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Determination of *Paronychiurus kimi* (Collembola: Onychiuridae) age structures by head width measurements with reference to cadmium toxicity

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ABSTRACT

The age structure of *Paronychiurus kimi* (Lee) was determined by analysis of the head capsule width distribution using a H_{CAP} program. The H_{CAP} program successfully separated five distinct peaks, which represented a single age group, with a low misclassification probability of 0.1. The mean head capsule widths for each age group were 0.123, 0.165, 0.258, 0.304 and 0.350 mm, respectively. The age groups of *P. kimi* could be subdivided into two postembryonic developmental stages, the juvenile stage (groups 1 and 2) and adult stage (groups 3–5), on the basis of stage at first reproduction of *P. kimi*. Based on this information, we examined the chronic effect of cadmium on the age structure of *P. kimi*. After 28-day chronic exposure to various cadmium concentrations, the total number of *P. kimi* individuals decreased in a concentration dependent manner. The proportions of the juvenile and adult stages to the total number of individuals in the cadmium treated soils were significantly different from those in the control. In addition, the overall proportions of age structures were affected by cadmium treatment. These results indicate that head capsule measurements can be used to determine the age group of *P. kimi* and chronic exposure of cadmium to *P. kimi* at the individual level may be translated and manifested at the population level, by changing the age structures of *P. kimi* populations.

1. Introduction

Collembola are among the most abundant of all soil-dwelling arthropods and they play an important role in conditioning detritus for microbial break down as well as maintaining soil microstructure (Chahartaghi et al., 2005). Moreover, they are important food sources for beetles, spiders, mites, and other soil-inhabiting biota. Because of these characteristics, they have been considered as a useful indicator species for assessing the environmental impact of a wide range of pollutants and have also been used to monitor the success of remediation of contaminated soils (Wiles and Krogh, 1998; Son et al., 2007).

The International Standards Organization (ISO) has published a protocol for the use of *Folsomia candida* Willem as an ecotoxicological test species that employs effects on reproduction as an endpoint (ISO, 1999) since reproduction is a more sensitive parameter and supplies more ecological information than mortality rate (Krogh and Petersen, 1995). There has been some criticism about the ecological relevance of classical toxicity endpoints because interpretation of the short-term endpoint response is almost always statistically based, with little appreciation for the ecological ramifications of the response observed. Many laboratory ecotoxicological tests have shown that soil contaminants can cause changes in all parameters of Collembola populations, including mortality, growth rate and reproduction, and as a consequence, density and population growth rates (Posthuma et al., 1993; Crouau et al., 2002; Son et al., 2007). Therefore, it must be emphasized that impact on population development is the result of effects on reproduction and on mortality of adults and juveniles (Crouau et al., 2002).

It has been widely acknowledged that age structural effects are crucially important in determining stability properties of many populations, but are widely neglected in ecotoxicological studies of soil invertebrates (Gurney et al., 1983; Donker et al., 1993). Despite the importance of age structures in ecotoxicological tests, no studies have been conducted to determine age structural effects on Collembola species in ecotoxicological studies. The reasons for this neglect appears to lie in the information and technical deficiencies of the method currently used to classify age structures of the soil invertebrates, especially in Collembola species. Hence, a study on the chronic toxicity tests may have higher ecological relevance when using the age structure of a soil invertebrate to assess the potential risks that link the effects of contaminants on individuals with population level consequences. This relevance is assured

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when the test results provide an estimate of contaminant concentration that is unlikely to produce chronic effects on soil invertebrates.

The measurement of head capsule width is the most frequently used method to determine the number of insect larval stages and to classify larvae on the basis of their instars (Dyar, 1890; Got, 1988; Logan et al., 1998; Klingenberg and Zimmermann, 1992). This method is based on the concept that there are discrete increases in head capsule width that are indicative of the larval instar (Dyar, 1890). Logan et al. (1998) provided a generalized computer program (H_{CAP}) for the analysis of larval head capsule data. Originally, the aim of the H_{CAP} program was to classify the instar distribution of coleopteran larva using the head capsule width distribution. It also gave a simple and rapid analytical tool to determine the instar of the insect larva. This program has also been successfully applied to other insect larvae (Godin et al., 2002; Sabbatini Peverieri and Faggi, 2005) that have distinct increases in head capsule width between adjacent instars. However, unlike most other insect, Collembola continue to molt after they reach sexual maturity; in some species as many as 40 times or more after attaining maturity (Mari Mutt and Soto-Adames, 1987). These characteristics make it difficult to classify every instar of Collembola.

