UNIVERSIDADE DE LISBOA
FACULDADE DE CIÊNCIAS
DEPARTAMENTO DE BIOLOGIA ANIMAL

Expansion of an invasive crayfish and decline of two native amphibians in the Grândola mountain range

Bruno Miguel Calhau Correia Pinto

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Professor Doutor Rui Rebelo

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“Tá-se bem no campo”

- Autor desconhecido

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Resumo

A biodiversidade está mais ameaçada que nunca, com um elevado número de espécies ameaçadas de extinção. A primeira fase no processo de extinção é a ocorrência de declínios populacionais que, caso não sejam revertidos, levam à extinção local das populações e, consequentemente, à alteração da sua distribuição. Uma das principais ameaças à biodiversidade é causada pelas espécies invasoras. Estas são definidas como espécies não-nativas que estabelecem populações capazes de produzir indivíduos dispersores, e com grande impacto nos ecossistemas invadidos. Uma das espécies invasoras de água doce com mais impactos descritos é o lagostim vermelho do Louisiana (*Procambarus clarkii* Girard, 1852), um crustáceo omnívoro e generalista, nativo do sudeste dos Estados Unidos da América e com alta capacidade de adaptação a diversas condições ecológicas. Apesar de preferir corpos de água permanentes, mais profundos e com condições mais estáveis, consegue invadir locais mais temporários e desfavoráveis. Este lagostim causa impactos fortes nas populações de anfíbios nativas, maioritariamente através da predação dos seus ovos e estádios larvares. Na Serra de Grândola, *P. clarkii* está presente desde o final do século XX. Nessa época, as duas espécies de anfíbios mais abundantes eram o sapo-parteiro-ibérico (*Alytes cisternasii* Boscá, 1879) e a salamandra-de-pintas-amarelas (*Salamandra salamandra* Linnaeus, 1758). Com o aumento dos avistamentos de *P. clarkii* e a recolha de cada vez menos provas de reprodução de *A. cisternasii*, este trabalho tem o objetivo de confirmar a expansão da espécie invasora e o declínio das espécies nativas, assim como analisar que variáveis ambientais influenciam a expansão do lagostim invasor entre 2000 e 2021. Há também uma tentativa de inferir que condições ambientais influenciam a permanência de cada espécie nativa neste período.

Para isso, indivíduos de lagostim e estádios larvares de ambas as espécies de anfíbio foram amostradas em 85 pegos distribuídos por duas ribeiras principais permanentes e quatro ribeiras secundárias temporárias ao longo de três sessões, entre Fevereiro e Abril de 2021, com recurso a passagens de camaroeiro na primeira sessão e a pesca elétrica nas seguintes. Diversas caraterísticas ambientais de cada pego foram recolhidas e a distância ao curso de água principal foi estimada. Os resultados, expressos como presencia/ausência foram comparados com dados de 2000. Foram utilizados testes de Mcnemar para inferir se a distribuição presente (2021) de cada espécie é significativamente diferente da sua distribuição passada (2000). Usando o *software* R, construímos modelos lineares generalizados com o objetivo de perceber que fatores influenciam a distribuição atual de *P. clarkii*. Foi utilizada a função *dredge* do *package* MuMin para modelar todas as combinações possíveis dos fatores ambientais considerados significantes. Aos melhores modelos resultantes ($\Delta AIC < 2$) foi aplicada a função *model average* do mesmo *package* para obter o modelo final que explica a variável dependente. O mesmo processo foi aplicado para a expansão do lagostim, utilizando a expansão para novos locais ou a manutenção da presença no local em 2021 como variável dependente; e para a manutenção ou desaparecimento de cada espécie de anfíbio entre 2000 e 2021.

As distribuições das três espécies estudadas mudaram significativamente entre 2000 e 2021, verificando-se a expansão do lagostim invasor para ribeiras secundárias e zonas mais altas das ribeiras principais, aumentando o número de pegos ocupados de 40 em 2000 para 51 em 2021. Verificou-se também o declínio coincidente de ambas as espécies de anfíbios nativos, com a queda do número de pegos onde *A. cisternasii* e *S. salamandra* estão presentes de 57 e 45 em 2000 para 13 e 31, respetivamente, em 2021. A expansão de *P. clarkii* é justificada pela sua grande capacidade de adaptação e tolerância, permitindo que invada locais com condições menos favoráveis. No entanto este invertebrado não foi encontrado em algumas ribeiras secundárias, provavelmente devido ao seu hidroperíodo mais curto. *A. cisternasii* sofreu um declínio generalizado, resistindo apenas em treze pegos, maioritariamente nas ribeiras principais, muito provavelmente devido ao aumento da pressão predatória do lagostim invasor, levando ao desaparecimento nos seus habitats de reprodução. As exceções corresponderão a pegos com

