

RESEARCH ARTICLE

Chironomidae Bloodworms Larvae as Aquatic Amphibian Food

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Different species of chironomids larvae (Diptera: Chironomidae) so-called bloodworms are widely distributed in the sediments of all types of freshwater habitats and considered as an important food source for amphibians. In our study, three species of Chironomidae (*Baeotendipes noctivagus*, *Benthalia dissidens*, and *Chironomus riparius*) were identified in 23 samples of larvae from Belgium, Poland, Russia, and Ukraine provided by a distributor in Belgium. We evaluated the suitability of these samples as amphibian food based on four different aspects: the likelihood of amphibian pathogens spreading, risk of heavy metal accumulation in amphibians, nutritive value, and risk of spreading of zoonotic bacteria (*Salmonella*, *Campylobacter*, and ESBP producing Enterobacteriaceae). We found neither zoonotic bacteria nor the amphibian pathogens *Ranavirus* and *Batrachochytrium dendrobatidis* in these samples. Our data showed that among the five heavy metals tested (Hg, Cu, Cd, Pb, and Zn), the excess level of Pb in two samples and low content of Zn in four samples implicated potential risk of Pb accumulation and Zn inadequacy. Proximate nutritional analysis revealed that, chironomidae larvae are consistently high in protein but more variable in lipid content. Accordingly, variations in the lipid: protein ratio can affect the amount and pathway of energy supply to the amphibians. Our study indicated although environmentally-collected chironomids larvae may not be vectors of specific pathogens, they can be associated with nutritional imbalances and may also result in Pb bioaccumulation and Zn inadequacy in amphibians. Chironomidae larvae may thus not be recommended as single diet item for amphibians. *Zoo Biol.* 33:221–227, 2014. © 2014 Wiley Periodicals, Inc.

Keywords: nutritional imbalances; amphibian pathogens; zoonotic agents

INTRODUCTION

Chironomidae are a family of nematoceran flies with a worldwide distribution. This large taxon of insects resembles mosquitoes but they lack the wing scales and elongate mouthparts of the Culicidae. In Europe, bloodworm is the common name for the aquatic larval stage of Chironomidae. However, this term is also attributed to Glyceridae (a family of polychaete worms) in some parts of the world [Kozloff, 1987].

Chironomids larvae can be found in almost any aquatic or semiaquatic habitat and are important food items for both vertebrates and invertebrates [Armitage, 1995; Bat and Akbulut, 2001].

Captive propagation of amphibians has gained attention in recent years, ex situ conservation being considered a

last resort to safeguard many species from extinction [Stuart et al., 2004]. Commercially available bloodworms are used in many aquatic amphibian species as important component of the captive diet. However, little is known about the nutrient composition of different species of chironomids larvae.

In addition, feeding bloodworms may compromise the

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Received 03 May 2013; Revised 10 January 2014; Accepted 27 January 2014

DOI: 10.1002/zoo.21122

Published online 17 February 2014 in Wiley Online Library (wileyonlinelibrary.com).

health of both the amphibians and their keepers. Indeed, since bloodworms feed on algae and detritus, they represent a possible reservoir of zoonotic agents (*Salmonella*, *Vibrio cholerae*, *Campylobacter jejuni*, and *Escherichia coli*) [Rouf and Rigney, 1993; Broza and Halpern, 2001; Moore et al., 2003] and of acquired antimicrobial resistance. Captive amphibian health may also be influenced by feeding bloodworms to amphibians, through possible transmission of *Ranavirus* virions and *Batrachochytrium dendrobatidis* zoospores (causal agent of chytridiomycosis) [Gleason et al., 2008; Gray et al., 2009].

Apart from infectious agents, bloodworms may contain high levels of heavy metals. Due to pollution and anthropogenic processes, the prevalence of heavy metals such as lead (Pb), zinc (Zn), cadmium (Cd), mercury (Hg), and copper (Cu) has increased in aquatic environments [Blaustein et al., 2003], resulting in possible accumulation in bloodworms [Sharley et al., 2004]. Amphibians are considered highly susceptible to heavy metal intoxications [Lefcort et al., 1998].

