Behavioural Responses of Iberian Midwife Toad Tadpoles (*Alytes cisternasii*) to an Introduced Exotic Predator, *Procambarus clarkii*

Respostas Comportamentais dos Girinos de Sapo Parteiro Ibérico (*Alytes cisternasii*) a um Predador Exótico, *Procambarus clarkii*

Vera Lúcia Ramos Gonçalves

Dissertação para obtenção do Grau de Mestre em
BIOLOGIA DA CONSERVAÇÃO

Orientador: Doutor Rui Miguel Borges Sampaio Rebelo (DBA, FCUL)
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A seguinte dissertação de Mestrado foi redigida sob a forma de dois artigos científicos, que formam um conjunto coerente em termos de objectivos e de linha de investigação:

Gonçalves, V., Amaral, S., Rebelo, R. (Submitted). Iberic midwife toad (*Alytes cisternasii*) tadpoles show behavioural modifications when faced with a recently introduced predator, *Procambarus clarkii.*

Gonçalves, V. & Rebelo, R. (Submitted). Behavioural responses of Iberic midwife toad tadpoles (*Alytes cisternasii*) to chemical cues of a natural predator (*Natrix maura*) and an exotic predator (*Procambarus clarkii*).

RESUMO

O principal alvo das investigações realizadas em conservação tem sido a perda de biodiversidade, tendo sido identificada a introdução de espécies exóticas como uma das principais causas do declínio e extinção de espécies a nível global.

Nas últimas décadas, foi relatado o desaparecimento repentino e a regressão na área de distribuição de numerosas espécies de anfíbios, a nível mundial. Entre os factores de ameaça mais apontados para este declínio também se encontra a introdução de espécies exóticas predadoras e competidoras. Na Península Ibérica, a introdução de *Procambarus clarkii*, que constitui um predador de ovos e larvas de anfíbios (especificamente de todas as espécies do sudoeste peninsular) poderá estar relacionada com a rarefacção e desaparecimento de algumas populações.

Contudo, a predação, por predadores nativos ou introduzidos, pode também ser uma importante força de selecção, resultando frequentemente na evolução de defesas antipredatórias.

Encontram-se descritos alguns comportamentos antipredatórios apresentados por larvas de anfíbios, que podem ser adoptados na presença de pistas químicas de predadores, e que parecem ser importantes para a coexistência dos anfíbios com os mesmos.

O objectivo desta dissertação foi: (1) avaliar quais as alterações comportamentais adoptadas pelos girinos de *Alytes cisternasii* perante um predador exótico recentemente introduzido, *Procambarus clarkii*, e avaliar se as diferenças na actividade sazonal de *P. clarkii* estarão relacionadas com alterações nos comportamentos antipredatórios adoptados pelos girinos; (2) verificar a existência de comportamentos antipredatórios nos girinos de *Alytes cisternasii*, perante o estímulo químico de *Procambarus clarkii*,

comparando-as com as respostas a um dos principais predadores nativos, a cobra-de-água-viperina, *Natrix maura*.

Os resultados obtidos neste trabalho demonstram que os girinos de *Alytes cisternasii* reagiram intensamente ao predador natural (*Natrix maura*). Contudo, já apresentaram também alterações comportamentais em resposta ao predador exótico, uma vez que submetidos ao estímulo químico de *Procambarus clarkii*, os girinos apresentaram uma diminuição de actividade e um aumento da utilização das zonas marginais, em condições nocturnas. De acordo com a estratégia de predação do lagostim, as respostas antipredatórias apresentadas pelos girinos poderão apresentar um carácter adaptativo.

Foi ainda possível verificar diferenças nas respostas comportamentais dos girinos, entre o Inverno e a Primavera. Contudo não foi encontrada evidência de que essas diferenças se encontrem relacionadas com as diferenças sazonais na actividade de *P. clarkii*.

Estes resultados dão-nos algumas pistas acerca da evolução de comportamentos antipredatórios em girinos de *A. cisternasii* face a um predador introduzido, *Procambarus clarkii*, o que pode ser importante para compreender o impacto que este predador tem nas suas populações e avaliar mais correctamente o grau de ameaça causado pela introdução desta espécie exótica.

ABSTRACT

The principal aim of the investigations carried out in conservation biology has been the loss of biodiversity; today, the introduction of exotic species has been clearly identified as one of the causes of the decline and extinction of species.

In the last decades, there were records of a rapid disappearance and regression in the distribution of numerous species of amphibians at a worldwide level. Similarly, the introduction of exotic predators and competitors is among the factors more frequently pointed for this decline. In the Southwest of the Iberian Peninsula, the introduction of *Procambarus clarkii*, which actively predates amphibian egg masses and larvae of all the Southwest Iberian amphibians may be related with the rarefaction and local extinction of some populations.

However, predation, by native or introduced predators, can also be an important force of selection, resulting in the evolution of antipredator defences.

There are several antipredator behaviours that can be adopted by larval of amphibians in the presence of predator chemical cues, and that seem to be important for the coexistence of amphibians with those predators.

The aims of this thesis were: (1) to assess the behavioural responses of *A. cisternasii* tadpoles when faced with a recently introduced exotic predator, *Procambarus clarkii*, and to evaluate if the seasonal differences in *P. clarkii*'s activity are related with alterations in the antipredator behaviours adopted by *A. cisternasii* tadpoles; (2) to verify, the existence of antipredator behaviours in tadpoles of *Alytes cisternasii* as a response to the chemical cues of the exotic predator, *Procambarus clarkii*, comparing them with the responses to one of its main native predators, the viperine water snake, *Natrix maura*.

The results obtained in this work show that the tadpoles of *Alytes cisternasii* reacted strongly to their native predator, *Natrix maura*, but also altered their behaviour in response to the exotic predator, as the tadpoles presented a reduction of activity and an increase of the use of marginal zones during the night, when submitted to the chemical cues of *P. clarkii*. The antipredator responses of the tadpoles apparently have an adaptive value, as they may protect the tadpoles from the predation strategy of this crayfish.

It was still possible to verify differences in the antipredator behaviours of the tadpoles, between winter and spring. However these differences were not related with the seasonal differences in the activity of *P. clarkii*.

These results may provide some cues on the evolution of antipredator behaviours in tadpoles of *A. cisternasii* when faced with an introduced predator, *Procambarus clarkii*, which is important to understand the future impacts that this predator will have in the populations of this species and evaluate more correctly the degree of threat caused by the introduction of this exotic species.

Índice Geral

Artigo I – Os girinos de sapo-parteiro-ibérico (*Alytes cisternasii*) apresentam modificações comportamentais em resposta a um predador recentemente introduzido, *Procambarus clarkii*

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A major goal of conservation biology is to understand the organization of the species and communities that compose the planet's ecosystems so that the biological diversity can be preserved (Pough *et al.*, 2004).

Amphibians warrant substantial conservation attention. They are considered valuable indicators of environmental quality, and they have multiple functional roles in aquatic and terrestrial ecosystems (Blaustein and Wake, 1990; Stebbins and Cohen, 1995) Furthermore, amphibians provide cultural and economic value to human society (Stebbins and Cohen, 1995; Reaser, 2000).

During the past decade, the amphibian decline issue has come to be regarded as an ecological emergency in progress (Stebbins and Cohen, 1995). The causes of amphibian population declines may include ultraviolet radiation, predation, habitat modification, environmental acidity and chemical pollution, diseases, changes in climate or weather patterns, and interactions among these factors (Alford and Richards, 1999; Blaustein *et al.*, 2003; Collins and Storfer, 2003).

The introduction of exotic species is a phenomenon responsible for worldwide biodiversity loss (Sakai *et al.*, 2001; Byers, 2002) and may have contributed to some cases of amphibian declines (Hecnar and M'Closkey, 1997; Lawler *et al.*, 1999; Knapp and Mathews, 2000; Hamer *et al.*, 2002; Kats and Ferrer, 2003). In particular, the introduction of predators in amphibian breeding habitats can have severe impacts, because predation is one of the main factors modulating amphibian larval communities (Azevedo-Ramos *et al.*, 1999).

Crayfish species have been introduced worldwide (Hobbs *et al.*, 1989), and there are few studies on their possible impacts on amphibians (Gamradt and Kats, 1996; Axelsson *et al.*, 1997; Nyström and Abjörnsson, 2000; Nyström *et al.*, 2001).

In Portugal, despite the absence of long-term monitorization data that would allow the evaluation of amphibian population tendencies, there are few evidences to suggest a large scale decline. However, some local extinctions are already documented, leading to an increasing fragmentation of populations (Almeida *et al.*, 2001), namely, as a result of the introduction of the red crayfish, *Procambarus clarkii* (Cruz *et al.*, 2003).

Procambarus clarkii was introduced in Portugal in the late 1970s and it expanded quickly by the center and south of the country, where there were no native crayfish, reaching high densities in some areas (Correia, 1995).

In conservation biology, there is a special concern regarding detrimental effects that introduced exotic predators can have on native communities (Primack, 1993 *in* Hecnar and M'Closkey, 1997). In order to evaluate the impact of this exotic and invading species in the communities of amphibians in Portugal, it was shown that this crayfish actively predates egg masses and larvae of all the Southwest Iberian amphibians (experiences carried in biotherium) (Cruz and Rebelo, 2005). Moreover this exotic species was probably responsible for a reduction in the abundance and local extinction of some species (namely *Pleurodeles waltl* and *Hyla arborea*) in Paul do Boquilobo Nature Reserve, Central Portugal, between the years of 1993 and 2001 (Cruz *et al.*, 2003).

Larval amphibians are extremely vulnerable to vertebrate and invertebrate predators (Alford, 1999 *in* Alford and Richards, 1999) and those that coexist with aquatic predators have evolved a range of antipredator mechanisms they may alter their life history (Skelly and Werner, 1990), behaviour (Petranka *et al.*, 1987; Kats *et al.*, 1988; Werner, 1991; Chovanec, 1992; Skelly, 1992; Relyea and Werner, 1999), or morphology (McCollum and Van Buskirk, 1996; Van Buskirk *et al.*, 1997; Van Buskirk and Relyea, 1998; Relyea, 2000, 2001).

However, widespread introductions of predators have increasingly exposed native amphibians to predators with which they have not previously interacted. Inappropriate

responses to novel predators increase mortality of native amphibians (Gamradt and Kats, 1996; Kiesecker and Blaustein, 1997), leading to significant depression of their populations.

If we understand the evolution of behavioural responses of amphibian larvae to an introduced species, we will be able to more correctly evaluate the degree of threat caused by the introduction of this exotic species on amphibian populations.

Thus, the objectives of this work are: (1) to assess the behavioural responses of *A. cisternasii* tadpoles when faced with a recently introduced exotic predator, *Procambarus clarkii*, and to evaluate if the differences in seasonal activity of *P. clarkii* are related with alterations in the antipredator behaviours adopted by *A. cisternasii* tadpoles (**Paper I**); (2) to verify, the existence of antipredator behaviours in tadpoles of *Alytes cisternasii* in response to the chemical cues of the exotic predator *Procambarus clarkii*, comparing them with the responses to one of its main native predators, the viperine water snake, *Natrix maura* (**Paper II**).