condições ideais para a sua sobrevivência ou para a expressão adequada de estratégias antipredatórias. Os girinos desta espécie têm uma elevada capacidade de fuga e são capazes de adaptar o seu comportamento para responder ao lagostim invasor, evitando-o. *S. salamandra* sofreu um declínio pequeno e localizado, desaparecendo essencialmente das ribeiras principais e mantendo a sua distribuição nas ribeiras secundárias, mesmo na maioria das secções invadidas pelo lagostim, indicando uma maior capacidade de coexistência com o invasor. Esta espécie tem também caraterísticas morfológicas e comportamentais que promovem a coexistência com a espécie invasora, como um período larvar curto e ovoviviparidade.

A expansão do lagostim nos últimos 21 anos foi direcionada para pegos mais rasos e de menores dimensões com menos manta morta no fundo, condições desfavoráveis à sua sobrevivência. Para além destes pegos manterem menos indivíduos, a manta morta funciona como refúgio e fonte de alimento. A expansão foi também influenciada negativamente pela cobertura arbustiva nas margens, em pegos com maior cobertura arbórea coincidentes com regiões intermédias das ribeiras secundárias. Diversas interações destas variáveis são também dadas como importantes para a expansão. No entanto, a falta de significância de variáveis no modelo final e o elevado número de interações incluídas revelam que o ajuste é fraco. Isto pode resultar da não preferência de *P. clarkii* por condições ambientais específicas, tentando invadir todos os locais disponíveis na área de estudo.

Em 2021, os girinos de *A. cisternasii* resistem em pegos largos com elevada cobertura de vegetação emergente e margens dominadas por herbáceas e pouca cobertura arbustiva. A maior largura e cobertura do pego por vegetação emergente definem locais mais amplos e com disponibilidade de refúgios, facilitando a evitação espacial de predadores. As margens abertas e dominadas por herbáceas são o habitat preferencial dos indivíduos adultos, que poderão beneficiar da proximidade ao pego para a deposição dos seus ovos. As interações significativas salientam a importância destes habitats terrestres circundantes para a manutenção de *A. cisternasii* na área de estudo.

A manutenção da distribuição de *S. salamandra* em 2021 foi influenciada negativamente pelo comprimento do pego e percentagem do fundo coberta por pedra e cascalho. Tanto quanto sabemos, nenhuma destas características é evitada por esta espécie, sendo substratos mais grosseiros até usados como refúgio. Estas condições são, no entanto, consideradas favoráveis para *P. clarkii*. Tendo em conta que substratos grosseiros podem ser benéficos para ambas as espécies por providenciarem refúgio, o urodelo pode deixar de usar estes microhabitats devido à ocupação pelo predador invasor.

É possível que outros fatores não avaliados neste estudo, tais como a variação intra e interanual das populações, as alterações climáticas e as doenças, tenham contribuído para a alteração da distribuição das espécies consideradas. São necessários estudos que avaliem a influência destes fenómenos na distribuição das espécies-foco deste trabalho.

Este trabalho confirma a expansão do lagostim vermelho do Louisiana na Serra de Grândola para as ribeiras secundárias a montante, com condições mais desfavoráveis à sua sobrevivência. Adicionalmente, confirma também o declínio de ambas as espécies de anfíbios, com a restrição de girinos de *A. cisternasii* a pegos específicos com condições favoráveis nas ribeiras principais, e a manutenção de larvas de *S. salamandra* em pegos das ribeiras mais temporárias. A invasão de locais com condições mais desfavoráveis realça a capacidade de invasão e adaptação de *P. clarkii*, sugerindo uma trajetória de invasão generalista e influenciada principalmente pela distância aos cursos de água principais. O sapo-parteiro-ibérico mostra preferência por pegos permanentes localizados nas ribeiras principais para o desenvolvimento larvar, contrastando com a salamandra-de-pintas-amarelas que parece preferir pegos temporários nas ribeiras secundárias. Com a expansão de *P. clarkii* para os pegos a montante, a sua pressão predatória irá aumentar, prevendo uma restrição do urodelo às secções mais

elevadas e não invadidas. Os nossos resultados realçam o impacto das espécies invasoras nos ecossistemas nativos e a importância do seu controlo de modo que sejam minimizados os seus impactos e prevenidos futuros declínios. Realçamos também a urgência de conservar o habitat favorável à reprodução de *A. cisternasii*, incluindo as zonas abertas circundantes, assim como as ribeiras temporárias com importância para a reprodução de *S. salamandra*.