In this study, we determined the identity and the nutritional value of a selection of bloodworms species originating from different countries, and the possible impact of the use of these bloodworms on human and amphibian health. Subsequently, their content of heavy metals and the presence of the following zoonotic and amphibian pathogens was determined: *Salmonella* spp., Extended-Spectrum Beta-Lactamase (ESBL) producing Enterobacteriaceae, *Campylobacter* spp., *Ranavirus*, and *B. dendrobatidis*.

MATERIALS AND METHODS

Bloodworms

Between October 2011 and April 2012, we collected chironomid larvae which were bought from a distributor in Belgium. This resulted in 23 bloodworm samples collected in Russia (18 samples), Poland (2 samples), Ukraine (1 sample), and Belgium (2 samples).

DNA Preparation

DNA was prepared for chironomid species identification and pathogen detection. We preserved all samples in 70% ethanol prior to DNA extraction. For the DNA preparation, the larvae were crushed to a fine powder in liquid nitrogen in a 1.5 ml Eppendorf tube and genomic DNA was extracted using the Bioline Isolate Genomic DNA Mini Kit (BIOLINE, London, UK). We stored extraction products at -20°C .

PCR Amplification to Identify the Chironomid Species

A 710-bp fragment of the mitochondrial cytochrome oxidase subunit (COI) was amplified as described by Sharley et al. [2004] using primers 911(5'TTTCTACAAATCAT-AAAGATATTGG3') as forward primer and HCO2198

(5'TAAACTTCAGGGTGACCAAAAAATCA3') as reverse primer.

The PCR products were purified by PCR purification kit (QIAGEN, GmbH, Hilden, Germany) according to manufacturer's instructions and sequenced with the same primers used in the PCR on an automated DNA sequencer (ABI3700, Applied Biosystems, California, USA). The sequence data were assembled and aligned using LICOR AlignIR Version 2. The obtained data were then compared to those published in GenBank through BLAST network service (<http://www.ncbi.nlm.nih.gov/BLAST>).

Isolation of *Salmonella*, *Campylobacter* and ESBL Producing Enterobacteriaceae

The fresh larvae were homogenized thoroughly by grinding and then divided into three subsamples for detection of *Salmonella* spp., ESBL producing Enterobacteriaceae and *Campylobacter* spp. We used the sterile procedures and instruments for each sample throughout the study to prevent the cross-contamination of the samples.

We investigated the presence of *Salmonella* spp. according to the EN ISO 6579:2002 standard method which was modified according to the recommendations of the Community Reference Laboratory for *Salmonella* in Bilt-hoven, the Netherlands. Briefly, pre-enrichment was done by incubation of the samples in buffered peptone water (Oxoid, Basingstoke, UK) during 18 hr at 37°C . From the pre-enrichment solution, three droplets were inoculated in a Rappaport-Vassiliadis broth (Difco, Becton, Dickinson and Co., New York) and Tetrathionate broth (Oxoid). Then they were incubated for 24 hr at 37°C . Samples from both broths were plated on Brilliant Green agar (Oxoid) and Xylose-Lysine-Deoxycholate agar (Oxoid), followed by incubation for 24 hr at 37°C .

For the isolation of ESBL producing Enterobacteriaceae, each homogenized sample was directly plated onto McConkey agar (Oxoid) containing $8\ \mu\text{g/ml}$ ceftiofur and was incubated for 24 hr at 37°C .

For detection of *Campylobacter* spp. 1 g of each sample was diluted in nutrient broth No.2 (Oxoid) supplemented with Modified Preston *Campylobacter*-selective supplement (Oxoid) and *Campylobacter*-specific growth supplement (Oxoid) and incubated for 24 hr at 42°C under microaerobic conditions. Then diluted samples were plated onto modified Charcoal Cefoperazone Deoxycholate agar (Oxoid) plates, supplemented with CCDA selective supplement (Oxoid), and *Campylobacter*-specific growth supplement (Oxoid). The plates were incubated for 48 h at 42°C under microaerobic conditions.