If *Alytes cisternasii* tadpoles are already able to recognize *P. clarkii* and to show specific antipredator behaviour towards the crayfish, this amphibian will probably be less vulnerable to the impacts of this predator, either direct (survival of individuals) or indirect (reduced fitness of the newly-metamorphosed toadlets and therefore of the adults).

So, this can be a good example to study the evolution of behavioural responses of amphibian larvae to an introduced species. By knowing and understanding these behavioural defences we will be able to more correctly evaluate the degree of threat caused by the introduction of this exotic species on the populations of *Alytes cisternasii*.

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IBERIC MIDWIFE TOAD (*ALYTES CISTERNASII*) TADPOLES SHOW BEHAVIOURAL MODIFICATIONS WHEN FACED WITH A RECENTLY INTRODUCED PREDATOR, *PROCAMBARUS CLARKII*

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I.1. ABSTRACT

Some studies have shown that exotic predators contributed to amphibian population declines. On the other hand, amphibian larvae that coexist with predators exhibit a variety of antipredator behaviours.

In this work we confirmed the existence of behavioural modifications in tadpoles of *Alytes cisternasii* in different developmental stages in response to the chemical cues of an introduced predator, *Procambarus clarkii*, by comparing the responses in two consecutive years and in different seasons. We also verified if the differences in seasonal foraging activity of this crayfish may change the type or intensity of behavioural alterations of the tadpoles.

According to the results obtained, *Alytes cisternasii* tadpoles present seasonal and circadian differences in their behaviour. These behavioural alterations can be the result of the chemical stimulus of the exotic predator, *Procambarus clarkii*, and in this case may be considered antipredator behaviours, or can result from others exogenous or endogenous factors.

I. 2. INTRODUCTION

Predation is one of the strongest selective forces in natural ecosystems. Experimental manipulations of predators have shown that they have important effects on prey individuals, populations and communities (Lima and Dill, 1990; Lima, 1998 a), and predation has resulted in the evolution of complex morphological, ecological and behavioural traits in prey in order to reduce vulnerability (Lima and Dill, 1990; Lima, 1998 a, b).

Because predators have both direct predatory effects as well as indirect consequences on prey behaviour, antipredator adaptations are under strong selection pressures (Sih, 1987 *in* Gamradt and Kats, 1996). Amphibian larvae that coexist with predators (e.g. fish, snakes, or insects) exhibit a variety of antipredator behaviours. The plastic behavioural responses include reduced activity (Anholt and Werner, 1995; Skelly, 1994), increased activity (Hewes, 1988), spatial avoidance of predators, and refuge use (Horat and Semlitsch, 1994; Kats *et al.*, 1988; Kiesecker *et al.*, 1996; Petranka *et al.*, 1987). These behaviours reduce vulnerability to predation (Lawler, 1989; Skelly 1992, 1994) and may be influenced by conditioning, the age of test animals, and genotype (Semlitsch and Reyer, 1992; Kats *et al.*, 1994; Bridges and Gutzke, 1997).

For larval amphibians, chemical stimuli released by their predators are usually more important in the elicitation of behavioural defences than visual or tactile stimuli (Petranka *et al.*, 1987; Stauffer and Semlitsch, 1993).

Kiesecker *et al.* (1996) verified that tadpoles of *Bufo boreas* recognized a predator on the basis of chemical stimuli and not on the basis of visual stimuli. Experimental works carried out with *Rana temporaria* showed that tadpoles increased the refuge use in the presence of chemical cues of the fish *Oncorhynchus mykiss* (Nyström and Abjörnsson, 2000), having the same pattern been found in amphibians of the family Ranidae, Hylidae, Ambystomatidae e Plethodontidae (Kats *et al.*, 1988).

According to Barr and Babbitt (2002), in the presence of the fish *Salvelinus fontinalis* the larvae of the salamander *Eurycea bislineata* increased the use of substratum, which presents interstitial spaces that may be used as a refuge in order to avoid predation, suggesting that the availability of refuges can be a important factor to allow the coexistence of these larvae with the predator fish.

Introduced predators are being increasingly implicated in amphibian declines (Fisher and Shaffer, 1996; Gamradt and Kats, 1996; Lawler *et al.*, 1999; Kats and Ferrer, 2003; Cruz *et al.*, 2006; Cruz *et al.*, *in press*). Native anurans that are able to reduce risky behaviours in response to cues from novel predators may be better able to persist after their establishment. Nonetheless, their ability to modify risky behaviours is likely a reflection of their behavioural plasticity and predator exposure over recent evolutionary time (Kats *et al.*, 1988; Kiesecker and Blaustein, 1997).

The time it takes for prey animals to acquire antipredator behaviours in response to a new predator can indicate the impact that the new predator can have on populations of these prey (Maloney and McLean, 1995). Once the behaviour has been acquired, the speed with which it dispersed in the population may be indicative of the intensity of predation pressure by the new predator (Kiesecker and Blaustein, 1997).

The American red crayfish, *Procambarus clarkii* (Girard, 1852), is an autochthonous species from the Northeast of Mexico and South Central US (Hobbs *et al.*, 1989), and the first occurrence of this species in Portugal dates from 1979 (Ramos and Pereira, 1981).

This species is a tactile nocturnal predator that actively searches the substratum of the water bodies (Harper *et al.*, 2002). It is able to colonize most types of freshwater habitats and may reach high densities, especially in temporary ponds (Cruz *et al.*, 2004).

Recently, Cruz and Rebelo (2005) pointed out this exotic species as a predator of egg masses and larvae of all the amphibian species of southwest Iberian Peninsula. Further studies demonstrated that the tadpoles of *Alytes cisternasii* (Boscá, 1879), an Iberian

endemism, altered their behaviour as a response to the chemical stimulus of *Procambarus clarkii* (Amaral, 2004). The antipredator behaviours presented by *Alytes cisternasii* tadpoles seemed to be in accordance with the circadian foraging activity of this crayfish, as the behavioural alterations were observed to be more intense during the night (Amaral, 2004), and it is in this period that *P. clarkii* presents greater predatory activity (Correia, 1998).

However, behavioural antipredator defences may change with tadpole developmental stage (Bridges and Gutzke, 1997) as the vulnerability to predators may not be the same in all life stages (Tejedo, 1993; Mathis *et al.*, 2003). On the other hand, in most natural systems predation risk varies temporally: predator activity patterns and densities vary both circadianly and seasonally, and such variation may have a profound impact on prey behaviour (Houston *et al.*, 1993; Clark, 1994; Lima and Bednekoff, 1999; Mirza *et al.*, 2006).

Procambarus clarkii also presents seasonal differences in foraging patterns. Feeding intensity increases over the spring and summer and declines during autumn and winter (Correia, 1998).

The objectives of this work are to verify, in experimental conditions, the existence of antipredator defences in tadpoles of *Alytes cisternasii* in different developmental stages in response to the chemical cues of *Procambarus clarkii,* by comparing tadpole responses in two different seasons, and to verify if the differences in seasonal foraging activity of this predator may change the type or intensity of behavioural alterations of the tadpoles.

I.2.1.Tadpole species studied

The Iberic midwife toad, *Alytes cisternasii* (Boscá, 1879), is an endemic species of the southwest of the Iberian Peninsula, being the major part of its distribution area situated in the Portuguese territory (Pargana *et al.*, 1998). In Portugal it has a   status of *"Near*

Threatened (NT) " and is protected by the Convention of Berne (annex II) and by the Habitats Directive (92/43CEE) (annex II,IV) (Almeida *et al.*, 2001). Although this species is not threatened in Portugal, the expansion of exotic predators may affect its distribution in the near future.

This species is adapted to hot and dry environments, especially in areas of sandy or coarse soils, normally in open, plane zones (Almeida *et al.*, 2001).

The larval life takes 110-140 days (Salvador and Garcia-París, 2001 *in* Marquéz, 2004) and the metamorphosis is in the spring, with few tadpoles occurring in the water bodies after May (Pargana *et al.*, 1998).

According to a study carried out at Grândola (SW Portugal), *A. cisternasii* has two reproduction peaks per year, one in the autumn and another in the spring (Rebelo and Crespo, 1999), and it reproduces preferentially in lotic habitats, where it coexists with some predators, as the recently introduced red swamp crayfish, *Procambarus clarkii* (Carreira, 2003).

I.3. METHODS

To compare the behavioural responses of *Alytes cisternasii* tadpoles to chemical cues of *Procambarus clarkii* in two different seasons (winter and spring), we used the results of Amaral (2004), obtained in the winter of 2004, and performed a similar experience in the spring of 2005 (table I.1). Both the experiments were performed with tadpoles from a population that is in contact with the exotic predator since the middle of the decade of 1990 and were carried out in the biotherium of the Centre of Environmental Biology (CBA) in Herdade da Ribeira Abaixo (Serra de Grândola - Alentejo, SW Portugal).

I.3.1. Capture and maintenance of the studied species

The tadpoles of *Alytes cistemasii* were captured in the Ribeira de Castelhanos (38°06'28.57"N; 8°34'14.56"W) and the temporary water lines that drain into this stream, in the Herdade da Ribeira Abaixo, with dip-nets (30 cm diameter; 2 mm green mesh). The tadpoles were kept in PVC aquariums (19 cm x 34 cm x 19 cm) filled with spring water and fed with cereal flakes.

Procambarus clarkii were captured in the Ribeira de Castelhanos, maintained in individual plastic containers filled with spring water and fed with commercial fish food. The crayfish were captured with funnel traps, baited with commercial cat food.

All the animals were maintained during the experiments in a 12:12 light-dark photoperiod.

I.3.2. Experimental unit

The experimental unit consisted of opaque aquariums of PVC (40 cm x 60 cm x 37, 5 cm), with the base covered with flat rocks. The rocks were placed in order to mimic the river bottom, with crevices that could function as shelter. Each aquarium was filled with 35 litres of spring water.

In the centre of each aquarium we placed a cage, suspended and slightly sunk near the surface. The cage was constructed with a 1, 5 litres plastic bottle, open at both sides, in the extremities of which was placed green net of fine mesh (2mm) and whose lateral walls were pierced, in order to allow for the circulation of water and the dispersion of the chemical stimulus.

On each experiment there were 2 treatments (the number of replicates was the same in both years):

(1) Empty cage (control) (9 replicas);

(2) Cage with an individual of *Procambarus clarkii* (10 replicas).

The experiment ran in two series: one diurnal one nocturnal. During the night, the tadpoles were observed with a low intensity light, having been verified in preliminary tests that this light intensity did not affect the behaviour of the tadpoles (Amaral, 2004).

