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Toxic effects of carbaryl on the histology of testes of *Bufotes variabilis* (Anura: Bufonidae)

Özlem Çakici

Ege University, Science Faculty, Biology Department, Zoology Section 35100 Bornova-İzmir, Turkey. E-mail: ozlem.cakici@ege.edu.tr

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Abstract. This study was designed to investigate histopathologic effects of carbaryl on the testes of adult toad, *Bufotes variabilis*. To that end, animals were exposed to carbaryl once by oral gavage (low dose: 50 µg/g, medium dose: 100 µg/g and high dose: 200 µg/g). After 96 h, toads were euthanized. In low-dose group, some seminiferous tubules lost their regular shape. Also, the enlargement of interstitial spaces among tubules and germ cell necrosis were determined. A weak hemorrhage was observed among some tubules. In medium-dose group, germ cell necrosis was detected in many seminiferous tubules. This time, a weak hemorrhage was detected within tubules. In the high dose group, an increase in the number of disorganized tubules were observed. Vacuolization and necrosis in germ cells of seminiferous tubules were frequently seen. According to these findings, carbaryl caused dose-related histopathological damage in testis of *B. variabilis*. Based on these findings, this study clearly shows that carbaryl affects male fertility in *B. variabilis*.

Keywords. Amphibia, Anura, Bufonidae, *Bufotes variabilis*, toad, carbaryl, testes, histopathology

INTRODUCTION

Wide range use of pesticides may cause serious problems in ecosystem. In fact, Rani et al. (2013) stated that even though pesticides are present in the environment in small quantities when compared to other contaminants such as industrial wastes and fertilizers, they are responsible for public and scientific concern because of their high biological activity, and many species are badly affected by this pesticide use. For instance, one of the biggest problem in conservation biology is the decline of many amphibian species (e.g. Alford et al., 2001; Stuart et al., 2004) and pesticides play an important role in these declines (Davidson, 2004; Punzo, 2005; Mann et al., 2009). According to Davidson (2004), cholin-esterase inhibiting pesticides such as organophosphates and carbamates have more effect in amphibian declines than other different pesticide classes. Carbaryl (1-Naphthyl-N-methylcarbamate) is included in carbamates and is used as a contact and systemic insecticide on agricultural products to control various kind of pests including moths, beetles, cockroaches, ants, ticks and mosquitoes (Tomlin, 2000; Murty et al., 2012). Garber et al. (2007) also reported that adverse effects of carbaryl on endangered anuran populations is relevant. To that end, a number of studies were conducted to determine the effects of carbaryl in the early developmental stages of amphibians. Punzo (2005) examined the time spent in activity (tail movement) and swimming speed of tadpoles of the Rio de Grande Leopard frog, *Rana berlandieri*. Author stated that time spent in activity and the swimming speed decreased after carbaryl exposure and this may influence an increased rate of predations. In addition, because of the slower growth rates, tadpoles needed more time to complete metamorphosis. Carbaryl had also teratogenic effects on the embryos of the Clawed frog, *Xenopus laevis* and caused abnormal tail flexure (Bacchetta et al., 2008). Similarly, Kang et al. (2010) reported that carbaryl was harmful in the Oriental fire-belleed toad, *Bombina orientalis* embryos and exposure to carbaryl caused some

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external deformities. Boone et al. (2013) stated that carbaryl could have long-lasting effects on the brain development when exposure occured at sensitive developmental stages. As a result of aquatic exposure to the carbaryl, both reduced survival and increased time to metamorphosis were observed in the aquatic phase of the American toad, *Bufo americanus* (Distel and Boone, 2009). The effects of a 24 hours exposure to carbaryl increased fatigue in juvenile Spotted salamander, *Ambystoma maculatum* (Mitchkash et al., 2014). Cholinesterase and carboxylesterases were strongly inhibited by carbaryl in the larvae of the toad *Rhinella arenarum* (Ferrari et al., 2011).