Detection of Zoonotic and Amphibian Pathogens using PCR

In parallel to bacteriological cultures, we tested all chironomid larvae samples for the presence of *Salmonella*, *Campylobacter*, *Ranavirus*, and *B. dendrobatidis* by PCR.

A PCR to detect the presence of *Salmonella* was performed as described by Rahn et al., [1992] using the *invA* gene primers 5'GTGAAATTATCGCCACGTTCCGGCAA3' and 5'TCATCGCACCGTCAAAGGAACC3'. A negative control (HPLC water) and positive control (DNA extracted from *Salmonella* Typhimurium DAB69) were used in each PCR run.

For the detection of *Campylobacter* a PCR was performed as described by Wegmuller et al. [1993] using *Campylobacter jejuni* *flaA* and *flaB* gene primers 5'GCTCAAAGTGGTTCT-TATGCNATGG3' and 5'GCTGCGGAGTTCATC TAA-GACC3'. A negative control (HPLC water) and a positive control (DNA extracted from *C. jejuni* KC40) were included in each PCR run.

For the detection of *Ranavirus*, a PCR reaction was performed following the protocol of Mao et al. [1997] using the major capsid protein (MCP) of FV3 gene primers 5'GACTTGGCCACTTATGAC3' and 5'GTCTCTGGA-GAAGAAGAA3'. Negative control (HPLC water) and positive control (DNA extracted from an infected and confirmed ranavirus-positive tadpole) served as controls for the PCR runs.

Amplification products were run on 1.5% agarose gel, immersed in TAE buffer (40 mM Tris-Acetate, 1 mM EDTA) stained with Ethidium Bromide 0.5 µg/ml and visualized with UV transilluminator.

The presence of *B. dendrobatidis* was investigated with quantitative PCR (qPCR) described by Boyle et al. [2004]. Subsequently, DNA samples were diluted 1:10 and qPCR assays were performed in duplicate on a CFX96 Real Time System (BioRad Laboratories, Hercules, CA). We used ITS1-3 Chytr (5-CCTTGATATAATACAGTGTGCCATATGTC-3) for the forward primer, 5.8S Chytr (5-AGCCAAGA-GATCCGTTGTCAA-3) for the reverse primer and Chytr MGB2 for the TaqMan Probe (5-6FAM CGAGTCGAA-CAAAAT MGBNFQ-3). One positive control sample containing *Bd* DNA (JEL423) and three negative control samples with HPLC water were included for each assay. To control and estimate inhibition, a subset of samples negative for the presence of *Bd* ($n = 20$) was retested under the same conditions as described above, but with an exogenous internal positive control (VICTM probe, Life technologies, Austin, TX) included as described by Hyatt et al. [2007].

Determination of Heavy Metal Content

We analyzed concentrations of Pb, Zn, Cd, Hg, and Cu using the method described by Tack et al. [2000]. For this purpose, 7 ml ultrapure 65% HNO₃ was added to 1 g of the larvae in a 100 ml Pyrex beaker covered with a watch-glass. The suspension was heated up to 150°C for 2 hr. Then it was treated with 4 ml 30% H₂O₂. After cooling, the solution was transferred to a 50 ml flask and diluted to the mark with distilled de-ionized water. Determination of Cd, Cu, Zn, and Pb in the solution was performed by inductively coupled plasma optical emission spectrometry (ICP-OES, Varian

Vista MPX, Palo Alto, CA) when results were below detection limit of ICP-OES. Mercury was measured using a cold vapor atomic absorption mercury analyzer (CV-AAS, CETAC QuickTrace M-7500, Omaha, NE).

Nutritional Analysis

All chironomid larvae samples were stored frozen at -20°C prior proximate analysis. To determine nutritional analysis, each sample was individually analyzed. We measured dry matter (DM) as performed by De La Noue and Choubert [1985]. Larvae were dried at 95°C for 24 hr until a constant weight was achieved. Ash contents were determined as described by Bogut et al. [2007] burning the larvae at 550°C for 4 hr in a muffle furnace. We determined crude protein (CP) content according to Kjeldahl × 6.25 methods using the Kjel-Foss, and crude fat (CF) content by Soxhlet methods. Neutral detergent fibre (NDF) and acid detergent fibre (ADF) were determined according to Association of Official Analytical Chemist [AOAC, 1990] methods.