Recently, *Paronychiurus kimi* (Lee), a collembolan species indigenous to Korean soils, was subjected to toxicity tests with the aim of identifying an alternative Collembola species to *F. candida* for examining the quality of Korean soil (Kang et al., 2001; Son et al., 2007). The suitability of *P. kimi* as a standard test species for metal toxicity was demonstrated using ISO artificial soil (Son et al., 2007). Recently, the OECD "Collembola reproduction test guideline" listed *P. kimi* as an alternative test species for *F. candida* (OECD, 2008). This species is a dominant Collembolan species in paddy fields of Korea and plays an important role in the soil ecosystem as a decomposer (Kang et al., 2001). Thus, the aims of this study were: (1) to determine the age groups of *P. kimi* based on frequency distributions of head capsule widths; (2) to relate body surface area with head capsule width; and (3) to test the chronic effect of cadmium on the age structures of *P. kimi* using a pre-determined age structure that was based on head capsule widths. The results provide ecological relevance of toxicity endpoints response in regards to understanding how classical toxicity endpoints can be incorporated into the age structural effects of *P. kimi*.

2. Materials and methods

2.1. *P. kimi* culture and image analysis

P. kimi individuals were collected from paddy fields using an extraction from soils by floating on water (Son et al., 2007). *P. kimi* were cultured in the laboratory using a method that was similar to the one developed for *F. candida* (Snider et al., 1969). *P. kimi* were cultured on a moist substrate of plaster of Paris and activated charcoal mixed in a volumetric ratio of 4:1. The mixture was filled to a depth of about 0.5 cm in plastic Petri dishes (9.5 cm in diameter, 1.5 cm in height) and kept in an incubator at 20 ± 1 °C under continuous darkness. Cultures were fed with diluted Brewers yeast once a week in small amounts to avoid spoilage by fungi.

To obtain a large number of synchronized cohorts, hundreds of adults were introduced into the breeding substrates separately and allowed to lay eggs for 3 days and then all the adults were removed. Several sets were prepared to ensure a large number of eggs were obtained. As the eggs were hatched ($\approx$ 14 days after egg laying), at least 100 digital images were generated in 1-week intervals for 13 weeks using a digital camera (Nikon Coolpix 4500, Japan) with an ocular micrometer (Kyowa, Japan) inserted into a binocular microscope (Olympus SZ30, Japan). The head capsule width (at the widest point, mm) and body surface area (mm^2) of each individual were measured using digital imaging analysis software (Inspector, Version 2.2, Matrox).

2.2. Head capsule measurement analysis

The data for the head capsule widths (total $n = 1860$) were analyzed using a H_{CAP} program developed by Logan et al. (1998). This program is based on the assumption that head capsule widths are normally distributed for each instar (McClellan and Logan, 1994); thus, head capsule widths were assumed to be normally distributed. The program determines a graphic of the frequency distribution of head capsule widths. The peak of each distribution was assumed to represent an age group and the lowest point between two subsequent peaks was used as the initial guesses of separation points between age groups, which were followed by the method of analysis described by McClellan and Logan (1994). The H_{CAP} program calculated mean and standard deviation of head capsule widths for each age group, and the probabilities of misclassification. Based on the estimated lower and upper limit of the head capsule width for each age group, raw head capsule width data, which fell into the categories based on their lower and upper limit for each age group examined for 13 weeks, were classified into each age group. Also, the head capsule widths were related to the corresponding body surface areas.