Palavras-Chave: *Procambarus clarkii*; *Alytes cisternasii*; *Salamandra salamandra*; Espécie invasora; Alteração de distribuição

Abstract

Biodiversity is more threatened than ever with Invasive Alien Species (IAS) being one of the most important drivers of population declines and extinctions. One of the most widespread and harmful freshwater IAS is the red swamp crayfish (*Procambarus clarkii*), a generalist and highly adaptive species that can establish in a wide range of water bodies with different conditions. This invasive crayfish has severe impacts on native communities, particularly on amphibians, by predating their eggs and larvae. In the Grândola mountain range, the expansion of crayfish in the last 30 years has been accompanied by decreases in the sightings of the two previously most abundant species of amphibians - Iberian midwife toad *Alytes cisternasii* and fire salamander *Salamandra salamandra*. From February to May 2021, we sampled 85 stream pools spread across two main permanent streams and four secondary temporary streams. On three sessions, and using dipnet sweeps and electrofishing, we sampled crayfish and amphibian larvae, in order to compare their presence along the river network with data registered in the year 2000. We also registered stream pool characteristics to estimate the abiotic factors influencing crayfish present distribution and expansion, as well as those influencing the maintenance of each amphibian species. Our results show a clear upstream expansion of *P. clarkii* to secondary streams, accompanied by a catastrophic decline in *A. cisternasii*, and a localized and smaller, though significant, decline in *S. salamandra*. These changes suggest that red swamp crayfish is the main cause of these declines. Coexistence probabilities between these species changed: the invader distribution in 2021 was favoured by wider and heavily shaded pools with high stone cover. According to our model, this species expanded to smaller and shallower pools with less shrub cover on the banks and less leaf litter, characteristics consistent with intermediate pools in secondary streams. *A. cisternasii* tadpoles only remain in wider stream pools highly covered by emergent vegetation near favourable adult habitats, on the main streams. These conditions may promote predator avoidance, allowing for coexistence. Smaller pools with finer substrates, characteristic of secondary streams, maintained *S. salamandra* larvae. Although other reasons for the declines still need to be excluded, *P. clarkii* seems to heavily impact these native amphibians, highlighting the need for conservation measures to be taken in order to mitigate the current impacts and prevent further expansion into critical habitats for amphibian reproduction.

Keywords: *Procambarus clarkii*; *Alytes cisternasii*; *Salamandra salamandra*; Distribution changes; Pool characteristics

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1. Introduction

1.1 Threats to biodiversity

Biodiversity is more threatened than ever, with many species facing the danger of extinction (Barnosky et al., 2011; Ceballos et al., 2020, 2015; Nic Lughadha et al., 2020; Sánchez-Bayo and Wyckhuys, 2019). Current extinction rates are many times superior to those before the evolution of humans, leading the scientific community to believe that a sixth mass extinction event is occurring (Barnosky et al., 2011; Ceballos et al., 2015; Le Roux et al., 2019; McCallum, 2015). Population declines are the first step in the process of extinction and tend to be an indicator of how threatened a species is (Ceballos et al., 2017). If not reversed, population declines lead to local extinctions that will alter a species' distribution and possibly influence its ecological role (Ceballos et al., 2017). This is happening to rare species as well as to common and widespread species, and across multiple different taxa, with important consequences to the ecosystem services that they provide (Bonardi et al., 2011; Ceballos et al., 2020, 2017; Gaston and Fuller, 2008; Inger et al., 2015; Petrovan and Schmidt, 2016; Sánchez-Bayo and Wyckhuys, 2019). The declines of common species can negatively affect ecosystem health because these constitute a high percentage of biomass necessary for correct ecosystem functioning (Gaston and Fuller, 2008).

Several factors can contribute to population declines, acting at different scales and intensities for different species and for populations of the same species (Blaustein et al., 2011; Grant et al., 2016). With several factors influencing a decline, it can be difficult to pinpoint the most impactful threats in each situation (Bonardi et al., 2011; Grant et al., 2016). Nowadays, the main drivers for vertebrate population declines are climate change, habitat loss and fragmentation, overexploitation of wild populations, pollution and the introduction of invasive species (Blaustein et al., 2011; Ceballos et al., 2020; Sánchez-Bayo and Wyckhuys, 2019).