Each tadpole and crayfish was used only one time in each replica; however the individuals of the diurnal series were used in the corresponding nocturnal series.

We used 5 tadpoles in each replica, to which we measured the head length (HL, mm) and identified the developmental stage (Gosner, 1960) (table I.1). Crayfish were measured along their cephalothorax length (mm) (table I.1) and sexed.

The choice of the parameters to record was decided based on the antipredator behaviours described in other species (Kats *et al.*, 1988; Kiesecker *et al.*, 1996; Keisecker and Blaustein, 1997; Nyström and Abjörnsson, 2000; Altwegg, 2002; Barr and Babbitt, 2002).

For the two treatments, after a 30 minute period of acclimatization of the tadpoles, the following parameters were registered every 15 minutes, for 3 times: refuge use (totally visible *vs.* not visible or partially visible), activity (active *vs.* inactive), vertical microhabitat use (substratum, water column or surface) and border use (wall of the aquarium or wall of the cage).

Table I.1. Comparison of the parameters of the two studies.

	Amaral (2004) (Winter)	Present study (Spring)
Months	February (2004)	April-May (2005)
Water temperature	10-14°C	16-18°C
Tadpoles (HL,mm) (mean±SD; N=45) (control)	17,71±1,81	16,36±1,67
Tadpoles (HL,mm) (mean±SD; N=50) (*P. clarkii*)	17,26±1,78	16,41±1,19
Tadpoles developmental stage* (N=45) (control)	25	41
Tadpoles developmental stage* (N=50) (*P. clarkii*)	25	41
***P. clarkii* (∗) (mean±SD; N=10)**	54,95±3,11	50,36±4,18

* The developmental stage of the tadpoles (Gosner, 1960) corresponds to the modal class.

(∗) The measures of *P. clarkii* correspond to the cephalothorax length.

I.3.3.Statistical analyses

The results referring to the three records (at 30, 45 and 60 minutes) of the number of tadpoles observed for each parameter (refuge use, activity, vertical microhabitat use, margins use), were added and utilized in the comparisons. To compare winter and spring experiments for both treatments we carried Two-way ANOVA's, one for the diurnal period and another for the nocturnal period.

To compare the head length (HL) and the developmental stage of the tadpoles, between corresponding treatments of both experiments we used t-tests for independent samples.

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The homogeneity of variances was tested and, when not met, the Wilcoxon nonparametric test was applied.

In all statistical analyses we considered a level of significance of $\alpha=0.05$; the analyses were performed with the program *STATISTICA (version 5.5)*.

I.4. RESULTS

In the comparison of tadpole head length and developmental stage between winter and spring experiments, significant differences were verified for both treatments, control and chemical cue of *P. clarkii* (table I.2).

Table I.2. Results of the t-test and Wilcoxon tests that compare between spring and winter samples for head length and developmental stage of tadpoles.

	Control		
	Teste Wilcoxon Z	*Teste-t* t_9	*P*
Tadpoles head length (mm)	–	-3.68858	0.000390
Developmental stage	5.669	–	0.0000
	Chemical cue of *P. clarkii*		
	Teste Wilcoxon Z	*Teste-t* t_9	*P*
Tadpoles head length (mm)	27.056	–	0.0068
Developmental stage	6.153	–	0.0000

I.4.1. Diurnal period

During the diurnal period, there were significant differences between the winter and spring results, regarding the behaviour "refuge use" ($F_{(1,34)}$=5,22;p=0,0257) (table I.3. (a)). In the spring, by day, the tadpoles increased the use of refuge (mean/spring=4,70;mean/winter=2,70) in both "control" and *"P. clarkii"* treatments, with no interaction between the categorical variables ($F_{(1,34)}$= 1,31;p= 0,2613) (table I.3 (a)) (fig. I.2 (A)).

There were also significant differences between seasons for the use of the microhabitat "surface" in the diurnal period ($F_{(1,34)}$=5,01;p=0,0318) (table I.3 (e)). During the spring the tadpoles of *A. cisternasii* were on average more observed at the surface than during the winter (mean/spring=4,50;mean/winter=1,86). Once more, there was no interaction between the factors (table I.3 (e)). In the diurnal period and in the spring, the tadpoles increased the use of the vertical microhabitat "surface" in the control treatment (mean/spring=4,00; mean/winter=1,11) and in the treatment with chemical cue of *P. clarkii* (mean/spring=5,00; mean/winter=2,60) (fig.I.1).
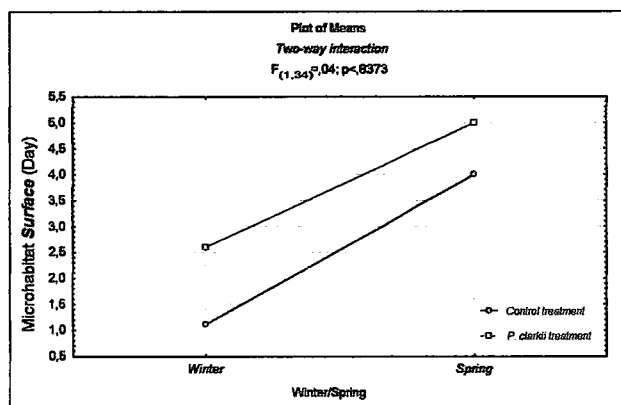
Figure I.1. Relation between seasons and treatments, for the use of microhabitat "surface", during the day.

Finally, there were significant differences between the results of winter and spring, regarding the behaviour "margins use" ($F_{(1,34)}$=4,87;p=0,0341) (table I.3 (f)). Once again, there was no interaction between the categorical variables ($F_{(1,34)}$=0,03;p=0,8732) (table I.3

(f)). The tadpoles increased the utilization of margins in the spring in both treatments, control (mean/spring=4,33; mean/winter=2,00) and chemical cue of *P. clarkii* (mean/spring=5,80; mean/winter=3,10) (fig. I.4 (A)).

Table I.3. Results of Two-way ANOVAs, testing for the effects of seasons (winter/spring), treatments (control/*P. clarkii*) and their interactions on all behavioural parameters measured during the day (significant *P* values at alpha < 0.05 are indicated by *).

			DAY			
Refuge use	**Source of variation**	**SS**	**df**	**MS**	**F**	**P**
(a)	Winter/Spring (A)	37,89474	1	37,89474	5,220507	**0,028684***
	Control/*P.clarkii* (B)	1,51579	1	1,51579	0,20882	0,6506
	A*B	9,473684	1	9,473684	1,305127	0,26126
	Error	246,8	34	7,258823		
Activity	**Source of variation**	**SS**	**df**	**MS**	**F**	**P**
(b)	Winter/Spring (A)	0,22924	1	0,22924	0,216237	0,644887
	Control/*P.clarkii* (B)	0,042105	1	0,042105	0,039717	0,843222
	A*B	0,018713	1	0,018713	0,017652	0,895087
	Error	36,04445	34	1,060131		
Water column	**Source of variation**	**SS**	**df**	**MS**	**F**	**P**
(c)	Winter/Spring (A)	1,688889	1	1,688889	0,679464	0,415517
	Control/*P.clarkii* (B)	0,010526	1	0,010526	0,004235	0,948495
	A*B	0,004678	1	0,004678	0,001882	0,965649
	Error	84,51111	34	2,485621		
Substratum	**Source of variation**	**SS**	**df**	**MS**	**F**	**P**
(d)	Winter/Spring (A)	3,410526	1	3,410526	0,247714	0,621893
	Control/*P.clarkii* (B)	2,583626	1	2,583626	0,187655	0,667612
	A*B	1,51579	1	1,51579	0,110095	0,742072
	Error	468,1111	34	13,76797		
Surface	**Source of variation**	**SS**	**df**	**MS**	**F**	**P**
(e)	Winter/Spring (A)	66,25029	1	66,25029	5,0135	**0,031802***
	Control/*P.clarkii* (B)	14,67134	1	14,67134	1,110256	0,299457
	A*B	0,566082	1	0,566082	0,042838	0,837265
	Error	449,2889	34	13,21438		
Margins use	**Source of variation**	**SS**	**df**	**MS**	**F**	**P**
(f)	Winter/Spring (A)	60,00263	1	60,00263	4,874766	**0,034099***
	Control/*P.clarkii* (B)	15,60263	1	15,60263	1,267597	0,268103
	A*B	0,318421	1	0,318421	0,025869	0,873172
	Error	418,5	34	12,30882		

I.4.2. Nocturnal period

During the nocturnal period, there were significant differences between the results obtained for winter and spring in the comparisons for the behavioural parameter "refuge use"($F_{(1,34)}$= 6,60;p=0,0148) (table I.4 (a)) and between the treatment control and the treatment with the chemical stimulus of crayfish ($F_{(1,34)}$= 11,39;p=0,0019) (table I.4 (a)).

In spring the tadpoles of *A. cisternasii* were on average more observed to use the refuges, than during the winter in both treatments; control (mean/spring=5,44;mean/winter=3,89) an in the treatment with the chemical cue of *P. clarkii* (mean/spring=3,30;mean/winter=1,10). However, in the presence of the chemical cue of *P. clarkii*, *A. cisternasii* tadpoles decreased their permanence in the refuges (mean/*P. clarkii*= 2,20; mean/control= 4,67). There was no interaction between the categorical variables ($F_{(1,34)}$= 0,19;p=0,6621) (table I.4 (a)) - the utilization of refuges was consistently lower in the chemical cue treatment than in the control (fig. I.2 (B)).

(A)

(B)
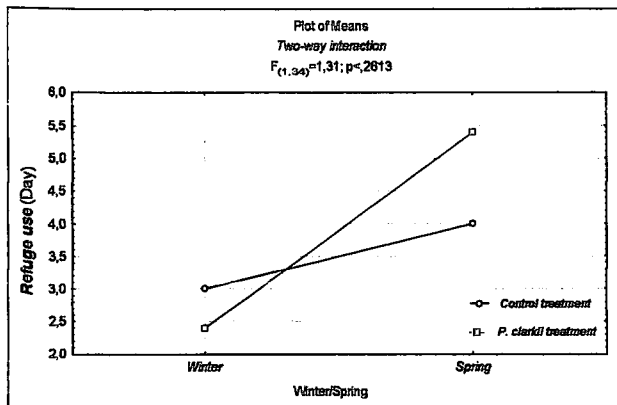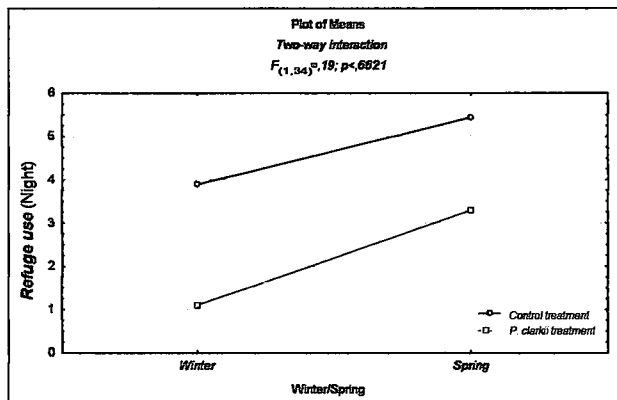
Figure I.2. Relation between seasons and treatments, for the behaviour "refuge use", during the day (A) and the night (B).