2.3. Relationship between development time and head capsule width

To determine the duration of each age group based on the head capsule growth, the relationship between head capsule width (S) and development time (t) was described using an asymmetrical sigmoid function (Berner and Blanckenhorn, 2007):

$$S(t) = \frac{S_{\max}}{\left(1 + \nu e^{-k(t-t_{\mathrm{m}})}\right)^{(1/\nu)}},$$

where t_{m} is the inflection point, $S_{\max}$ is the asymptotic (final, maximal) adult size, and k and ν are the two constants that determine the curvature and degree of asymmetry of the function, respectively.

2.4. Effect of cadmium on the age structures in *P. kimi*

To test the effect of cadmium on the age structures of the *P. kimi* population, the *P. kimi* populations were exposed to various concentrations of cadmium, and allowed to produce progeny for 4 weeks. The reproduction test was initiated with age group 3 (6-week old) *P. kimi* to allow rapid population growth within a short time frame. This was selected because the first reproduction of *P. kimi* was observed at this age group (Kang et al., 2001).

In this test, 20 synchronized *P. kimi*, which were 6 weeks old, were exposed per container containing 30 g of wet weight soil. The artificial soil used for the test was prepared according to OECD guideline 207 (OECD, 1984), which was comprised (by dry weight) of 10% finely ground *Sphagnum* peat, 20% kaolin clay and 70% sand, with the pH adjusted to 6.0 ± 0.5 by addition of calcium carbonate. Tests were carried out at a soil moisture content of 50% of the water holding capacity. Cadmium was added as an aqueous solution of chloride salt ($CdCl_2 \cdot 2(1/2)H_2O$; CAS No. 7790-78-5; 98% purity). Nominal concentrations of cadmium were 0, 24.61, 49.23 and 98.46 mg/kg with four replicates per concentration. During exposure, the test vessels were kept in continuous darkness at 20 ± 1 °C. The content of the soil moisture was adjusted weekly by replenishing the weight loss with the appropriate amount of deionized water. Granulated Brewer's yeast was added biweekly to the soil surface as food. At the end of the test, both adults and juveniles were counted

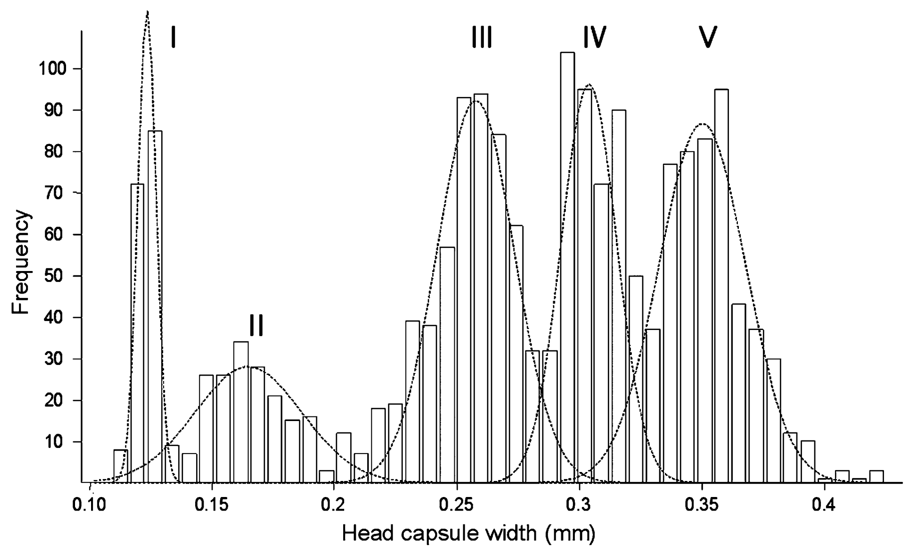

Fig. 1. Frequency distributions of head capsule widths fit with normal distributions (dotted line) of *Paronychiurus kimi* reared at 20 °C. I, II, III, VI and V denote age groups 1, 2, 3, 4, and 5, respectively.

after floatation and pooled for further image analysis. The collected individuals were categorized into five age groups based on the head capsule widths (Fig. 1 and Table 1).