1.2 Invasive Species

Invasive alien species (IAS) can be defined as the subset of non-native species that were able to establish breeding populations capable of dispersion and with negative ecological, economic and/or health related impacts in the invaded range (Blackburn et al., 2011; Essl et al., 2018; Perkins et al., 2011; Pyšek et al., 2020). IAS are one of the major causes for population declines and extinctions worldwide (Bellard et al., 2016; Blackburn et al., 2019). They are the most common driver for animal and plant extinctions (Blackburn et al., 2019) and are projected to increase in the future (Seebens et al., 2021, 2017). IAS are a human created problem potentialized by globalization, human history and culture, and increasing trade, the latter being the most important pathway for new invasions (Early et al., 2016; Seebens et al., 2018; Turbelin et al., 2017). Major independent and external influences (like climate change) also impact the relations between IAS and native ecosystems, potentiating new invasions or aggravating effects of already established non-native species (Carreira et al., 2017; Perkins et al., 2011). The invasion process has different stages (transport, introduction, establishment and spread) (Blackburn et al., 2011). Several successful IAS (i.e. species that reach the final stage) are highly adaptable generalists that can thrive in multiple environments (Carreira et al., 2017; Lodge et al., 2012; Lowe et al., 2000)

Invaders can affect native species directly (Falaschi et al., 2020), outcompeting them for resources (Carmo et al., 2018; Damas-Moreira et al., 2020; Zwerschke et al., 2018), hybridizing with closely related species (Hirashiki et al., 2021; Mesgaran et al., 2016) or, most commonly in animal species, predating on taxa occupying a lower trophic level (Gurevitch and Padilla, 2004). Indirect effects on native species can also occur (Falaschi et al., 2020) by being potential vectors for pathogens and diseases (Najberek et al., 2022; Oficialdegui et al., 2019b) or changing the invaded ecosystem (Walsh et al., 2016). An IAS may even promote the invasion of other species (Simberloff and Von Holle, 1999). They can also have a significant direct impact on mankind by altering ecosystem services (Ogden et al., 2019;

Paini et al., 2016; Walsh et al., 2016) and are associated with increasingly higher economic costs (Cuthbert et al., 2022; Diagne et al., 2021). The impact of IAS depends on the trophic level and abundance in relation to the native species, as well as the stage of the invasion process (Blackburn et al., 2011; Bradley et al., 2019; Robertson et al., 2020) and can take some time to become noticeable (Crooks, 2005).

1.3 The invasive crayfish

Freshwater ecosystems are particularly threatened by IAS due to the easiness of dispersion of individuals (Sala et al., 2000). Aquatic invertebrates, especially freshwater crayfishes, are some of the most widespread invasive taxa (Lodge et al., 2012; Loureiro et al., 2015). The red swamp crayfish (*Procambarus clarkii* Girard, 1852) is a North American crayfish species that has invaded every continent except Antarctica and Oceania (Lodge et al., 2012; Loureiro et al., 2015). Its establishment in non-native areas was due to intentional release or accidental escape from farming, particularly for human consumption (Lodge et al., 2012; Loureiro et al., 2015; Oficialdegui et al., 2019a). This species is considered the most invasive crayfish in the world with many impacts to trophic webs and environmental conditions in its invaded range (Cruz et al., 2008, 2006; Souty-Grosset et al., 2016; Twardochleb et al., 2013). Although it prefers lotic and stable watercourses with extended hydroperiods, it is a highly adaptable generalist (Ficetola et al., 2012a; Gherardi, 2006; Loureiro et al., 2018) that can survive in shallower and more temporary habitats (Alcorlo et al., 2009; Bernardo et al., 2011; Cruz and Rebelo, 2007; Ficetola et al., 2012a). Due to its high abiotic tolerance, this invader is able to efficiently disperse through water or over dry land (Banha and Anastácio, 2014; Bernardo et al., 2011; Cruz and Rebelo, 2007; Dörr et al., 2020; Kerby et al., 2005) or to build burrows to take refuge and endure unfavorable conditions (Barbaresi et al., 2004b). Besides its high phenotypic plasticity, it is also a prolific and adaptable breeder, able to achieve high densities and adapt its life cycle to the environmental conditions of its invaded range (Alcorlo et al., 2009; Chucholl, 2011). As an omnivore, it feeds on aquatic invertebrates and particularly amphibian eggs and larvae, as well as on plant matter and detritus (Carreira et al., 2017; Correia, 2003). Juveniles tend to be more carnivorous and adults more herbivorous and detritivorous (Correia, 2003). All these characteristics culminate in its success as an IAS with very significant impacts on native amphibians through predation of their larval stages and eggs, with local species extinctions documented (Cruz et al., 2008, 2006; Nunes et al., 2010). *P. clarkii* can also act as a vector and reservoir for pathogens that affect native taxa (Mazza et al., 2018; Oficialdegui et al., 2019b), as well as an ecosystem engineer, altering environmental conditions in its favor (e.g. burrowing) (Barbaresi et al., 2004a, 2004b). External factors like climate change might alter the impact of *P. clarkii* in the future, changing its diet and favoring an increase in its distribution (Bellard et al., 2013; Carreira et al., 2017).

