Schuster, 2005), date of arrival in a black redstart population also advanced over a 34-year period (1970-2003). Contrary, the arrival of black redstart in eastern Poland (Biaduń *et al*., 2011) did not change during the research period, 1970-2009. The advances in arrival dates over time in black redstart are similar to those seen previously in some other bird species in Croatia (*e.g.* Dolenec and Dolenec, 2010a; Dolenec, 2013), but not all (*e.g.* Dolenec, 2012). Some authors in other countries also haven't found trend of earlier arrival dates. For example, in Poland (Biadun *et al*., 2009) only two out of 15 bird species demonstrated a statistically significant trend in earlier arrivals. Likewise, in Russia (Sokolov and Gordienko, 2008), only two out of 16 bird species demonstrated significant trend. According to Biaduń *et al.* (2009) results are consistent with hypothesis that birds living interior a continent are more influenced by climate than the ones living on periphery. The benefits of arriving early enough to successfully claim a territory for nesting (Kokko, 1999).

Some organisms may be facile changing their seasonal progressions in relation to climate changes, whereas others are less able to respond (Bradley *et al*., 1999). According to Visser (2008), the pivotal question in the debate on the ecological effects of climate change is whether species will be able to adapt fast enough to keep up with their changing environment.

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