Statistical Analysis

We evaluated the overall difference between species for each variable by using either an ANOVA or the non-parametric Kruskal-Wallis test. For each variable, an ANOVA model was built. Normality of the residuals for each model was evaluated using a QQ plot. Models without normally distributed residuals were disregarded, and a Kruskal-Wallis test was used instead for those variables. When applicable, a Dunnett T3 pairwise comparison test was carried out as well. This post-hoc test could deal with unequal variances and sample sizes, while being more conservative than, that is, the Games-Howell post-hoc test. It must be noted that due to the unequal sample sizes, the Kruskal-Wallis test might be overly optimistic [Zimmerman, 2000].

RESULTS

Identification of the Larvae

Fourteen samples originating from Russia and the only sample from Ukraine consisted of *Baeotendipes noctivagus*. The samples from Belgium (two samples), Poland (two samples) and one sample from Russia contained the species *Chironomus riparius*. Three samples from Russia belonged to the species *Benthalia dissidens*.

Zoonotic and Amphibian Pathogens

Cultures for *Salmonella*, *Campylobacter*, and ESBL producing Enterobacteriaceae were all negative. The negative results for *Salmonella* and *Campylobacter* were confirmed by PCR. The PCR's for the amphibian pathogens *B. dendrobatidis* and *Ranavirus* were all negative.

Heavy Metal Content

For the three species identified, *B. noctivagus* ($n = 15$), *C. riparius* ($n = 5$), and *B. dissidens* ($n = 3$), the concentrations of heavy metals varied between 0.0007 and 0.0790 $\mu\text{g/g}$ for Hg, 2–17 $\mu\text{g/g}$ for Cu, <0.003–0.351 $\mu\text{g/g}$ for Cd, 0.6–9.9 $\mu\text{g/g}$ for Pb, and 9–59 $\mu\text{g/g}$ for Zn on a dry matter basis (DMB).

The residual analysis of the ANOVA models showed for all metals a moderate to strong deviation from normality. Therefore, we evaluated our data using Kruskal–Wallis. The Hg concentrations differed significantly between groups ($P = 0.007$). The boxplot (Fig. 1) points towards lower concentrations for *C. riparius* compared to *B. noctivagus*. In addition, *C. riparius* contained higher concentrations of Zn compared to the two other species ($P = 0.007$). The distribution of the measurements within each species is given in Figure 1.

Nutritive Value

The QQ plots showed in general an acceptable normality for the residual values. Hence, we could use the ANOVA models for comparison of the different species. Differences between species were found for CP ($P < 0.001$), ADF ($P = 0.007$), CF ($P < 0.001$), and Ash ($P = 0.001$). According to the post hoc tests, *C. riparius* differed from *B. noctivagus* and *B. dissidens* by a significantly higher content of both CP and CF ($P < 0.05$) and a significantly lower content of ADF ($P < 0.05$). *B. dissidens* contained significantly less CF than the other two species ($P < 0.05$). However, due to the low numbers of *B. dissidens*, it should be careful drawing firm conclusions from these results. The distribution of the nutrient values per chironomid species are illustrated in Figure 2.

DISCUSSION

In our samples, commercially available “bloodworms” comprise at least three different species of Chironomidae larvae (*B. noctivagus*, *C. riparius*, and *B. dissidens*) with *B. noctivagus* as the predominant species. Patterns of species diversity can vary over spatial scales and the pattern observed in this study may differ from those found in other parts of the world.

In contrast with previous studies [Rouf and Rigney, 1993; Broza and Halpern, 2001; Moore et al., 2003], none of the tested samples were positive for *Salmonella*, *Campylobacter*, ESBL producing Enterobacteriaceae, *Ranavirus*, or *B. dendrobatidis*. Thus, the use of bloodworms appears to pose a minor risk regarding spread of these pathogens to humans or amphibians. However, samples used in this study only represent a snapshot of possible contamination, not taking into account for example seasonal fluctuations in disease dynamics [Bosch et al., 2007; Todd-Thompson, 2010].