1.4 Amphibians: reasons for their decline

One of the main consequences of *P. clarkii* invasion is its impacts in native amphibians (Cruz et al., 2008, 2006). Amphibians are the Earth’s most endangered group of vertebrates, with about 32,5% of the world’s species threatened with extinction (Stuart et al., 2004). Being ectothermic animals, this taxa has a high dependence on environmental conditions to survive and reproduce, such as comfortable temperatures and high humidity levels (Blaustein et al., 1994). Amphibians have complex life cycles, going through a transition between ecologically distinct habitats - terrestrial and aquatic – usually marked by metamorphosis (Blaustein et al., 1994). All these characteristics, together with their tendency to show philopatry, increase extinction risk by limiting the ability to recolonize prior-occupied areas (Blaustein et al., 1994). The main factor contributing to amphibian declines is habitat destruction but this group is also threatened by pollution, climate change, invasive species, overexploitation and, particularly, pathogens (Blaustein et al., 2011; Bucciarelli et al., 2014; Collins, 2010; Nyström et al., 2007; Pounds et al., 2006). *Batrachochytrium dendrobatidis* (Bd) and *B. salamandrivorans* (Bsal) (fungus responsible for the disease chytridiomycosis) as well as different *Ranavirus* sp. (viruses that affect ectothermic vertebrates) are amphibian pathogens responsible for declines and local extinctions around the world (DiRenzo and Campbell Grant, 2019; Duffus et al., 2015; Skerratt et al., 2007). Temperature and humidity shifts, besides altering environmental conditions and promoting changes in distributions (Enriquez-Urzelai et al., 2019), may favor the incidence and magnify the effects of these pathogens, leading to populations declines (Cohen et al., 2019a, 2019b; Pounds et al., 2006); these changes in environmental conditions are caused by climate change, another major threat to amphibian populations. Invasive species constitute a major threat for this group (Falaschi et al., 2020), given their ability to impact amphibians in multiple ways. In this work, we will try to relate the spread of an invasive species with the decline of two native amphibians.

1.5 The two native amphibians

The Iberian midwife toad (*Alytes cisternasii* Boscá, 1879) is a small semi-fossorial anuran endemic to the Iberian Peninsula. Its distribution is restricted to southern and eastern Portugal, and the central and western portion of Spain (IUCN SSC Amphibian Specialist Group, 2020; Maravalhas and Soares, 2017). It is associated with warm cork oak (*Quercus suber*) and holm oak (*Quercus ilex*) woodlands with a low-density shrub layer, as well as open meadows (Correia and Santos-Reis, 1999; IUCN SSC Amphibian Specialist Group, 2020; Rebelo and Crespo, 1999). This species is a nocturnal carnivore, feeding on a wide variety of terrestrial invertebrates (Maravalhas and Soares, 2017). It can remain inactive for long periods of time while waiting for favorable environmental conditions. There may be two reproductive peaks – one in autumn and other in spring (Michaels et al., 2016; Rebelo and Crespo, 1999). After a complex amplexus culminating in external fertilization, the males of this species carry the eggs in their hind legs during the incubation period, being able to carry the eggs of multiple females (Maravalhas and Soares, 2017; Marquez and Verrell, 1991). After an incubation period of about 3 weeks, males travel to a watercourse where deposition of eggs and synchronized hatching of tadpoles occurs (Maravalhas and Soares, 2017; Marquez, 1992). This species can use watercourses with a wide variety of characteristics for egg deposition but tends to prefer stream habitats (IUCN SSC Amphibian Specialist Group, 2020; Rebelo and Crespo, 1999). Tadpoles can reach 70 mm long and take several months to complete metamorphosis (Almeida et al., 2001; Ribeiro and Rebelo, 2011). During this stage, diet consists of plant matter and aquatic invertebrates (Almeida et al., 2001). They are preyed upon by invasive carayfish, salamander larvae, water snakes and freshwater invertebrates (Almeida et al., 2001). One of the main threats for this Iberic endemism is predation by invasive species, namely *P. clarkii*, and loss of terrestrial and aquatic habitat (Gonçalves et al., 2011; IUCN SSC Amphibian Specialist Group, 2020). This species might also be susceptible to infection by Bd. (Baláž et al., 2014; IUCN SSC Amphibian Specialist Group, 2020; Rosa et al., 2013).