Based on above-mentioned studies, it can be concluded that carbaryl have severe adverse effects on early life stages of amphibians. Naturally, early life development stages are usually considered to be the most sensitive to the adverse effects of toxic compounds. However, it is also important to analyse the effects of pesticides in adult amphibians because they may have a potential to irreversibly damage the organs. For this reason, studies are needed to determine the effects of carbaryl on the gonadal structure of adult amphibians. Considering all, histopathologic effects of carbaryl on the testis of Levantine frog, *Pelophylax bedriagae* were examined by Çakıcı (2013). Several histopathologic findings indicated that carbaryl had the potential to affect germ cell development and possibly to have negative effects on spermatogenesis. Accordingly the present study aimed at the evaluation, on the basis of tissue histopathology, of the negative effects of carbaryl in the Variable toad, *Bufotes variabilis* a species widely distributed throughout Turkey (Başoğlu et al., 1994).

MATERIAL AND METHODS

Animal groups and experimental design

Adult individuals of *B. variabilis* were caught around Bornova, İzmir Province in Turkey. Before experiments, toads were acclimatized to the laboratoty conditions at a 12 - h dark/light cycle, at 22 ± 3° C temperature and 45 ± 5 % relative humidity for 15 days. Then, the toads were randomly assigned to either the carbaryl-treated groups (low, medium and high dose groups) or to the control group, each group consisting of 8 mature male toads. As formerly mentioned by DuRant et al. (2007), contaminated insects are an important way of exposure to pesticides for insectivorous vertebrates inhabiting areas that receive pesticide application. As adult toads fed on insects, they may exposed to carbaryl by eating prey that's covered in carbaryl. However only one study made by Fair et al. (1995) directly quantified carbaryl residues of terrestrial invertebrates and determined that grasshoppers had mean residues of 17 μg/g two days following rangeland application of 0.5 kg active ingredient/ha. By means of this insect residue data and carbaryl application rates, which can vary from 1.12 to 22.42 kg active ingredient/ha (EPA 2004), DuRant et al. (2007) stated that a 10 g lizard consuming 1 g prey could ingest close concentrations ranging between 3.9-78.5 μg/g 2 days following carbaryl application and factoring the short-half life into their estimates, scientists selected three doses that encompassed the entire range of concentrations that they believed lizards could encounter in the environment. Therefore, these results were used to calculate the concentrations of carbaryl used in this study and the author is confident that these concentrations of carbaryl are the ones which toads could encounter in their environment. In addition, Durant et al. (2007) stated that studies related to the acute exposure of carbaryl are ecologically relevant because carbaryl have a short half-life.

Experimental groups contained low (50 μg/g), medium (100 μg/g) and high doses (200 μg/g) of carbaryl insecticide (purity 98 %) supplied by AgroBest Grup (İzmir, Turkey). Carbaryl was dissolved in acetone and prior to experiments, acetone was used as control and no important histologic effects were observed in testicular tissue of the toads. Carbaryl was administrated once by oral gavage to experimental groups.

Histological analyses

Following 96 h the exposure to carbaryl, toads were euthanized and their testes were quickly removed. After fixation in Bouin for 24 h, testis samples were dehydrated in ethanol, cleared in xylol and embedded in paraffin. Tissues were serially sectioned at 5 μm, then stained with Mayer's Haematoxylin and Eosin and photographed with Olympus CX31 (Tokyo/Japan).

RESULTS

During this study, there was no mortality in the experimental *Bufotes variabilis* after exposure to carbaryl for 96 h. According to light microscopic examinations, spermatogenesis appeared normal in the control group animals (Fig. 1A), with germ cells in cystic organizations of a seminiferous tubule were easily observed (Fig. 1B).

This investigation showed that carbaryl treatment caused dose-related histopathologic changes in *B.variabilis*. In the low-dose group, some seminiferous tubules lost their regular shape and enlargement of interstitial spaces among tubules was clearly observed (Fig. 2A). In addition, germ cell necrosis within seminiferous tubules was also detected (Fig. 2B), with a weak hemorrhage that was present among some tubules (Fig. 2C).

In the medium-dose group, germ cell necrosis was clearly present in many seminiferous tubules (Figs 3A and B), again with a weak hemorrhage present within tubules (Fig. 3C).