We compared the heavy metal concentrations in the bloodworms with the maximum metal content of Hg (0.1 $\mu\text{g/g}$) (The EFSA Journal, 2008), Cu (35 $\mu\text{g/g}$) (European Commission Directive 70/524/EEC, 2003), Cd (2 $\mu\text{g/g}$) (EU in Directive 2005/87/EC), Pb (5 $\mu\text{g/g}$) (EU in Directive 2005/87/EC), and Zn (250 $\mu\text{g/g}$) (European and Directive 70/524/EEC, 2003) in fish (as typical aquatic animals) diet. Our results revealed that the concentration of Pb in two samples exceeded the maximum metal levels. However, four samples contained a lower concentration of Zn than the minimum requirement level (15 $\mu\text{g/g}$) in fish diet [Watanabe et al., 1997]. The excessive levels of Pb and low contents of Zn render the sole feeding of commercial bloodworms to captive amphibians questionable. Hence,

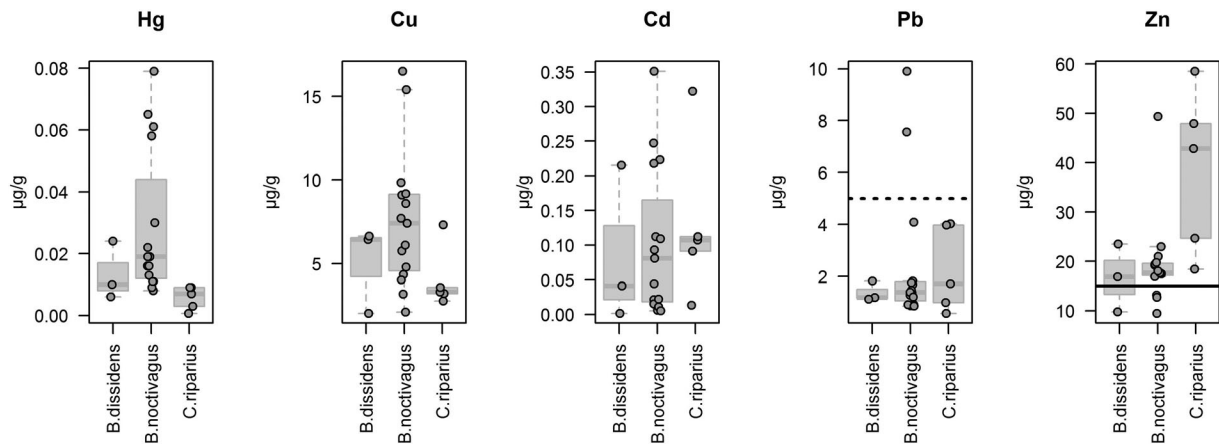


Fig. 1. Box- and point plots for trace metals (Zn and Cu) and xenobiotic heavy metals (Hg, Cd, and Pb) concentrations ($\mu\text{g/g}$) per chironomid species on a dry matter basis (DMB). The point line indicates the maximum Pb levels for fish (as typical aquatic animals) food (5 $\mu\text{g/g}$) and exhibits two samples are above the maximum limits. The black line indicates the minimum Zn requirement as trace metal (15 $\mu\text{g/g}$) in fish diet and shows four samples are below the limit. Other metal values were in acceptable limits in fish food.