In contrast with *A. cisternasii*, the fire salamander (*Salamandra salamandra* Linnaeus, 1758) is a common European urodele widespread throughout the Portuguese mainland and characterized by its black and yellow adult coloration (IUCN SSC Amphibian Specialist Group, 2022; Maravalhas and Soares, 2017). It prefers wet and cold forest habitats with proximity to streams and shelter, but can tolerate less than ideal conditions such as more urban areas (Burgstaller et al., 2021; IUCN SSC Amphibian Specialist Group, 2022; Kiss et al., 2022). This species is a crepuscular and nocturnal carnivore, feeding on a wide variety of terrestrial invertebrates (Maravalhas and Soares, 2017; Marques et al., 2022). Like *A. cisternasii*, it may have two activity peaks – one in autumn and one in spring (Maravalhas and Soares, 2017) – but this is dependent of abiotic factors like humidity and temperature (Degani and Warburg, 1978). Activity tends to be related with reproduction, culminating with the deposition in streams, ponds or still waters of fully developed larvae (Almeida et al., 2001; IUCN SSC Amphibian Specialist Group, 2022; Maravalhas and Soares, 2017; Rebelo and Crespo, 1999). At this stage, diet consists of aquatic invertebrates (Almeida et al., 2001; Degani, 2013). *S. salamandra* larvae can be preyed upon by water snakes, invertebrates and aquatic birds, fish and, even, conspecifics (Almeida et al., 2001). It can take from about one to several month in the aquatic habitat to achieve full metamorphosis and become terrestrial (Almeida et al., 2001; IUCN SSC Amphibian Specialist Group, 2022). This species is mainly threatened by habitat destruction, pollution, collection and invasive predators of its larvae (e.g., fish and *P. clarkii*) (IUCN SSC Amphibian Specialist Group, 2022). Recently, chytridiomycosis caused by *Batrachochytrium salamandrivorans* has been responsible for population declines and local extinctions in several parts of Europe (IUCN SSC Amphibian Specialist Group, 2022). This fungus is particularly deadly for *S. salamandra* and its spread could become an even bigger threat (Martel et al., 2014).

1.6 Objectives and predictions

This research was motivated by the decrease in the frequency of sightings of *A. cisternasii* at the Grândola mountain range, matched with the increasing sightings of *P. clarkii*, which hints at a possible expansion (Rui Rebelo personal comm.). Our objective is to study the possible impact of invasive *P. clarkii* in *A. cisternasii* and *S. salamandra* populations at the Grândola mountain range, comparing their distributions along the main streams between 2000 (early stage of *P. clarkii* invasion) and 2021 (late stage of *P. clarkii* invasion). We also analyze what abiotic factors influence the 2021 distribution of *P. clarkii* and what factors influence the change in distribution of each species - expansion of invader and maintenance of natives - as we expect the invasive species to expand its distribution into less favorable habitats due to its adaptive generalist behavior. In contrast, we anticipate that both native amphibians have suffered a decline, remaining in non-invaded stream pools with near optimum conditions. So, this work aims to answer the following questions:

1) Did the analyzed species distributions in the study area change from 2000 to 2021?

2) Did the distributions change in relation to the distance to the main, permanent, water course?

3) What factors influence *P. clarkii* present distribution?

4) What factors influence *P. clarkii* expansion between 2000 and 2021?

5) What factors influence *A. cisternasii* maintenance between 2000 and 2021?

6) What factors influence *S. salamandra* maintenance between 2000 and 2021?

2. Methods

2.1 Study Area

This study was conducted in the Grândola mountain range, Baixo Alentejo, Portugal. This mountain range extends for 8113 km^2 and is subject to a Mediterranean climate with some Atlantic influence, characterized by warm and dry summers and rainy winters (Correia and Nisa, 1999; Correia and Santos-Reis, 1999; Costa et al., 2010a). The mean annual temperature for the area is 17° C with rainfall values varying between 600mm in the lowlands and 1050mm on the hilltops (Fernandez et al., 2020). Smaller streams only maintain water flow in the wet and rainy season (usually from October to May), drying in late spring (Reis, 2019). The (litho)soils are mostly composed of schists with low permeability (Correia and Santos-Reis, 1999; Costa et al., 2010a; Fernandez et al., 2020). All sampled water courses are part of the Grândola stream watershed, which in turn belongs to the Sado watershed.

We mainly focused on the Ribeira Abaixo homestead (HRA), where the field station of the Faculty of Science of the University of Lisbon/ Centre for Ecology, Evolution and Environmental Changes (cE3c) is situated, as well as the surrounding areas. This 221 ha homestead is located 7 km south of the town of Grândola and is one of the sites of the LTsER Montado Platform. Several intermittent streams cross the property, flowing into the main Castelhanos stream, which is the only permanent watercourse and the eastern limit of the homestead (Correia and Santos-Reis, 1999; Rebelo et al., 2009). This streamflow is dependent on precipitation levels and varies throughout the year. In the winter months, it can have high streamflow; in the warmer months, the water level is significantly reduced, sometimes drying out in certain sections (Reis, 2019). After flowing north past the homestead, it converges with the permanent Cortilhões stream into the Grândola stream, the most probable source of the *P. clarkii* invasion (Cruz and Rebelo, 2007).