As to tadpole activity, the results of the comparison between spring and winter were "nearly significant" during the night ($F_{(1,34)}$= 3,49;p=0,0702) (table I.4 (b)) - the tadpoles were less active in the spring than in the winter (mean/spring=0,43;mean/winter=1,08).

In the control treatment, on average the number of *A. cisternasii* tadpoles observed in activity, during the night was similar for both seasons (mean/winter=0,56;mean/spring=0,56). In the treatment with chemical cue of *P. clarkii*, the tadpoles were less observed in activity in the spring than in the winter (mean/spring=0,30;mean/winter=1,60) (fig.I.3). The results were not similar for both treatments, with a nearly significant interaction ($F_{(1,34)}$= 3,49;p=0,0702) (table I.4 (b)), thus, the reduction in activity that was verified in the spring could be related with the chemical cue of *Procambarus clarkii*.
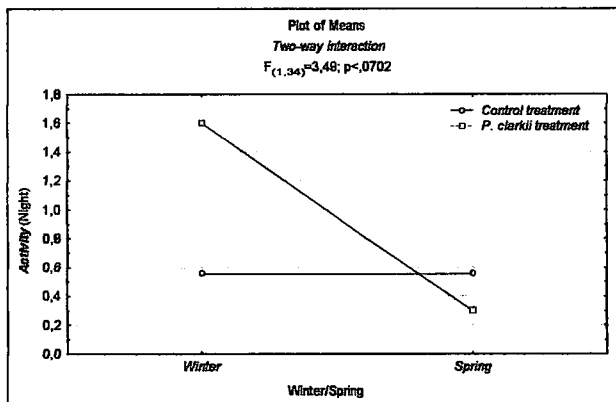
Figure I.3. Variation between seasons and treatments, for the behaviour "activity", during the night.

There were significant differences between treatment control and treatment with chemical stimulus of crayfish ($F_{(1,34)}$= 5,09;p=0,0306) (table I.4 (f)) for the use of the microhabitat "margins".

During the night the tadpoles were on average more observed using the margins when submitted to the treatment of the chemical stimulus of *P. clarkii* (mean/*P.clarkii*=5,15;mean/control=2,61).

The tadpoles of *A. cisternasii* used the margins more frequently in the spring in the control treatment (mean/spring=3,00;mean/winter=2,22) and in the treatment with crayfish (mean/spring=5,50;mean/winter=4,80) (fig.I.4 (B)), without interaction between the categorical variables ($F_{(1,34)}$= 0,00;p=0,9726) (table I.4 (f)) – the use of margins was consistently higher in the treatment with *P. clarkii*.

(A)

(B)
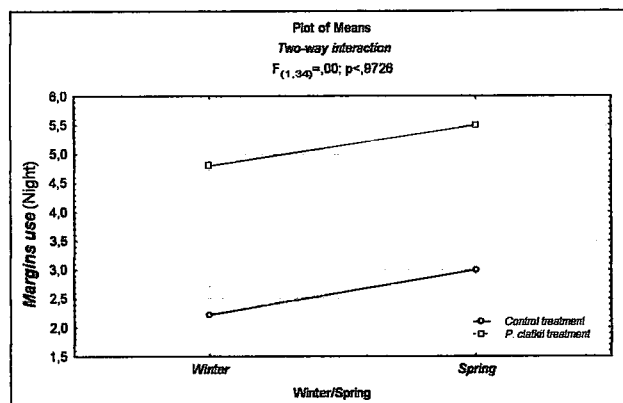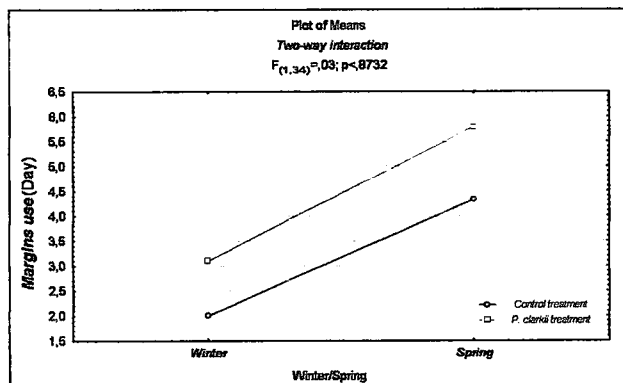
Figure I.4. Relation between seasons and treatments, for the behaviour "margins use", during the day (A) and the night (B).

Table I.4. Results of Two-way ANOVAs, for seasons (winter/spring), treatments (control/*P. clarkii*) and their interactions on all behavioural parameters measured during the night (* significant *P* values at alpha < 0.05; * nearly significant).

				NIGHT		
Refuge use	**Source of variation**	**SS**	**df**	**MS**	**F**	**p**
(a)	Winter/Spring (A)	33,40468	1	33,40468	6,598988	**0,014765***
	Control/*P.clarkii* (B)	57,64211	1	57,64211	11,38701	**0,001861***
	A*B	0,983626	1	0,983626	0,194312	0,662142
	Error	172,1111	34	5,062091		
Activity	**Source of variation**	**SS**	**df**	**MS**	**F**	**p**
(b)	Winter/Spring (A)	4,002632	1	4,002632	3,494452	0,070208 *
	Control/*P.clarkii* (B)	1,473977	1	1,473977	1,286838	0,264566
	A*B	4,002632	1	4,002632	3,494452	0,070208 *
	Error	38,94444	34	1,145425		
Water column	**Source of variation**	**SS**	**df**	**MS**	**F**	**p**
(c)	Winter/Spring (A)	7,864327	1	7,864327	2,806068	0,103083
	Control/*P.clarkii* (B)	3,162573	1	3,162573	1,128437	0,295596
	A*B	4,495906	1	4,495906	1,604183	0,21392
	Error	95,28889	34	2,802614		
Substratum	**Source of variation**	**SS**	**df**	**MS**	**F**	**p**
(d)	Winter/Spring (A)	12,40819	1	12,40819	1,512952	0,227132
	Control/*P.clarkii* (B)	16,01053	1	16,01053	1,952192	0,171403
	A*B	0,618713	1	0,618713	0,075441	0,785236
	Error	278,8445	34	8,201307		
Surface	**Source of variation**	**SS**	**df**	**MS**	**F**	**p**
(e)	Winter/Spring (A)	1,778947	1	1,778947	0,152738	0,698371
	Control/*P.clarkii* (B)	25,27368	1	25,27368	2,169963	0,149928
	A*B	0,515789	1	0,515789	0,044285	0,83458
	Error	396	34	11,64706		
Margins use	**Source of variation**	**SS**	**df**	**MS**	**F**	**p**
(f)	Winter/Spring (A)	5,172222	1	5,172222	0,431383	0,515733
	Control/*P.clarkii* (B)	61,06696	1	61,06696	5,093213	**0,030559***
	A*B	0,014327	1	0,014327	0,001195	0,972626
	Error	407,6555	34	11,98987		

I.5. DISCUSSION

According to the results obtained, *Alytes cisternasii* tadpoles present seasonal and circadian differences in their behaviour and these behaviours can be altered by the chemical stimulus of *Procambarus clarkii*, a recently introduced exotic predator.

In the diurnal period, the responses of the tadpoles were different between winter and spring for the use of refuge and of surface and margins microhabitats. In all cases the tadpoles used these microhabitats more often in the spring. However these seasonal differences were apparently not related with the seasonal feeding activity of *P. clarkii* as no interaction was verified between the treatments and seasons: in both treatments the tadpoles manifested a more intense response in the spring.

The increase of the permanence of the tadpoles in the refuge and in the microhabitats "surface" and "margins" in the spring during the diurnal period may be related with the differences verified between the two studies: body size and developmental stage of tadpoles or water temperature.

In the spring the tadpoles presented an advanced developmental stage (E41; Gosner, 1960), close to metamorphosis. Thus, the most intense behaviours in the spring can be related with the tadpoles developmental stage; if they are considered antipredator behaviours, this means that this kind of responses could be more intense close to metamorphosis.

The use of behavioural antipredator defences may change with development (Bridges and Gutzke, 1997), and the behavioural response to temporal variation in predation risk may be stage-dependent (Laurila *et al.*, 2004). In some cases, increased antipredator behaviour close to metamorphosis is in accordance with the asset protection principle (Clark, 1994): the larger the current asset in terms of fitness, the more important its protection becomes and the stronger is the sensitivity for predation risk. Hence, tadpoles close to the end of the larval stage may be less willing to take risks (Laurila *et al.*, 2004).

In the spring and during the night, the tadpoles of *Alytes cisternasii* increased the use of refuges. Behavioural responses such an increase in refuge use have been reported for many amphibian species exposed to predation risk (Kats *et al.*, 1998; Teplitsky *et al.*, 2003).

Once again the responses are similar in both seasons, and in the spring the responses of the tadpoles were more intense. However, these behavioural alterations can not be justified by the increased spring activity of crayfish as the tadpoles responded similarly in both treatments (control and chemical stimulus of *P. clarkii*), significantly increasing their refuge use in the spring. Furthermore, the increase of the refuge use can not be considered an antipredator behaviour towards *Procambarus clarkii* due to its predation strategy.

When submitted to the chemical cue of the exotic predator during the night, the tadpoles decreased refuge use and increased their permanence close to the margins.

These responses can be considered antipredator behaviours, because they protect the tadpoles against the predation strategy of crayfish, which is the constant tactile exploration of the environment close to the bottom, mainly under rocks (Amaral, *pers. obs.*), and mainly in the night (Correia, 1998). By exhibiting these behaviours during the night, the tadpoles of *A. cisternasii* decreased the permanence in the bottom of the aquaria and consequently decreased the probability of meeting the predator. Therefore the tadpoles of *Alytes cisternasii* apparently recognize and respond to the chemical cue of *Procambarus clarkii*, according with the predation strategy and circadian foraging activity of this predator.

In both seasons (winter and spring) it was verified that the use of margins was a very intense response to the chemical cue of crayfish (fig. I.4) during the nocturnal period. These results suggest that tadpoles realize that there is something potentially dangerous in the deepest zones, and that its mechanisms of behavioural response towards this type of stimulus include the increase of the use of marginal zones.

The results of the alterations of activity levels are nearly significant. In the spring, and during the night, the tadpoles decreased their activity. Since there were no differences between the two seasons in the control treatment, but in the treatment with chemical cue of the exotic predator the tadpoles decreased strongly their activity in spring, this nearly significant difference can be associated with the presence of crayfish. *Procambarus clarkii* feeds more intensely in the spring, during the night (Correia, 1998), when the decrease in activity of the tadpoles may decrease the number of meetings with the predator.