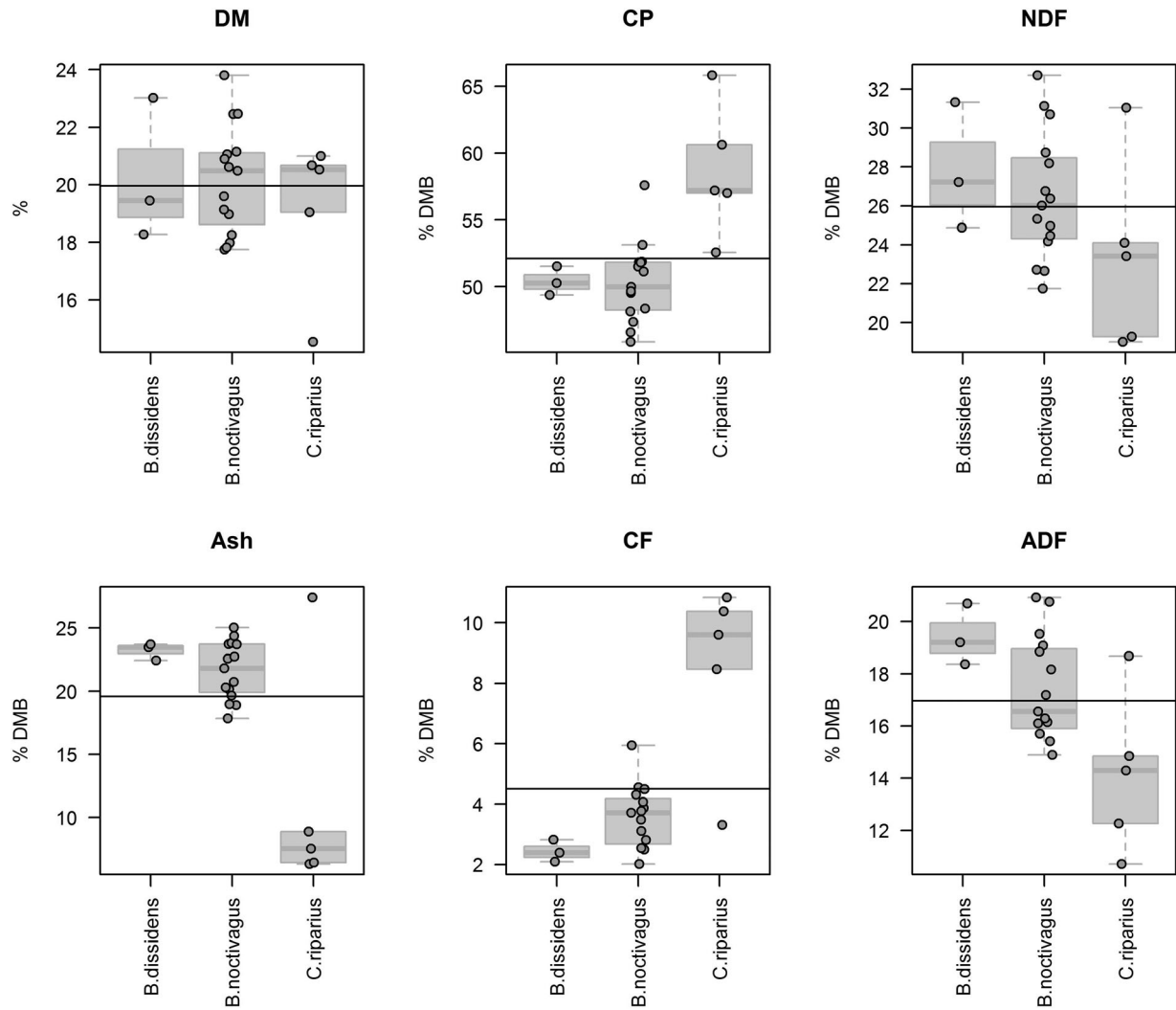


Fig. 2. Box- and point plots for nutrients measures per chironomid species as percent on a dry matter basis (DMB) within species. Black lines indicate the average value of each nutrient including 19.95 ± 2.06 , 52.11 ± 4.73 , 16.96 ± 2.65 , 19.58 ± 6.19 , 4.50 ± 2.68 , and 25.95 ± 3.78 for DM, CP, ADF, Ash, CF, and NDF, respectively. DM: Dry Matter, CP: Crud Protein, ADF: Acid Detergent Fiber, CF: Crud Fat, NDF: Neutral Detergent Fiber.

feeding a variety of prey items is advisable to overcome Zn inadequacy and to maintain balanced physiological metals content for amphibians.