Sampling for this work included the small temporary streams in and around HRA as well as the permanent Castelhanos and Cortilhões streams. Secondary streams are labeled as presented in Figure 1: Secondary stream 1 (SS1); Secondary stream 2 (SS2); Secondary stream 3 (SS3); Secondary stream 4 (SS4). SS2 and SS4 have a shorter hydroperiod than SS1 and SS3.

Figure 1 - Study area with the location of the most important watercourses

The HRA landscape is an adequate representation of the Grândola mountain range landscape (Rebelo et al., 2009). The homestead is mostly dominated by Montado, a semi-natural and human-dependent agroforestry system of cork oak (*Quercus suber*) woodlands. It is typically characterized by a sparse and open tree layer (mainly *Q. suber* e *Quercus ilex subsp. rotundifolia*) and an understory layer composed of *Cistus spp.* dominated shrublands and/or herb-dominated pastures (Correia and Nisa, 1999; Correia and Santos-Reis, 1999; Rebelo et al., 2009). There are riparian galleries with different degrees of development along the streams (Correia and Nisa, 1999).

Climate change affects the study area, promoting changes in landscape due to the increase in frequency and intensity of extreme climate phenomena (Correia and Santos-Reis, 1999; Lionello et al., 2014; Lionello and Scarascia, 2018; Safford and Vallejo, 2019). In addition, *Q. suber* has been experiencing a decline in the Montado ecosystem of central and south Portugal in the last two decades due to environmental and anthropogenic factors such as drought and land abandonment (Costa et al., 2010b; Rebelo et al., 2009). Furthermore the area has been experiencing an increase in abnormally dry years when the dry season lasts until January (IPMA, 2022; Nunes and Lourenço, 2015).

According to Rebelo & Crespo (1999), in the late 20th century *A. cisternasii* and *S. salamandra* were the most abundant amphibian species in the HRA, being widely spread throughout the area. After invading the southeastern part of Portugal in the 1990s (Cruz and Rebelo, 2007), *P. clarkii* was already present in the Castelhanos stream in 1999, coexisting with *A. cisternasii* tadpoles. Invaded streampools already had less tadpoles than non-invaded streampools (Rebelo and Crespo, 1999). After the Autumn reproductive event, *A. cisternasii* tadpoles could be found in all waterbodies in the homestead except those with smaller dimensions, shallower water level and shorter hydroperiod. Like *A. cisternasii* tadpoles, *S. salamandra* larvae were present in all waterbodies in HRA, but unlike the toad, these larvae could even be found in the most temporary pools.

2.2 Sampling

2.2.1 Animal capture and handling

We sampled 85 different stream pools along 3 sessions between February and April 2021. Sampled stream pools are presented in Figure 2. The 1st session lasted from 16/02/2021 to 02/03/2021 and sampling consisted of dipnet (1mm mesh) sweeps for a minimum of 10 minutes. For the 2nd and 3rd session (18/03/2021-23/03/2021 and 11/04/2021-16/04/2021 respectively) we used electrofishing for at least 5 minutes, aided with dipnet sweeps. All *P. clarkii*, *A. cisternasii* and *S. salamandra* individuals seen but not caught were registered as well. GPS coordinates for all sampling points were gathered using a *Garmin eTrex10* GPS. Capture and handling of all animals in this study was conducted under the 719/2021/CAPT license. Because *P. clarkii* is an invasive species, all sampled individuals were euthanized.