The reduction of the activity level in the face of predators that hunt based on prey movement is considered an adaptative behaviour, since it reduces the probability of the prey to be detected, as well as diminishing the probability of the prey to occupy the immediate area where the predator is found (Lawler, 1989; Skelly and Werner, 1990; Werner, 1991; Skelly, 1992; Skelly, 1994). However, these results were not conclusive, and it would be interesting to further test the alteration of the behaviour "activity".

These results may provide some cues about the evolution of antipredator behaviours in tadpoles of *A. cisternasii* face to an introduced predator, *Procambarus clarkii*, which can be important to understand the impact that this predator has in the populations of this species. The tadpoles of *A. cisternasii* already presented behavioural alterations in response to the exotic predator, but it remains to evaluate in which degree this response confers them some advantages (or disadvantages).

Also it would be interesting to compare the behavioural responses between this and other populations with different histories of contact with *P. clarkii*, which would allow a better perception of the evolution of antipredator responses.

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BEHAVIOURAL RESPONSES OF IBERIC MIDWIFE TOAD TADPOLES (*ALYTES CISTERNASII*) TO CHEMICAL CUES OF A NATURAL PREDATOR (*NATRIX MAURA*) AND AN EXOTIC PREDATOR (*PROCAMBARUS CLARKII*)

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II.1. ABSTRACT

Recently significant declines of amphibian populations were registered at a worldwide level, being one of the causes the introduction of exotic predators and competitors, such as the red swamp crayfish (*Procambarus clarkii*), a species introduced in Portugal.

However, predation by native or introduced predators can also be an important force of selection, resulting in the evolution of antipredator defences in amphibian larvae.

The aim of this work was to compare, the antipredator defences of the tadpoles of *Alytes cisternasii*, when faced with the chemical stimulus of an exotic predator, *Procambarus clarkii*, with the responses to one of its main native predators, the viperine snake, *Natrix maura*.

The alteration in the use of the vertical microhabitat seemed to be the antipredator behaviour more frequently used as a response to the chemical cues of both predators.

The tadpoles of *A. cisternasii* reacted more intensely to the natural predator than to the introduced predator. However, the facts that the behavioural responses were observed in nocturnal conditions and that the tadpoles presented a reduction of activity when subjected to the introduced predator, suggest that the tadpoles recognize something strange in the environment when in presence of *P. clarkii*, and that its mechanisms of behavioural responses before alterations of the environment include the reduction in activity levels.

II.2. INTRODUCTION

In the last decades, there were records of a rapid disappearance and regression in the distribution of numerous species of amphibians, at a worldwide level (Blaustein and Wake, 1990; Wake, 1991; Blaustein *et al.*, 1994; Blaustein and Wake, 1995; Alford and Richards, 1999).

The causes more frequently pointed for this decline are global warming (climate change), increase of the levels of ultraviolet radiation, acid precipitation, diseases, habitat loss and modification, and contamination of the water bodies (Alford and Richards, 1999; Blaustein *et al.*, 2003; Collins and Storfer, 2003). Recently, several declines were associated with the introduction of exotic predators and competitors (Fisher and Shaffer, 1996; Gamradt and Kats, 1996; Hecnar and M'Closkey, 1997; Knapp and Matthews, 2000; Kats and Ferrer, 2003), including the American red crayfish (*Procambarus clarkii*). In Portugal, studies carried out with the aim to evaluate the impact of this exotic and invading species in the communities of amphibians, revealed that this crayfish actively predates egg masses and larvae of all the Southwest Iberian amphibians (Cruz and Rebelo, 2005).

Predation is considered by various authors to be the main biotic factor structuring larval anuran communities (Gómez-Mestre and Keller, 2003).

Predation, by native or introduced predators, can thus be an important force of selection, resulting in the evolution of antipredator defences (Lima and Dill, 1990; Lardner, 2000). In the larval amphibians these defences include changes in life history, morphology, behaviour, alterations in use of different components of the habitat and variations in the reproductive strategies (Kats *et al.*, 1988; Lawler, 1989; Skelly and Werner, 1990; Skelly, 1992; Stauffer and Semlitsch, 1993; Warketin, 1995; Smith and Van Buskirk, 1995). These responses are often shown to increase survivorship (Van Buskirk *et al.*, 1997; Van Buskirk and McCollum, 1999; Teplitsky *et al.*, 2005).

Behavioural defences can be induced rapidly and are more easily reversible than are morphological or life-history defences (Tollrian and Dodson, 1999). Several antipredator behaviours have been described in larval amphibians, such as the reduction of activity levels, alterations of the use of different microhabitats or increase of refuge use (Holomuzki, 1986; Petranka *et al.*, 1987; Kats *et al.*, 1988; Skelly and Werner, 1990; Anholt and Werner, 1995; Kiesecker *et al.*, 1996; Kiesecker and Blaustein, 1997; Altwegg, 2002). These antipredator behaviours can be adopted in the presence of chemical cues of predators (Petranka et al., 1987; Kats *et al.*, 1988; Stauffer and Semlitsch, 1993; Kiesecker *et al.*, 1996; Kiesecker and Blaustein, 1997; McCollum and Leimberger, 1997; Altwegg, 2002), and seem to be important for the coexistence of the amphibians with those predators (Kats *et al.*, 1988; Keisecker and Blaustein, 1997; Rundio and Olson, 2003). However, associated with these antipredator behaviours, there may be some costs, such as the reduction in feeding efficiency related with the reduction of growth rates and consequently of survival (Lawler, 1989; Skelly, 1992).

For larval amphibians, chemical stimuli released by their predators are usually more important in the elicitation of behavioural defences than visual or tactile stimuli (Petranka *et al.*, 1987; Stauffer and Semlitsch, 1993).

The ability of prey to recognize predators may be genetical (Hobson *et al.*, 1988; Riechert and Hedrick, 1990; Van Damme *et al.*, 1995), learned, or both (Chivers and Smith, 1994).

Prey that have not been in contact with predators or that have only been in contact with native predators may not have developed efficient defences against the introduced predators (Kiesecker and Blaustein, 1997; Nyström *et al.*, 2001), or the antipredator responses exhibited, having evolved as a response to native predators may not be efficient against the introduced predators (Gamradt and Kats, 1996).

Kiesecker and Blaustein (1997) showed that tadpoles of *Rana aurora* of two different populations, one that had been in contact with larvae and adults of *Rana catesbeiana* and another that had not, presented behavioural differences in the presence of this predator, introduced in their study area (Oregon) in the beginning of the decade of 1930. They also verified that the tadpoles with previous experience avoided more efficiently predation by *Rana catesbeiana* than tadpoles without experience. These authors established, in this way that in little more of 60 years the tadpoles of *Rana aurora* recognized and developed strategies of defence against the introduced predator.

Frequently the prey species does not use the same antipredator responses towards all predators, but uses specific strategies of defence for each predator (Sih, 1987 *in* Schmidt and Amézquita, 2001; Relyea, 2001a), because predators differ in how well they can encounter, capture, handle, and consume prey, as well as in their predation strategies (Relyea, 2001a). Failure of a prey to recognize and respond to a specific predator increases the probability that it will be captured during an interaction with it (Kiesecker and Blaustein, 1997).

The objective of this work is to verify, in experimental conditions, the existence of antipredator defences in tadpoles of *Alytes cisternasii*, in response to the chemical cues of an exotic predator, *Procambarus clarkii,* comparing them with the responses to one of its main native predators, the viperine water snake, *Natrix maura*.

The direct predation of *Procambarus clarkii* on tadpoles of *Alytes cisternasii* was demonstrated in recent studies carried out in mesocosms, with a reduction in the survival of the tadpoles of *A. cisternasii* in the presence of *P. clarkii*, even when alternative food was provided (Cruz and Rebelo, 2005).

While it is expected that the tadpoles of *Alytes cisternasii* will present behavioural responses to one of its main natural predators, the type and intensity of its responses to

a predator recently introduced are unknown, and its description will permit an evaluation of the future of this species in areas invaded by *P. clarkii*.

If the behavioural responses are similar for both predators, they probably evolved against the native predator and may be not efficient against the introduced species, which has a different predation strategy. On the other hand, if *A. cisternasii* is already able to recognize and to develop specific strategies of defence against *P. clarkii*, this process will have take place in about 30 years, since *P. clarkii* was introduced in Portugal, and this species will probably be less vulnerable to the impact of this predator, either direct (survival of individuals) or indirect (reduce fitness of the newly-metamorphosed toadlets and therefore of the adults).

II.2.1. Studied Species

The Iberic midwife toad, **Alytes cisternasii** (Boscá, 1879), is an endemic species of the southwest of the Iberian Peninsula, being the major part of its distribution area situated in the Portuguese territory (Pargana *et al.*, 1998). In Portugal it has a status of "*Near Threatened (NT)* " and is protected by the Convention of Berne (annex II) and by the Habitats Directive (92/43CEE) (annex II,IV) (Almeida *et al.*, 2001). Although this species is not threatened in Portugal, the expansion of exotic predators may affect its distribution in the near future.

This species is adapted to hot and dry environments, especially in areas of sandy or coarse soils, normally in open, plane zones (Almeida *et al.*, 2001).

The tadpoles can reach 70 mm of total length (Barbadillo *et al.*, 1999; Almeida *et al.*, 2001). The dorsal coloration is variable; generally brownish with dark or silver spots on the body and tail (Almeida *et al.*, 2001).

The larval life takes 110-140 days (Salvador and Garcia-París, 2001 *in* Marquéz, 2004) and the metamorphosis is in the spring, with few tadpoles occurring in the water bodies after May (Pargana *et al.*, 1998).

According to a study carried out at Grândola (SW Portugal), *A. cisternasii* has two reproduction peaks per year, one in the autumn and another in the spring (Rebelo and Crespo, 1999), and it reproduces preferentially in lotic habitats, where it coexists with some predators, as the recently introduced red swamp crayfish, *Procambarus clarkii* (Carreira, 2003).

The viperine snake (***Natrix maura***), is one of the main predators of larval amphibians in Iberian freshwater ecosystems (Barbadillo *et al.*, 1999; Almeida *et al.*, 2001). It is a small snake that measures around 65 to 70 cm of total length. The cylindrical body is covered dorsally with carinal scales (Barbadillo *et al.*, 1999; Almeida *et al.*, 2001).

In Portugal, this species is distributed widely in all the country. It is an animal of aquatic habits, being found generally in the proximities of any water body (Pargana *et al.*, 1998). It may hibernate between November and February (Hailey and Davies, 1987; Almeida *et al.*, 2001) but in the south of Portugal this species seems to be active during all the year (Malkmus, 1996 *in* Santos, 2004). Essentially diurnal, exhibits crepuscular and nocturnal activity during the hottest periods of the summer. In the arid zones of the south of the Iberian Peninsula, this species is active during the night (Santos, 2004).