2.5. Statistical analysis

The data acquired in this study were analyzed using the statistical software SAS (SAS, 1999). Chi-square Goodness-of-fit (GOF) tests were used (1) to determine whether the observed proportion of juvenile and adult stages of *P. kimi* for the cadmium treated group was significantly different from the expected proportion in the control and (2) to examine whether the proportion of each age group was different between the control and cadmium treated groups. In addition, Dyar's rule (1890) was used to analyze the age group number (indicated by the H_{CAP} program) and the natural log of the mean head capsule widths. Finally, a log–log linear regression analysis was used to determine the relationship between surface area and head capsule widths. The probability level used as statistically significant was $P < 0.05$.

3. Results

3.1. Head capsule width analysis

The frequency distributions of the head capsule widths for the sample of 1860 *P. kimi* individuals are given in Fig. 1. The head capsule width of *P. kimi* measured for 13 consecutive weeks ranged from 0.109 to 0.425 mm (Table 1), with five distinct and separate mean peaks at 0.123, 0.165, 0.258, 0.304 and 0.350 mm. Each peak observed in this study most likely represents age groups that include several instars, rather than a single instar, because it is known that collembolans continue to molt throughout their entire life span unlike most other insect species. For this reason, the term 'age group' was adopted, rather than the term 'instar'.

The dividing points of each peak and the probability of misclassifying age groups illustrate that the chance of misclassification was low. The probabilities of misclassification ranged from 0.012 to 0.094 (Table 1). The highest probability of misclassification was observed for age group 4 ($P = 0.094$) where the dividing points overlapped with the two adjacent age groups. In fact, all individuals can be classified into one of the five age groups with a probability of error less than 0.1. The program also calculated mean, standard deviation, and size range for each age group.

These results were also supported by Dyar's rule (Dyar, 1890), which hypothesized a geometric progression of head capsule size with instar. Although the age group was not initially expected to follow the Dyar' rule, when the log-transformed mean head capsule widths were plotted against each age group, a linear regression equation fit at a highly significant level ($P < 0.0041$, $r^2 = 0.955$; Fig. 2). The highest increment of Dyar's ratio was observed at age group 3, which was the transitional period from the juvenile to adult stage. When body surface area was related to head capsule widths using a log–log regression, a linear relationship was observed at a highly significant level ($P < 0.001$, $r^2 = 0.990$; Fig. 3), indicating that body surface growth can be used to classify the age group.

3.2. Time dependent increase of head capsule width of *P. kimi*

The head capsule width of *P. kimi* increased as a function of time in a sigmoidal pattern, which had an r^2 value of 0.897 (Fig. 4). The inflection point of this relationship was observed only a few days (2.55 (1.66–3.44)) after egg hatching, indicating that the fastest incremental increase in head capsule width occurred when *P. kimi*

Table 1
Head capsule means, ranges, and misclassification probabilities for five age groups of *Paronychiurus kimi* estimated by H_{CAP} program.

Age group	*n* (estimated)	Head capsule width (mm)			Probability of misclassifying		
		Lower	Mean ± SD	Upper	*i* as *i* − 1	*i* as *i* + 1	Total
1	153	–	0.123 ± 0.004	0.132	–	0.012	0.012
2	219	0.132	0.165 ± 0.022	0.214	0.066	0.012	0.078
3	524	0.214	0.258 ± 0.016	0.285	0.003	0.045	0.048
4	388	0.285	0.304 ± 0.011	0.322	0.043	0.051	0.094
5	548	0.322	0.350 ± 0.018	–	0.058	–	0.058