Finally, in the high-dose group, an increase in the number of disorganized tubules was observed (Fig. 4A),

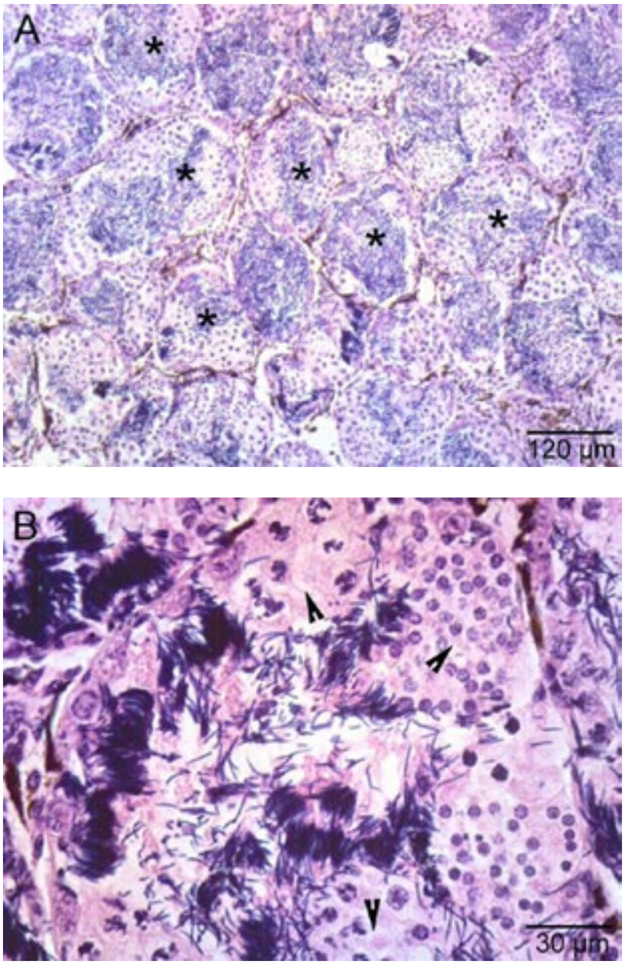

Fig. 1. Histologic section of testis in control group. A) General view of testis. Normal appearance of seminiferous tubules (asterisk); B) Normal spermatogenesis in seminiferous tubules (asterisk) in the control group. Note the germ cells in a cystic organization (arrowhead).