Hailey and Davies (1986) verified that *N. maura* presents two predation behaviours: in one its is immovable in different points of the water column, locates the prey visually and capture it as it approaches; in the other there is an active exploration, searching for chemical cues of the prey.

According to Rebelo (*pers.com.*), the strategies of predation of this species may consist in an exploratory behaviour of crevices and shelters in the bottom of ponds and river banks, to confine potential prey like tadpoles.

Procambarus clarkii (Girard, 1852) is a crayfish native of the Northeast of Mexico and South Central US (Huner and Barr, 1991), that was introduced in Portugal due to the expansion of the population that became naturalized in Badajoz, Spain (Correia, 2002). Ramos and Pereira (1981) designate the year of 1979 as the year of arrival of the red crayfish to Portugal, concretely to the Caia river, an affluent of the Guadiana, having expanded rapidly since then (Anastácio, 1993). In the first half of the decade of 1980, abundant populations were detected in the Tejo (Ferreira, 1985, Reiner, 1985 *in* Gutiérrez-Yurrita *et al.*, 1999) and Guadiana river basins (Adão, 1991); in 1987, this species was found in the Mondego river (Marques *et al.*, 1992 *in* Gutiérrez-Yurrita *et al.*, 1999); and in 1990, it reached the Sado river (Adão, 1991).

Procambarus clarkii is a tactile nocturnal predator that actively searches the substratum of the water bodies (Harper *et al.*, 2002). Its high ability to adaptat to new habitats and a good tolerance to a huge range of environmental conditions (Fidalgo *et al.*, 2001), are associated with an elevated growth rate and a successful reproductive strategy, responsible for the development of abundant populations (Anastácio and Marques, 1995). It is considered a generalist species and, therefore, its introduction has had ecological impacts, with the reduction of the complexity of the habitats, due to the consumption of macrophytes (Gherardi *et al.*, 2001) and of large quantities of detritus (Harper *et al.*, 2002), and even of invertebrates and vertebrates, being the juveniles of this crayfish particularly carnivorous (Gutiérrez-Yurrita *et al.*, 1998).

Freshwater crayfish can, generally, modify the aquatic communities and reduce the availability of places for laying their egg masses as well as of shelters for amphibians (Axelsson *et al.*, 1997); in particular, *P. clarkii* constitutes a predator of eggs, larvae and adults of amphibians (Gamradt and Kats, 1996; Cruz and Rebelo, 2005).

II.3. METHODS

To test the behavioural responses of the tadpoles of *Alytes cisternasii* to chemical cues of the two predators, we performed an experience with a population that is in contact with the exotic predator since the middle of the decade of 1990.

The experience was carried out in the biotherium of the Centre of Environmental Biology (CBA) in Herdade da Ribeira Abaixo (Serra de Grândola - Alentejo, SW Portugal).

II.3.1. Capture and maintenance of the individuals used

The tadpoles of *Alytes cisternasii* were captured in the Ribeira de Castelhanos (38°06'28.57"N; 8°34'14.56"W) and the temporary water lines that drain in this stream, in the Herdade da Ribeira Abaixo, with dip-nets (30 cm diameter; 2 mm green mesh).

They were kept in PVC aquariums (19 cm x 34 cm x 19 cm) with spring water and fed with cereal flakes.

Procambarus clarkii were captured in the Ribeira de Castelhanos and maintained in individual plastic containers with spring water and fed with commercial fish food. The crayfish were captured with funnel traps, baited with commercial cat food.

Natrix maura were captured with funnel traps in the Ribeira de Castelhanos, kept in a PVC aqua-terrarium (19 cm x 34 cm x 19 cm), and fed with tadpoles.

All the animals were maintained during the experiences in a 12:12 light-dark photoperiod. This experience took place between the months of April and May of 2005

II.3.2. Experimental unit

The experimental unit consists of opaque PVC aquariums (40 cm x 60 cm x 37, 5 cm), with the floor covered with rocks, which were placed in order to mimic the river bottom, with crevices that could function as shelters. Each aquarium was filled with 35 litres of spring water.

In the centre of each aquarium we suspended a cage slightly sunk at the surface. The cage was constructed with a plastic bottle of 1, 5 litres, open at both sides, the extremities of which were covered with green net of fine mesh (2 mm) and whose lateral walls were pierced, in order to allow for the circulation of water and the dispersion of the chemical stimulus.

These cages equally allow the passage of visual stimuli that could also influence the behaviour of the tadpoles. However, in preliminary tests (Cruz and Rebelo, *pers. com.*), it was verified that with or without visual stimulus, the behavioural responses were similar. Thus we opted to use these cages, of more economic production.

In each experience we carried out 3 treatments:

  (1) Empty cage (control) (15 replicas);

  (2) Cage with an individual of *Procambarus clarkii* (15 replicas);

  (3) Cage with an individual of *Natrix maura* (15 replicas).

The experiment ran in two series: one diurnal one nocturnal. During the night, the tadpoles were observed with a low intensity lantern, having been verified in preliminary tests that this light intensity did not affect the behaviour of the tadpoles (Amaral, 2004).

The water temperature was kept between the 16-18°C.

Each tadpole and crayfish was used only one time in each replica; however the individuals of the diurnal series were used in the corresponding nocturnal series.

Natrix maura individuals were repeated in several replicas.

In each replica we used 5 tadpoles, to which we measured the head length (HL, mm) and identified the developmental stage (Gosner, 1960).

The choice of the behaviours to record was decided based on what was described for other species (Kats *et al.*, 1988; Kiesecker *et al.*, 1996; Keisecker and Blaustein, 1997; Nyström and Abjörnsson, 2000; Altwegg, 2002; Barr and Babbitt, 2002).

After a 30 minute period of acclimatization of the tadpoles, we recorded, for 3 times, at 30, 45 and 60 minutes, the following parameters: refuge use (totally visible *vs.* not visible or partially visible), activity (active *vs.* inactive), vertical microhabitat use (substratum, water column or surface) and margin use (wall of the aquarium or wall of the cage). The tadpoles were considered active if they were moving in the moment when the observer saw them for the first time.

II.3.3. Statistical analyses

The results referring to the three records of the number of tadpoles observed for each parameter were added and utilized in the comparisons.

Within each treatment, the comparison for each of the parameters between the results of the two periods of observation (day and night) was carried out through the application of t-tests for dependent samples. To investigate the homogeneity of variances the Levene test was applied, and when that was not verified we carried out the nonparametric Wilcoxon test.

To compare the different parameters among the three treatments, we carried out two univariate ANOVA's, for the day and night results, respectively. When there were significant differences between the treatments, a LSD post hoc test was used.

The normality of data and homogeneity of variances were tested, and when those criteria were not met, the Kruskal-Wallis nonparametric test was applied.

Head length (HL) of the tadpoles used in the three treatments was compared with a Kruskal-Wallis test, since homogeneity of variances was not verified.

All statistical analyses were performed with Program *STATISTICA (version 5.5)*, α was set at 0.05 for all comparisons.

II.4. RESULTS

The sizes and developmental stages of the tadpoles used in each treatment are presented in Table II.1. There were no significant differences between the three treatments, in the head length of the tadpoles (H $_{(9, 68)}$ =0.00, P= 1.00).

Table II.1. Head length and developmental stages of the tadpoles used in each treatment.

	Treatment		
	Control (a)	Chemical cue of *P. clarkii* (b)	Chemical cue of *N. maura* (c)
Tadpole Head Length (HL, mm) (mean±SD;N=75)	15.9±1.9	15.8+1.8	15.3+2.1
Tadpole Developmental Stage* (N=75)	41	41	37

* The developmental stage of the tadpoles (Gosner, 1960) corresponds to the modal class.

II.4.1. Circadian behavioural alterations

Table II.2 shows the results of the *t* or Wilcoxon tests that compared the differences between day and night, for the parameters registered in the three treatments.

In the control treatment, significant differences were found for the use of microhabitats substratum, surface and margins (table II.2 $_{(a)}$). During the day, the tadpoles were on

average more observed at the surface (mean/day=6.3; mean/night=3.3) (fig. II.1 (A)), and at the margins of the aquaria (mean/day=6.3;mean/night=4.7) (fig. II.1 (B)). This meant that during the day they were observed more frequently away from the substratum (mean/night=8.8;mean/day=6.5) (fig. II.1(C)).
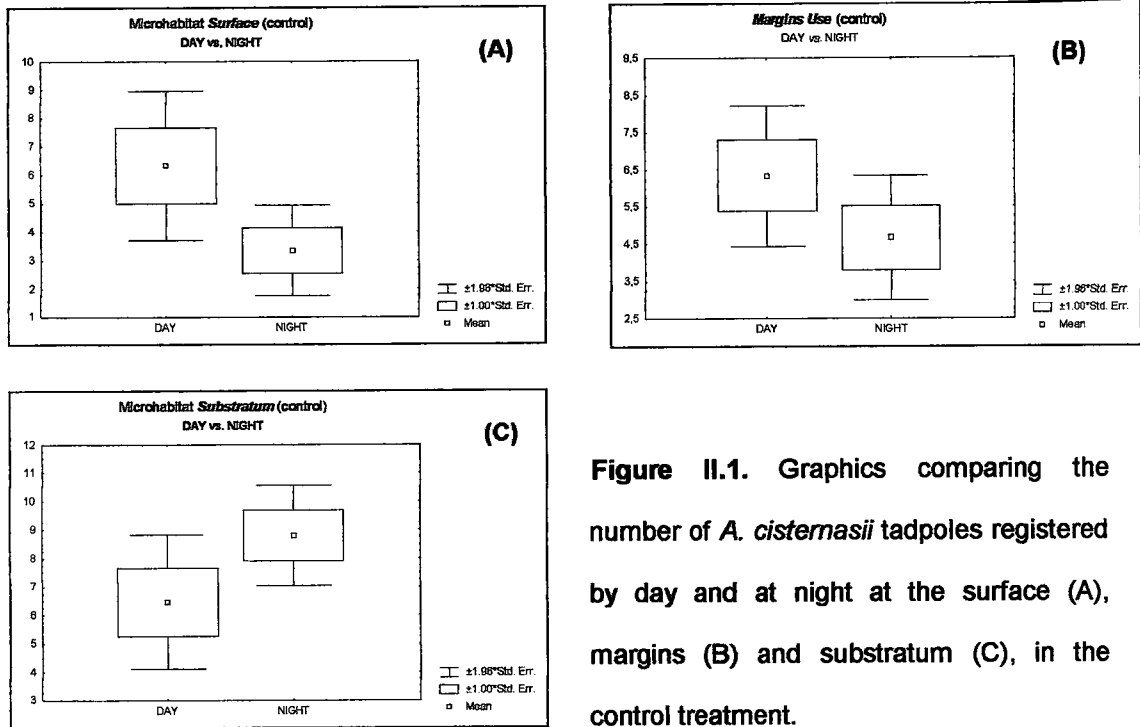
Figure II.1. Graphics comparing the number of *A. cisternasii* tadpoles registered by day and at night at the surface (A), margins (B) and substratum (C), in the control treatment.