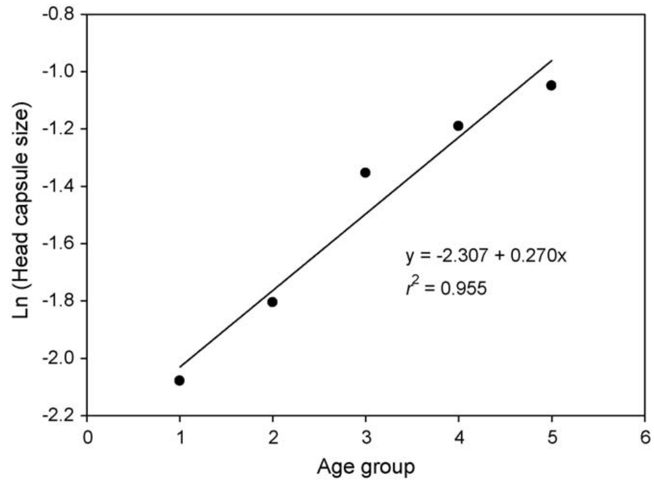

Fig. 2. Linear regression between the log-transformed mean head capsule widths and the age group of *Paronychiurus kimi* reared at 20 °C.

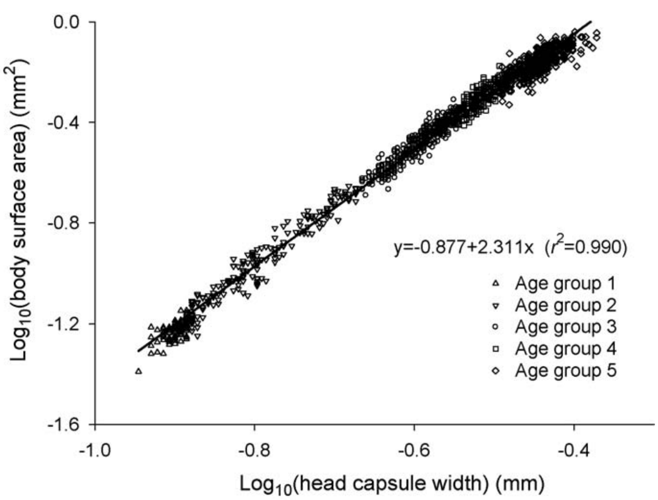

Fig. 3. Regression of the log–log scale between the body surface area and the head capsule width of *Paronychiurus kimi* for each age group determined by the H_{CAP} program. Data was collected for 13 weeks after egg hatching reared at 20 °C.

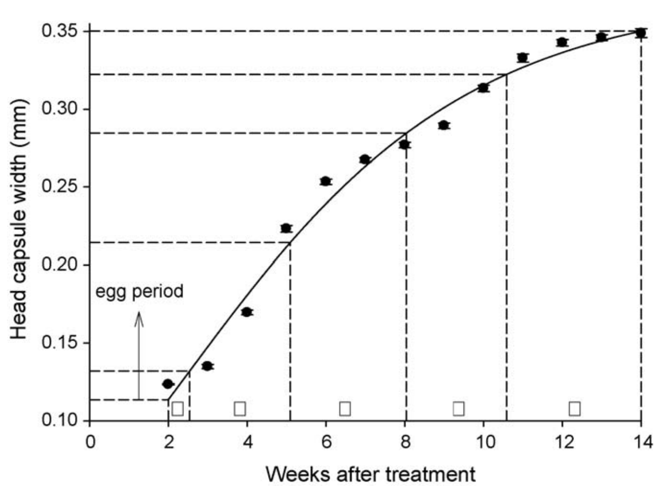

Fig. 4. Time-dependent development of head capsule width of *Paronychiurus kimi*. Developmental periods of each age group were estimated using lower and upper limits of each age group summarized in Table 1. I, II, III, VI and V denote age groups 1, 2, 3, 4, and 5, respectively.

transitioned from age group 1 to age group 2. A developmental period of each age group in weeks was estimated on the basis of the lower and upper limits of the head capsule width (Table 1) and recorded as 0.54, 2.55, 2.94, 2.54, and 3.42 for age groups 1–5, respectively. Even though *P. kimi* reached sexual maturity 4 weeks after egg hatching, the head capsule width gradually increased and reached a maximum at 0.374 (0.364–0.384) mm, indicating that *P. kimi* continued to molt after reaching sexual maturity. The reason for stating "sexual maturity 4 weeks after egg hatching" will be discussed in Section 4.