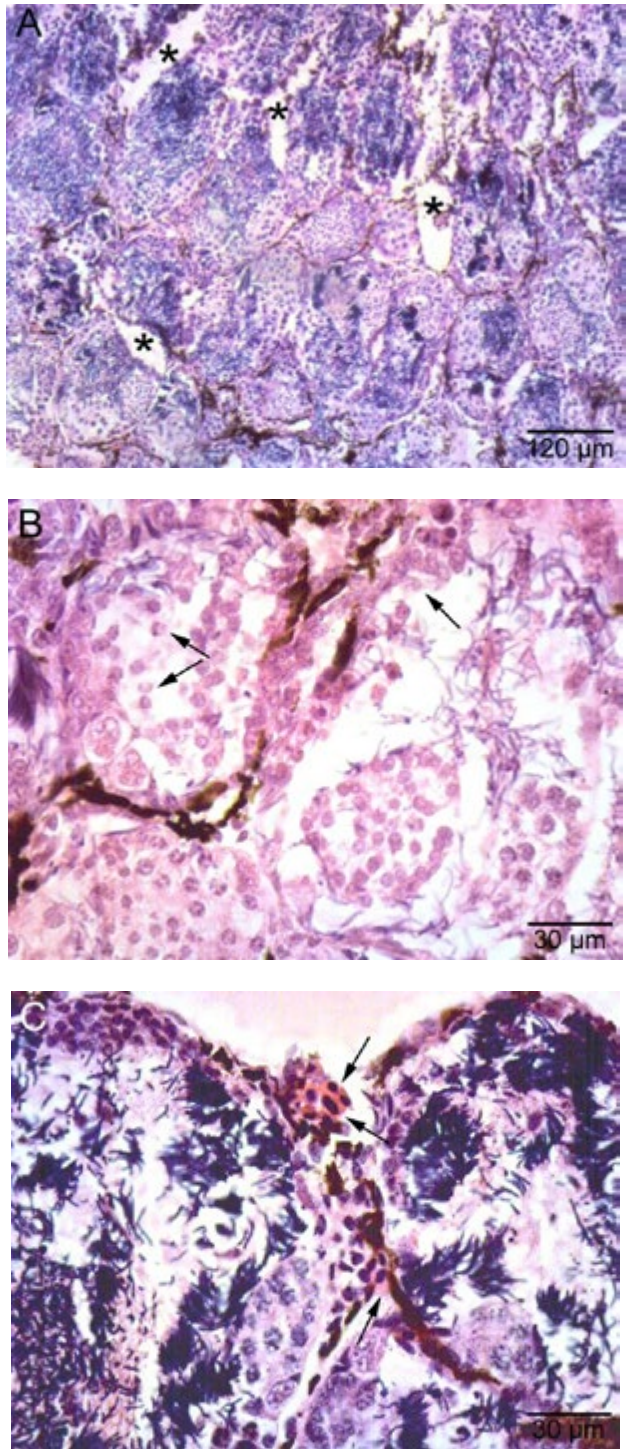

Fig. 2. Histologic section of testis in low dose group. A) General view of testis. The enlargement of interstitial spaces (asterisk); B) Germ cell necrosis (arrow); C) Note the weak hemorrhage (arrow) between two disorganized seminiferous tubules.

while vacuolization in germ cells was also detected in many seminiferous tubules (Fig. 4B) and evident germ cell necrosis was conspicuous (Fig. 4C).

DISCUSSION

It is a common characteristic of male amphibians that germ cells in the same stage of differentiation constitute clusters in arranged in cystic structures with a synchronism development (Lofts, 1974; Rastogi, 1976; Jorgensen et al., 1986; 1988; Rastogi et al., 1988; Oliveira et al., 2002). For example Carezzano et al (2013) stated that seminiferous locules distinguished in cysts with spermatogenic cells at different stages of development

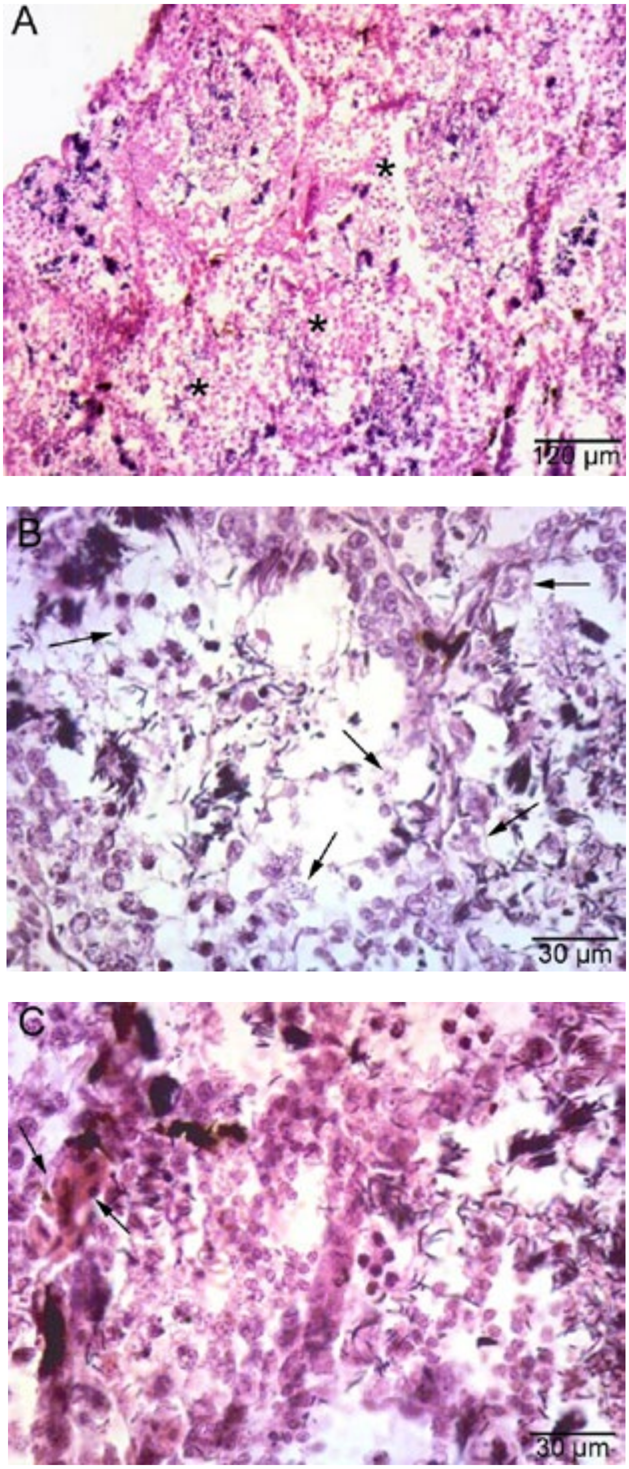

Fig. 3. Histologic section of testis in medium dose group. A) General view of testis. Note the disorganized tubules (asterisk); B) Prominency in germ cell necrosis (arrow); C) A weak hemorrhage (arrow) within seminiferous tubules.