In the treatment with the chemical cue of *P. clarkii*, the circadian differences in the use of surface or margins of the aquaria were not observed (table II. 2 (b)), but the tadpoles still increased their permanence close to the substratum during the night (mean/night=8.6;mean/day=6.3). Additionally, the tadpoles decreased their activity during the night (mean/night=0.2;mean/day=0.8) (fig. II.2).
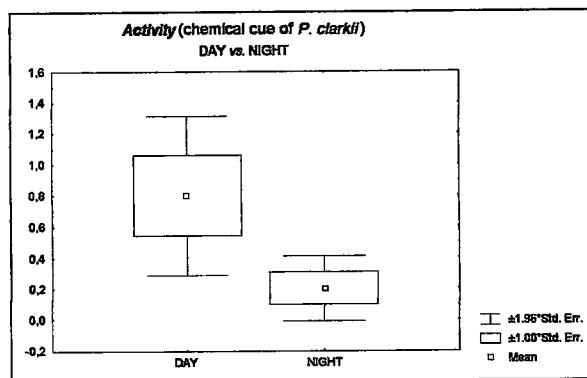
Figure II.2. Graphics comparing the number of *A. cisternasii* tadpoles registered by day and at night in activity, in the treatment with chemical cue of *P. clarkii*.

For the treatment with the chemical cue of *Natrix maura,* during the day, the tadpoles also increased the use of margins (mean/day=9.3;mean/night=6.3) and decreased the permanence in the substratum (mean/day=3.6;mean/night=5.7). However, there were no differences in the use of the surface or in the activity levels, and there was as a decrease in the use of the water column during the day (mean/day=0,3;mean/night=1,2) (fig. II.3).
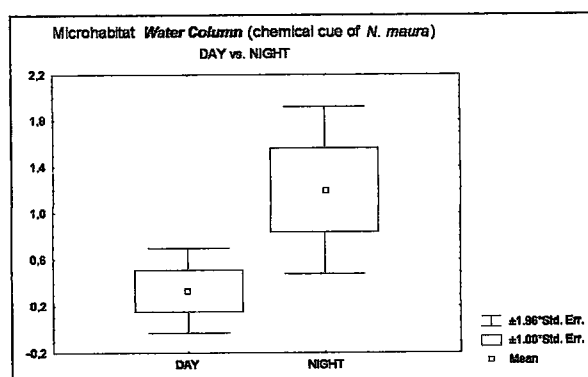
Figure II.3. Comparison of the number *A. cisternasii* tadpoles registered by day and at night in the water column, in the treatment with chemical cue of *N. maura*.

Table II.2. Results of the t-test and Wilcoxon test, that compare circadian differences between day and night, for the parameters registered in the three treatments (significant *P* values at alpha < 0.05 are indicated by *).

| Treatment	Parameter	*Wilcoxon test* Z	*t-test* t_{14}	*P*
Control (a)	Refuge use	—	-1.12	0.28
	Activity	—	1.32	0.21
	Water column	—	-0.75	0.46
	Substratum	**2.55**	—	**0.011***
	Surface	—	**3.35**	**0.005***
	Margins use	—	**2.27**	**0.04***
Chemical Cue of *P. clarkii* (b)	Refuge use	0.28	—	0.78
	Activity	—	**2.2**	**0.045***
	Water column	—	0.31	0.76
	Substratum	—	**-3.09**	**0.008***
	Surface	—	2.03	0.06
	Margins use	1.09	—	0.27
Chemical Cue of *N.maura* (c)	Refuge use	—	-1.73	0.11
	Activity	—	-0.81	0.43
	Water column	—	**-2.58**	**0.02***
	Substratum	—	**-2.22**	**0.04***
	Surface	—	2.08	0.06
	Margins use	—	**4.69**	**0.00035***

A univariate ANOVA was used to compare the three treatments during the day, and showed significant differences for the parameters use of microhabitat "water column" and "margins" (table II.3 (a)).

Applying a LSD test it was verified that the results of the *N. maura* chemical cue differed from both the control (*P*=0.011) and the *P. clarkii* chemical cue (*P*=0.010), with a higher utilization of the margins in the treatment with the viperine snake than in the other two (mean/control=6.3;mean/*P.clarkii*=6.5;mean/*N.maura*=9.3) (fig. II.4).

The same occurred in the use of the microhabitat "water column", the results of the treatments with *N. maura* differing from the ones of the control ($P < 0.01$) and of the *P. clarkii* chemical cue ($P < 0.05$) and the tadpoles remaining less in this microhabitat (mean/control=1.1;mean/*P.clarkii*=0.9;mean/*N.maura*=0.3) (fig. II.5).
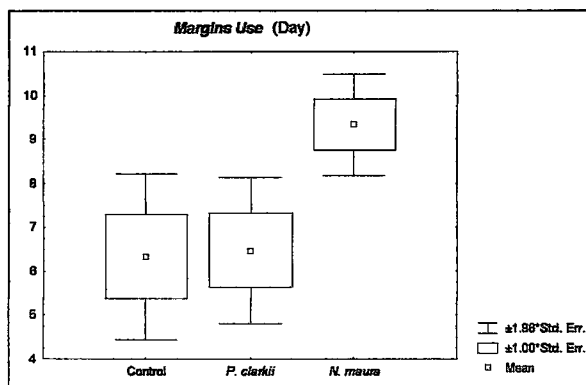
Figure II.4. Comparison of the three treatments, diurnal series, for the behaviour "margins use".
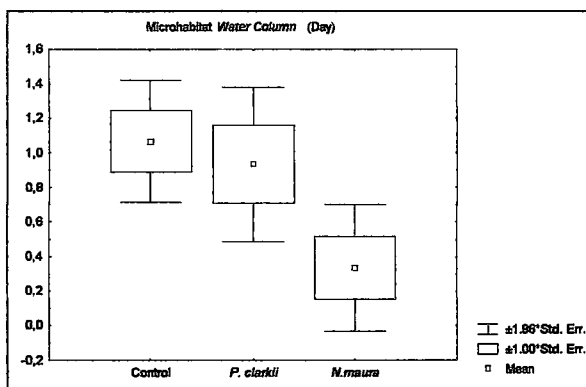
Figure II.5. Comparison of the three treatments, diurnal series, for the behaviour "use of the microhabitat water column".

Table II.3. Results of the Kruskal-Wallis test and the univariate ANOVA comparing the treatments control, chemical cue of *P. clarkii* and chemical cue of *N. maura*, for the parameters registered by day and at night (significant *P* values at alpha < 0.05 are indicated by *).

Series	Parameter	KWU $H_{(2,43)}$	ANOVA $F_{(2,42)}$	*P*
	Refuge use	—	2.44	0.11
	Activity	3.54	—	0.17
Day	Water column	—	4.55	0.019*
(a)	Substratum	—	3.17	0.057
	Surface	10.56	—	0.31
	Margins use	—	5.51	0.0096*
	Refuge use	—	0.47	0.63
	Activity	—	0.45	0.64
Night	Water column	—	0.65	0.53
(b)	Substratum	10.21	—	0.07
	Surface	—	6.63	0.0044*
	Margins use	—	1.17	0.33

The Kruskal Wallis test did not find significant differences for the parameters "activity" and "use of the microhabitat surface" (table II.3 (a)).

As for the nocturnal series, the assumptions of the ANOVA were not met for the use of substratum (table II.A2 – Annex II). The Kruskal-Wallis test did not find differences among the treatments (H $_{(2,43)}$ =10.21; *P*=0.07) (table II.3 (b)).

A univariate ANOVA was applied to the remaining parameters and showed differences in the behaviour of the tadpoles in the use of the microhabitat "surface" (table II.3 (b)). Applying an LSD test it was verified that the results of the treatment of the *N. maura* chemical cue differed from both the control (*P*=0.003) and the *P. clarkii* chemical cue (*P*=0.03), with the tadpoles using more the microhabitat "surface" in the *N. maura* chemical cue than in the other treatments

(mean/control=3.3;mean/*P.clarkii*=3.9;mean/*N.maura*=6.7) (fig. II.6).
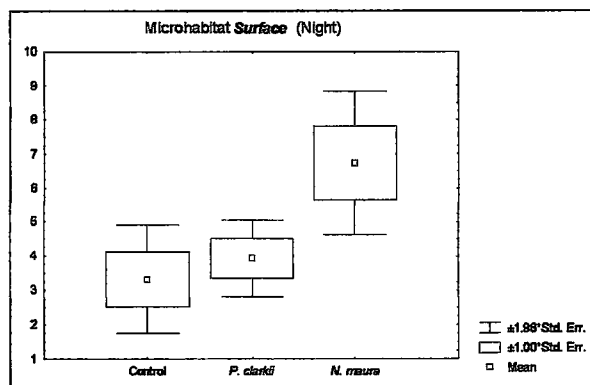
Figure II.6. Comparison of the three treatments, nocturnal series, for the behaviour "use of the microhabitat surface".

II.5. DISCUSSION

The alteration of the use of the vertical axis of the aquaria seems to be the most common antipredator behaviour used as a response to the chemical cues of the predators.

As anticipated, the tadpoles of *Alytes cisternasii* presented strong antipredator responses to their natural predator, *Natrix maura*, and significant differences were verified between the control and the *N. maura* chemical cue treatments in both the nocturnal and diurnal period.

In the diurnal period the tadpoles altered the use of the vertical microhabitat, fleeing from the water column and increasing its presence close to the margins, whereas in the nocturnal period the tadpoles modified the use of the vertical microhabitat, being more frequent at the surface.

These behaviours can be considered adaptive, considering that *Natrix maura* presents nocturnal and diurnal activity, and that this activity is developed by day mainly close to the substratum and at night mainly close to the margins (Scali *et al.*, 2001).

With these behavioural alterations, the tadpoles diminish the probability of being found in the same microhabitat that their natural predator. Similarly, Holomuzki (1986) verified that the larvae of *Ambystoma tigrinum nebulosum,* altered their microhabitat use when faced with a high risk of predation, and that the flexibility in the alteration of the microhabitat use depended on the intensity of predation risk.

Comparing the *Natrix maura* chemical cue with the *P. clarkii* chemical cue treatments, in the two series of experiences, the majority of the responses were qualitatively similar, as there were same-sign differences in the use of the water column, margins and the surface. However, the responses were always stronger to the chemical cue of *N. maura* than to the chemical cue of *P. clarkii.*

When the behavioural results were analyzed in the same group, we did not find differences between the control and the *P. clarkii* chemical cue treatments. However, if the data are analyzed without the *N. maura* treatment, the tests reveal differences between the control and the *P. clarkii* treatment (Gonçalves *et al, unpublished*). Therefore, probably the strong response to *N. maura* confounds the ability of the tests to detect the weaker responses to *P. clarkii.*

Comparing the crayfish chemical cue treatment in the two periods of observation, the behavioural responses of the tadpoles were a reduction of the activity and an increase of its permanence close to the substratum during the night.