3.3. Effect of cadmium on the age structures in P. kimi

After 4 weeks of exposure to cadmium, the total number of *P. kimi* individuals decreased in a concentration dependent manner, which ranged from 1225 in the control to 148 at a cadmium concentration of 98.46 mg/kg (Table 2). Although the initial population consisted of adults in age group 3, some individuals remained in the same age group after 4 weeks in all treatments conditions including the control. However, these individuals did not constitute a considerable portion of the surviving initial population except at highest cadmium treatment conditions (≈63%). The proportion of juvenile and adult stages to the total number of individuals for each cadmium treatment was significantly different from that of the control (χ^2 = 13.37, df = 1, $P < 0.0003$ at 24.61 mg/kg; χ^2 = 11.83, df = 1, $P < 0.0006$ at 49.23 mg/kg; χ^2 = 7.79, df = 1, $P < 0.0052$ at 98.46 mg/kg). Furthermore, the overall proportions of the age structure for each cadmium treatment were also significantly different from the control (χ^2 = 68.22, df = 4, $P < 0.0001$ at 24.61 mg/kg; χ^2 = 32.78, df = 4, $P < 0.0001$ at 49.23 mg/kg). These results clearly demonstrate that chronic exposure of cadmium to *P. kimi* can decrease reproduction and adult survival, in conjunction with changing the age structures.

4. Discussion

Correctly determining the instar is basic to any life history study on Collembola, for only then can one resolve such problems as the duration of each instar, the number of stages before sexual maturity, the total number of molts in the life of the species and the changing patterns in population structure throughout the year. Unlike other insect species, the adult Collembola continue to molt after reaching maturity, in some species this can be as many as 40 times or more (Mari Mutt and Soto-Adames, 1987). In addition, the distinction between successive instars of Collembola is very small and thus, it is difficult to determine the instar number, especially in the field. These biological traits have made it difficult to study the age structure and population dynamics of Collembola species.

Head capsule width has been frequently used as an indicator to determine the instars of various insect species (Salas and Frank, 2001; Godin et al., 2002). Coombs et al. (1997) reported that measurements of head capsule size were far less variable than other morphological characteristics, and resulted in a much lower number of misclassifications of instars. Unlike other morphological characteristics of insects, head capsule sizes discretely expand between age stages (Chapman, 1998). The measurement of head capsule size is relatively easy and robust because the head capsule widths are less influenced by insect body posture and amount of food consumed. Based on the results presented in this study, it seems that the head capsule widths of *P. kimi* individuals could be used to reliably determine age groups, which is indicated by the low probabilities of misclassification (Table 1). However, the H_{CAP} program used in this study was only capably of classify 5 life stages of *P. kimi*, which is far less than expected because it is known that the number of molts required for sexual maturity of Collembola

Table 2
Effect of cadmium toxicity on compositions (%) of age groups when *Paronychiurus kimi* were exposed to various concentrations of cadmium for 28-day exposure.

Concentration	Total no. of individuals	Composition of each age group (%)				
		Juvenile stage		Adult stage		
		1	2	3	4	5
0	1225	51.4 (629)[a]	39.7 (486)	2.9 (36)	2.1 (26)	3.9 (48)
24.61	1058	53.1 (562)	34.7 (367)	7.0 (74)	2.1 (22)	3.1 (33)
49.23	564	44.3 (250)	42.6 (240)	5.1 (29)	4.6 (26)	3.4 (19)
98.46	148	54.1 (80)	30.4 (45)	10.8 (16)	4.7 (7)	0.0 (0)

[a] Values in parenthesis indicate the number of individuals belong to each age group.

species varies from three to twelve and a large majority require four to six molts (Christiansen, 1964). This could be attributable, in part, to the fact that one or more sub-instars, which have different distributions, may have been categorized into one certain age group and/or the head capsule increase between adjacent instars was too small to differentiate.