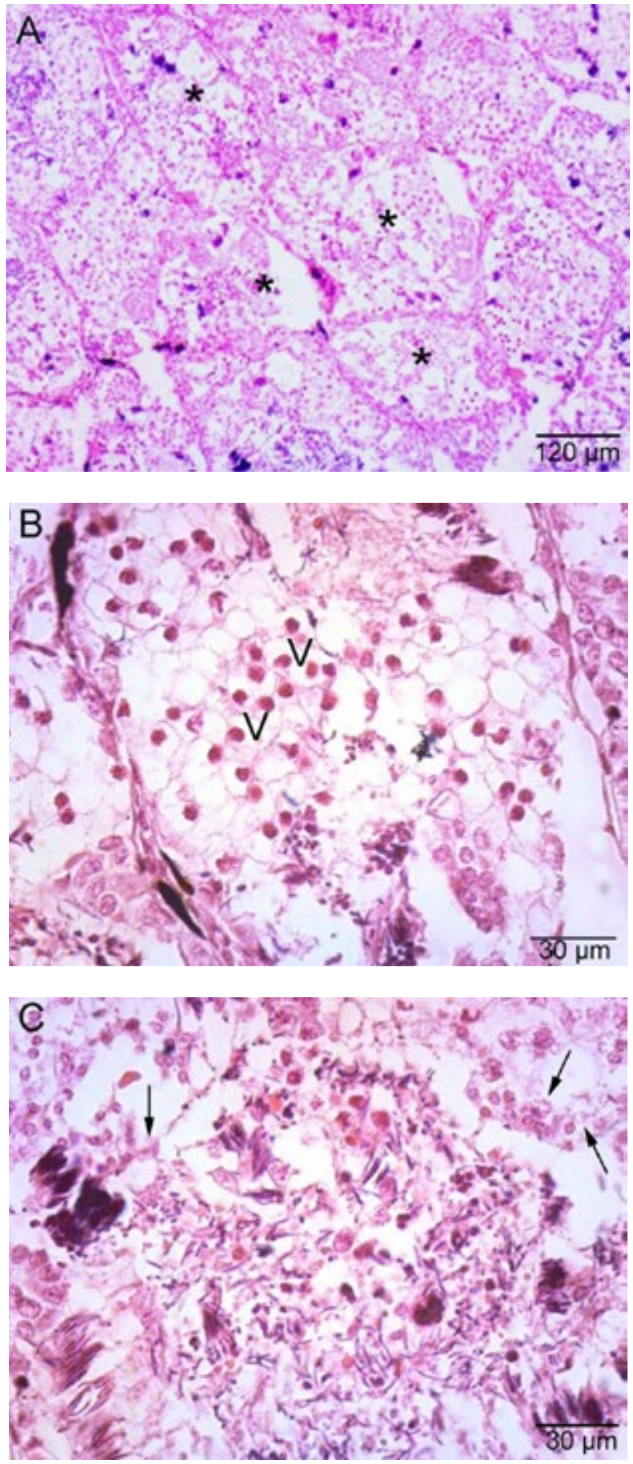

Fig. 4. Histologic section of testis in high dose group. A) General view of testis: An increase in the number of disorganized tubules (asterisk); B) Vacuolization (V) in germ cells; C) Prominent germ cell necrosis (arrow) in a seminiferous tubule.

were present in the testes of the Argeentine horned frog, *Ceratophrys ornata*. Ferreira et al. (2008) described the testis structure of the Harlequin frog *Pseudis limellum*. These authors stated that germ cells were organized as cysts and spermatogenesis is similar to other anurans, but with some peculiarities related to organization of the germ cells (Ferreira et al., 2008). Santos and de Oliveira (2008) reported that in the male tree frog *Dendropsophus minitus*, spermatogenesis occured in seminiferous tubule where germ cells were arranged in spermatogenic cysyts, and each cyst included cells in the same stage of differentiation. In other words, sperm maturation followed a similar pattern in many amphibian species and, in genereal terms, *Bufotes variabilis*' spermatogenesis appeared similar to the above mentioned species.