It is in the nocturnal period that *P. clarkii* presents higher predatory activity (Correia, 1998), and the reduction in tadpole activity can thus diminish their vulnerability to predation and increase their probability of survival. However, simultaneously, their increased permanence close to the substratum during the night may increase their vulnerability to the crayfish. Many authors affirm that active tadpoles increase their meeting rate with predators and, in this way, their probability of capture (Skelly, 1994;

Laurila *et al,* 1997; Kiesecker and Blaustein, 1997; Nyström and Abjörnsson, 2000). Therefore, the reduction of the activity level is considered an adaptative behaviour, as it both reduces the probability of the prey to be detected, and diminishes the probability of the prey to occupy the immediate area where the predator is found (Lawler, 1989; Skelly and Werner, 1990; Werner, 1991; Skelly, 1992; Skelly, 1994).

The facts that the behavioural responses to the introduced predator were observed in nocturnal conditions and that the tadpoles presented a reduction of activity, suggest that the tadpoles recognize something strange in the environment when in presence of *P. clarkii,* and that their behavioural responses towards alterations of the environment include the diminution of activity.

In this sense, the tadpoles present already behavioural alterations in response to the exotic predator, but it remains to evaluate the degree in which that response confers them advantages or disadvantages.

The tadpoles already exhibited behavioural alterations in response to the exotic predator, but in this work we also verified that some of them were different from the ones adopted in response to a natural predator. Therefore, these tadpoles discriminate among predators and respond accordingly, as it was observed in other studies (Eklöv and Werner, 2000; Eklöv, 2000; Nyström and Abjörnsson, 2000; Relyea, 2001b; Van Buskirk, 2001, Teplisky *et al.,* 2005), where tadpoles expressed specific plastic responses to different predator types.

On the other hand, the increase of the use of the microhabitat "substratum", during the nocturnal period cannot be considered an antipredator behaviour, as it favours the predation strategy of crayfish, which is to constantly explore tactically the environment close to the bottom of the waterbodies, mainly under rocks (Amaral, *pers. obs.*). However, the presence near the substratum during the night may be related to feeding, and the tadpoles may be compromising between the need to feed themselves and the risk of predation.

It is easy to agree that the survival of prey is directly influenced by predation. However, survival can also be influenced, in an indirect way, by the antipredator behaviour developed as response to predation (Kiesecker and Blaustein, 1997). Identifying potential costs associated with prey responses to alarm cues contributes to a more accurate understanding of the effects of predators on prey populations and whole communities (Lima, 1998).

Experiences carried out in natural habitats with tadpoles of *Hyla versicolor* showed that in the not-lethal presence of the natural predator, *Ambystoma tigrinum*, the tadpole's behavioural response to the predator was to reduce the level of activity (Skelly, 1992), and this was associated with the reduction of the growth and development rates (Skelly, 1992; Nyström *et al.*, 2001). Similarly, when tadpoles alter the use of different microhabitats to avoid predation, as in the case of the *Alytes cisternasii* tadpoles of the present study, there is a reduction in the rate of food intake (Skelly, 1992; Holomuzki, 1986) that can decrease the rate of growth and the size at metamorphosis (Skelly, 1992; Kiesecker and Blaustein, 1997). This diminution of the size at metamorphosis may reduce the probability of the newly metamorphosed froglets to survive to the adult state (Smith, 1987). The tadpoles of *A. cisternasii*, by modifying the use of the microhabitat and moving away from its feeding places, generally close to the substratum, in the presence of *N. maura,* may also have a reduction in their rate of growth and size at metamorphosis.

Our results may give some insight into the evolution of antipredator behaviours in tadpoles of *A. cisternasii* facing new predators, what can be important to understand the impact that different predators have in the populations of this species. It is necessary, however to conduct similar experiment in the field in order to verify if, in natural conditions, the tadpoles of *A. cisternasii* exhibit the same type of behavioural responses that in aquaria. It would also be interesting to study if these responses imply costs to the fitness of the individuals.

The spread of exotic species is a global phenomenon that poses critical problems for many ecosystems. This study demonstrates the importance of understanding the behavioural and context-dependent responses of prey to predators, and how they can influence the outcome of interactions between species. To better understand the results presented by this population, it is necessary to know the behavioural responses of naïve tadpoles of *Alytes cisternasii* that never have been in contact with *P. clarkii*. Further work is of course needed to differentiate between individual experience and genetic factors in predator recognition by Iberic midwife toad tadpoles.

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The results obtained in this work show that *Alytes cisternasii* tadpoles presented seasonal and circadian differences in their behaviour. Some of these behavioural alterations were the result of the chemical stimulus of the exotic predator, *Procambarus clarkii,* but others can result from other exogenous or endogenous factors, such as the body size of tadpoles, developmental stage or water temperature.

When submitted to the chemical cue of *Procambarus clarkii,* the tadpoles presented a reduction of activity and an increase of the use of the marginal zones, during the night. These antipredator responses of the tadpoles were similar to those presented to their natural predator, *Natrix maura,* and apparently have an adaptive value, as they may protect the tadpoles from the predation strategy of this crayfish.

These results therefore suggest that *Alytes cisternasii* is already able to recognize and to develop specific strategies of defence against *P. clarkii* and that this process took place in about 30 years, since *P. clarkii* was introduced in Portugal. So, probably, this species will not be highly vulnerable to the impact that the crayfish can have on amphibian tadpoles.

However, field work will have to be carried out to verify if in natural conditions the tadpoles of *Alytes cisternasii* exhibit the same type of behavioural responses than in laboratory, as the quantification of prey responses to predator cues in a natural context may allow for the development of more realistic hypotheses regarding the complex effects of predators on prey species, including non-lethal effects.

Another suggestion to consider for the future would be to continue to test the ability of *Alytes cisternasii* to show other antipredator defences, such as modifications in life history or morphology, when submitted to the stimuli of *Procambarus clarkii.* It would also be interesting to evaluate the ability of other species of Southwest Iberian amphibians

apparently more vulnerable to the introduced predator (e.g. *Pleurodeles waltl* and *Hyla arborea*) (Cruz *et al.*, 2003), to develop such antipredator mechanisms.

To perceive the ability of the tadpoles to present antipredator defences and to know what these defences are may yet not be enough, as survival can also be influenced, in an indirect way, by the antipredator behaviour adopted in response to predation. As it was evidenced in some studies, the tadpole behavioural response to predators may be to reduce the level of activity or to increase the permanence in a refuge (Skelly, 1992; Lima, 1998). In these cases, there is a reduction in the rate of food intake (Skelly, 1992; Holomuzki, 1986), than can decrease the rate of growth and the size at metamorphosis (Skelly, 1992; Kiesecker and Blaustein, 1997).This diminution of the size at metamorphosis reduces the probability of the newly metamorphosed froglets to survive up to the adult state (Smith, 1987). Thus it would be interesting to evaluate the indirect effects of the antipredator defences, in the tadpoles of *A. cisternasii*.

A relatively important aspect in the analysis of the antipredator behaviour towards an introduced predator is the comparative examination of the behaviour of different populations, since this can provide important insights on their history of contact with the predator. In order to better understand the results presented by this population, it will be necessary to study the behavioural responses of naïve tadpoles of *Alytes cisternasii* that never have been in contact with *P. clarkii*. Further work is therefore needed to differentiate between individual experience and genetic factors in predator recognition by Iberic midwife toad tadpoles.

To recognize and understand the antipredator mechanisms of larval amphibians can be important to know the impact of predation and thus to plan conservation measures for these species. Considering the known impacts of *P. clarkii* on all the Southwest Iberian amphibians, associated with the investigations of the antipredator defences here presented, it is necessary to propose and apply crayfish management measures in order conserve amphibians.

Some biologists have suggested that eliminating exotic predators from habitats in which they are established is not necessarily a good idea (Hayes and Jennings, 1986). The best solution would be to increase and preserve the number of aquatic habitats that are free of exotic predators. This objective would be accomplished most effectively by two approaches: provide for more careful regulation of the spread of introduced species and propose land management decisions that include purchasing and preserving habitats free of exotic predators.

Because exotic species are usually found in permanent waters, some authors suggested more emphasis on protecting ephemeral habitats for amphibians (Adams, 1999).

According to Cruz *et al.* (2004) by utilizing knowledge of the locomotion abilities of *Procambarus clarkii*, it would be possible to create physical barriers to protect against the colonization of ephemeral and isolated water bodies, thereby protecting these important habitats from crayfish invasion.

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Table I.A1. Results of the Levene test used to test the assumption of homogeneity of variances of the spring and winter data, for the head length and developmental stage of the tadpoles (significant P values at alpha < 0.05 are indicated by *).

	Control		
	df	F	P
Tadpoles head lenght (mm)	8,36	1.348	0.252
Developmental stage	10,34	4.739	0.000291*
	Chemical cue of *P. clarkii*		
	df	F	P
Tadpoles head lenght (mm)	9,40	2.821	0.0115*
Developmental stage	6,43	3.61	0.0055*

Table II.A1. Results of the Levene test used to test the assumption of homogeneity of variances of the nocturnal and diurnal observations, for the three treatments (* significant P values at alpha < 0.05).

Treatment	Parameter	$F_{(1,28)}$	P
	Refuge use	4.773	0.281
	Activity	2.598	0.115
Control	Water column	0.928	0.485
(a)	**Substratum**	**5.787**	**0.012***
	Surface	2.094	0.164
	Margins use	2.151	0.183
	Refuge use	**5.787**	**0.012***
Chemical	Activity	0.363	0.557
cue	Water column	3.405	0.067
P. clarkii	Substratum	1.305	0.383
(b)	Surface	0.514	0.811
	Margins use	**5.607**	**0.015***
	Refuge use	1.356	0.336
Chemical	Activity	2.308	0.142
cue	Water column	0.909	0.467
N.maura	Substratum	2.487	0.197
(c)	Surface	1.029	0.516
	Margins use	1.146	0.417

Table II.A2. Results of Levene test utilized to test the assumption of homogeneity of variances for the univariate ANOVA that compares the results obtained during the day/night for the treatments control, chemical cue of *P. clarkii* and chemical cue of *N. maura* (* significant P values at alpha < 0.05).

Series	Parameter	$F_{(2,42)}$	P
Day	Refuge use	1.700	0.230
	Activity	**4.695**	**0.049***
	Water column	1.789	0.209
	Substratum	2.186	0.178
	Surface	—	—
	Margins use	1.734	0.223
Night	Refuge use	2.182	0.152
	Activity	3.600	0.596
	Water column	1.586	0.249
	Substratum	—	—
	Surface	3.214	0.106
	Margins use	2.304	0.136