According to a report by Choi et al. (2002), who conducted experiments under the same environment conditions as used in this study, the first reproduction of *P. kimi* occurred 4 weeks after hatching and reproduction lasted for 20 weeks at 20 °C. The corresponding mean head capsule width to the first reproduction period was 0.258 ± 0.016 mm (mean $\pm$ SD). Thus, based on the reproducibility of the organism, the age groups of *P. kimi* could be divided into two postembryonic developmental stages: (1) the juvenile stage (age groups 1 and 2) and (2) the adult stage (age groups 3–5).

The growth pattern of the head capsule width over time was described by sigmoidal curves, which converged to asymptotic values on the 10th week after egg hatching (Tukey test, $P < 0.05$). These results suggest that the adult stage of *P. kimi* could be subdivided into two stages; postmaturity growth (age groups 3 and 4) and senile molts (age group 5). This is in line with a study conducted by Lindenmann (1950), who reported that the Collembola pass through three phases of postembryonic growth; juvenile, postmaturity growth, and senile molts and during the last period no significant growth takes place, even though many molts occur as observed in earlier development.

The significant linear relationship between head capsule width and number of age groups observed in this study indicates that growth of the head capsule width of *P. kimi* follows a geometrical progression (Gaines and Campbell, 1935). The excellent fit to the linear model indicates that no age groups were overlooked in this study. Interestingly, the net increase in the head capsule width of *P. kimi* during the juvenile stage tended to be greater than that in the adult stage and measurable changes occurred when the age group changed from 2 to 3. This may be attributed to the fact that molting is more frequent in the juvenile period since it is the period of faster growth. Britt (1951) found that measurable changes in the head capsule of *Achorutes armatus* Nic. occurred when they changed from the juvenile to adult stage.

The age structures of *P. kimi* differed significantly among cadmium treatments (Table 2). When soils become contaminated with metals, the species composition of soil invertebrate fauna around sources of metal pollution change (Bengtsson and Tranvik, 1989), but when such effects are observed, it is often too late for remedial measures. Demographic population attributes, such as age distribution and brood size, will respond to environmental pollution at an earlier stage, and these demographic traits can be used as an important endpoint of toxic effect (Stark and Wennergren, 1995; Spromberg and Meador, 2005; Landis and Kaminski, 2007). Donker et al. (1993) reported that a woodlouse (*Porcellio scaber* Latreille) population inhabiting a metal contaminated site differed from a reference population in demographic parameters such as age structures and reproduction, reflecting effects of cadmium and zinc on mortality and body size. The age structure has high biological relevance for age structured populations that is likely to serve as population-scale endpoints in ecotoxicological studies. Knowing the age structure, together with the survivorship and reproductive characteristics of each age class, allows prediction of future dynamics and a forecast of ecological services (Landis and Kaminski, 2007). Two types of age structure are important for population-level assessment in ecotoxicological studies (Landis and Kaminski, 2007); (1) the 1st type of age structure is the realized or sampled age structure and (2) the 2nd type is the stable age structure. Stark and Vargas (2003, 2005) showed that the stable age distribution of *Daphnia pulex* (leydig) changed systematically with exposure concentrations of insecticides. In addition, Stark and Banken (1999) demonstrated that the starting population structure of *Tetranychus urticae* Koch would affect the outcome exposure to a toxicant.

In conclusion, the work presented here shows that the head capsule width is a good indicator to describe the population structures of *P. kimi* and can serve as a basis for understanding the population dynamics and demography of *P. kimi*. Estimation of age groups using head capsule width can be used for ecotoxicological studies with *P. kimi*. The importance of measuring toxicological effects at the population level has been emphasized by Ferson et al. (1996). Information on age structure enables us to understand what the life cycle as well as the age structure of *P. kimi* will be under environmental and chemical stresses. Population structures are the product of selection for tolerance, as well as toxic effects on growth and reproduction (Donker et al., 1993). Therefore, populations with different age structures do not behave the same way after exposure to pollutants, which indicates that the way in which the effects of pollutants on organisms are viewed must change. Population structure should also be given careful consideration when evaluating the effects of pollutant on *P. kimi* population.

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