Due to the limited research about the effects of carbaryl on gonadal structure of fish, amphibians or reptiles, the present research can only be compared with few other studies. Çakıcı (2013) studied the histopathologic effect of carbaryl on the testes of Levantine frog, *Pelophylax bedriagae*. In that study, three doses of carbaryl, corresponding to 50, 100 and 200 μg/g, respectively, were used and carbaryl caused dose-related histopathologic changes in the testes of the experimental animals were observed (Çakıcı (2013). Similarly, dose-related histopathologic findings were determined in the toad *Bufotes variabilis*, as reported in the present study. In particular, shrinkage of seminiferous tubules and disintegration in germ cells were determined in the low dose group when compared to the control one. Cakici and Akat (2012) also studied the toxic effect of carbaryl on testes of the Snake-eyed lizard *Ophisops elegans*. These authors used low (2.5 μg/g), medium (25 μg/g) and high (250 μg/g) carbaryl doses, finding no important histopathologic defects on seminiferous tubules in the low dose group of animals. Cakici and Akat (2012) observed the tubular asymmetry and blood cell infiltration in interstitial space of tubules in the testes of *O. elegans*, while Çakıcı (2013) stated that most tubule lost their normal appearance in high dose carbaryl treated group of *P. bedriagae*. Similarly, germ cell necrosis was conspicuous in *B. variabilis*. Cakici and Akat (2012) determined the vacuolization in germ cells in the testis of *O. elegans* but this effect was much more commonly seen in *B. variabilis*. A study made by Sanchez et al. (2014) investigated the connection between male gonadal abnormalities and the effects of agricultural activities in two anuran species, the toad *Rhinella fernandezae* and the frog *Dendropsophus sanborni*. These authors determined some abnormalities such as poorly developed seminiferous tubules and reduction in number of germ cells in both species. In view of their findings, they concluded that agrochemical use may have negative effects on the reproductive success on these two species.

Saxena and Mani (1987) examined the effects of some pesticides (fenitrothion and carbofuran) on the testicular recrudescence in the freswater murrel, *Channa punctatus*. According to this study carbofuran, a carbamate insecticide, delayed the formation of spermatids and sperms. In addition, necrosis of spermatogonia and spermatocytes were also observed. Harilal and Sahai (1990) determined that carbaryl accumulated in the gonadal tissues even after 4 days exposure in the catfish *Heteropneustes fossilis*. Jyothi and Narahan (1999) examined the toxic effects of carbaryl on gonadal structure of a freshwater fish, *Clarias batrachus* and according to this study, exposure to carbaryl caused some histopathologic changes, such as vacuolation and necrosis in gonads, while basement membrane thickness was also observed in the testis. Similarly, vacuolization and necrosis were determined in the testis of *Bufotes variabilis*. Lauan and Ocampo (2013) determined that exposure to carbaryl caused some histopathologic alterations in Nile tilapia, *Oreochromis niloticus* such as hemorrhagic necrosis detected in the peripheral and interlobular interstitium of the seminiferous lobules. In addition, they stated that as the treatment doses increased, disorganization of the testes structure also appeared to become more serious (Lauan and Ocampo, 2013). Mensah et al (2012) reported that sublethal doses of carbaryl in the environment may have an adverse effect on the reproductive success of zebrafish.

According to Toppari et al. (1996), to affect spermatogenesis, pesticides acts by hormonal or genotoxic pathways by passing through the blood testis barrier, while Fattahi et al. (2012) asserted that pesticides could also have direct effects on testicular tissue. To better understand this relevant issue, more comprehensive studies should be made to clarify the physiologic mechanisms of insecticides on the male reproductive system in particularly in amphibians.

In summary, all the above studies showed that the pesticide carbaryl may have severe negative impacts on the germ cell development in the testes of various terrestrial and freshwater animals. Similarly, many histopathologic findings presented in this study indicated that carbaryl affected the development of germ cells and had negative effects on the spermatogenesis in *B. variabilis*. As it is known, decline of many amphibian species is a global problem and the negative effects of wide range use of pesticides on the viability and reproduction of amphibians should be better investigated, in particular on adult individuals.

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