Since dietary zinc has an antagonistic effect on Cu absorption [Evans et al., 1970] leading to decreased Cu status in organisms, the high concentrations of Zn in *C. riparius* suggests a decreased bio-availability of Cu in this species.

The different load of heavy metals in chironomids larvae most probably correlates with content of metals in water and sediments. The synergistic effect of different physical and chemical factors such as temperature and hardness of water, sediment pH, exposure time, chemical form, and availability of metals might promote accumulation of heavy metals into chironomids larvae [Bhattacharya et al., 2006; Lagrana et al., 2010]. To eliminate or reduce the environmental impacts (e.g., heavy metals load), captive rearing of bloodworms in controlled environments is

advisable, though there are great difficulties and laborious in keeping cultures going and failures can be occurred betimes [Galtsoff et al., 1937].

Although amphibians can detoxify both physiological (such as zinc and Cu) and xenobiotic (such as cadmium, mercury, and lead) heavy metals through metallothionein proteins, excess quantities of metals can induce toxicity (acute or chronic) in amphibian species [Muller et al., 1993; Saint-Jacques and Séguin, 1993; Lance et al., 2012]. Pb intoxication can lead to malformations, delayed developmental rate and decreased muscle tone [Horne and Dunson, 1995].

When comparing our results with those from previously published work [Sugden, 1973; Armitage, 1995; Habib et al., 1997; Habashy, 2005; Bogut et al., 2007; Thipkonglars et al., 2010; Rajabipour et al., 2011; Pasmans et al., 2012] the protein content of chironomid larvae varies markedly,

between 31% and 67% on a DMB. Similarly, fat and ADF content showed considerable variation between the different studies [Armitage, 1995; Bernard et al., 1997; Habib et al., 1997; Habashy, 2005; Bogut et al., 2007; Thipkonglars et al., 2010; Pasmans et al., 2012], from 3 to 14% and 4 to 17% on a DMB, respectively. The nitrogen in chitin (exoskeleton) estimated by ADF is likely not contributing to the dietary protein supply of insectivore amphibians, hence the ADF content indicates how much of the analysed CP is actually non-protein nitrogen. It is therefore likely that *C. riparius* provides more dietary protein due to the lower percentage of ADF in comparison with the two other species ($P < 0.05$).

Differences within or between species or studies may be caused by intrinsic differences in composition and/or by the environmental context. Indeed, the proximate composition of larvae samples may depend on the larvae age, composition of earthen pond water, food availability, and food quality and quantity [Mackey, 1977; Vos et al., 2000; Habashy, 2005; Bogut et al., 2007; Rajabipour et al., 2011]. In addition, environmental factors such as temperature, photoperiod, pH, oxygen content, and biotic interaction can affect growth and consequently nutritional composition in bloodworms [Maier et al., 1990; Tokeshi, 1995].

While there is a great paucity of information on the zoo diets fed to amphibians, the information that exists is basically limited to very few numbers. McWilliams [2008] reported that a suitable diet for insectivorous amphibians might contain 30% to 60% proteins [McWilliams, 2008]. In comparison with the previous study, our analysis shows chironomid with the average protein value $52.1 \pm 4.73\%$ to be high in protein content. Whereas the dietary fat fractions for amphibians naturally range from less than 10% to more than 30% [McWilliams, 2008] the obtained low fat contents in this (4.5%) and other studies (between 3% and 14%) indicates that feeding environmentally-collected chironomids larvae might not comply needs for amphibian diet program. Since lipid content largely determines the energy content, hence low lipid content might not ensure the needed dietary energy density. However, McWilliams [2008] provided basic information on what to feed captive amphibians, this area of study is still lagging behind the disciplines of mammalian and avian nutrition and further studies should fortify knowledge of amphibian nutrition involving different species and different developmental growth stages.

CONCLUSION

In our study we found that although chironomid larvae can consider as food with high protein concentration and with low risk of transmission of infectious organisms, relatively high levels of Pb and low levels of Zn combined with low fat content renders feeding environmentally-collected chironomids larvae as single diet item to amphibians unsuitable.

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