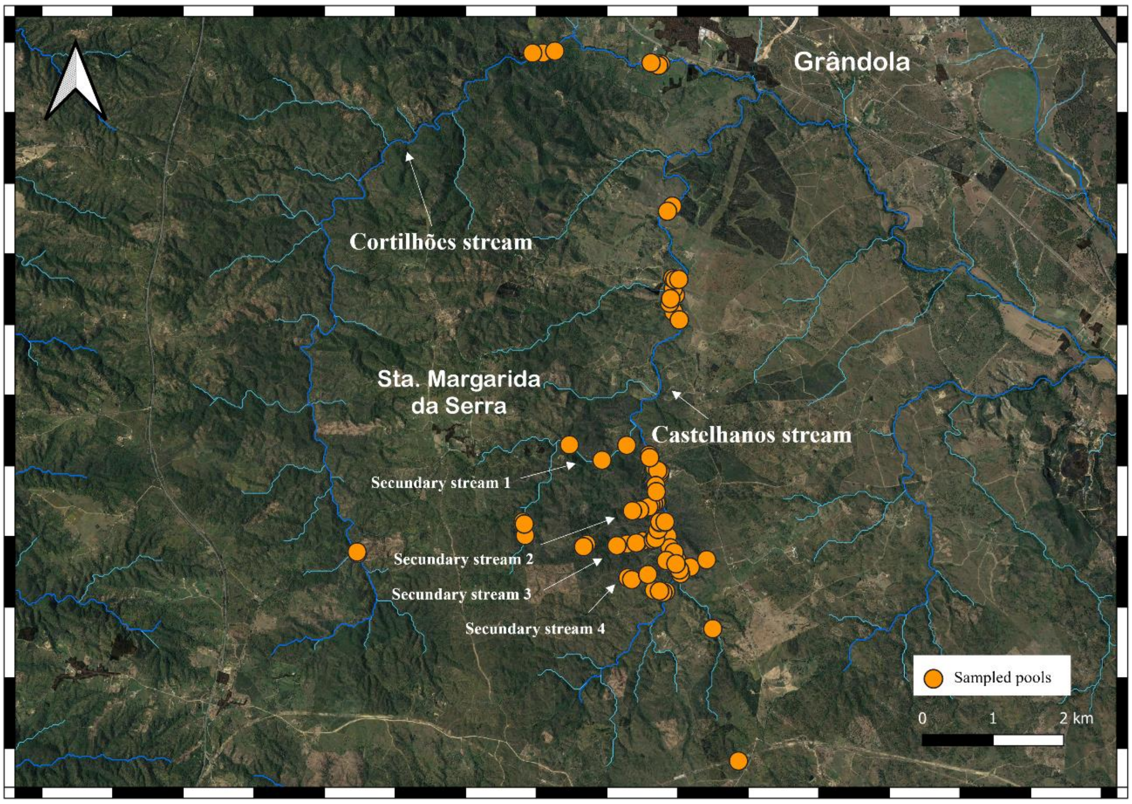

Figure 2 - Map of all sampled stream pools

2.2.2 Data collection of abiotic variables

2.2.2.1 Physical variables

In each stream pool, we measured several physical variables such as length, width (both in meters), as well as depth (cm) of 5 different points spread throughout the pool. Waterflow was estimated by dropping a dry leaf as close as possible to the middle of the pool (so that the pool margins have the least possible influence on the measurement) and recording how long it took to move a known distance. Water temperature was also recorded using a YSI 556 MPS temperature probe. All variables were measured at first arrival in each stream pool.

2.2.2.2 Margin variables

We defined the margin as the 10-meter area adjacent to each side of the stream pool. We recorded several habitat characteristics on each margin. Tree, shrub and herb cover were estimated by visual assessment, following the guidelines of (Direcção de Unidade de Gestão Florestal, 2009). The margin slope was also categorized visually in a 0-2 scale: 0 corresponded to flat margins, while those categorized with 1 and 2 had a gentle and steep slope, respectively.

2.2.2.3 Landscape variables

We defined the landscape of the surroundings of each stream pool as the 100-meter radius area adjacent to it and estimated tree and shrub cover using the same methodology mentioned before (Direcção de Unidade de Gestão Florestal, 2009).

2.2.2.4 Aquatic vegetation variables

We estimated percent cover of the stream pool by 3 types of aquatic vegetation: underwater vegetation, emergent vegetation and margin vegetation. These variables were estimated using a 2500 cm^2 quadrat divided in 25 squares, each with 100 cm^2. Cover of each category was calculated by the percentage of squares where it was present. In large stream pools, several quadrats were used to sample the entire pool.

2.2.2.5 Substrate variables

To estimate the cover by different types of substrate in the bottom of the stream pools, we used the same method (quadrat) used for the aquatic vegetation. The 6 different categories used were stone (>30 cm), rubble (7,5-30 cm), gravel (2-7,5 cm), sand (0,5-2 mm), silt (<0,5 mm) and leaf litter.

2.3 Statistical analysis

2.3.1 Data organization and variable creation

For each pool we calculated the mean values for tree, shrub and herbaceous cover using the values of both margins. The same was done to the landscape variables (tree and shrub cover) as well as for the aquatic vegetation and substrate variables on those pools where several values were recorded. Mean depth of each stream pool and its standard deviation was calculated. Mean depth’s standard deviation was used as an indicator of stream pool heterogeneity. The explanatory variables used in the modeling process are depicted in Table 1.

Table 1 - Explanatory variables

Variable Type	Variable	Units
Physical	**Length**	m
	Width	m
	Mean Depth	cm
	Mean Depth Standard Deviation	cm
	Water Flow	ms^{-1}
	Temperature	ºC
Margin	**Margin Tree Cover**	% cover
	Margin Shrub Cover	% cover
	Margin Herb Cover	% cover
	Margin Slope	% cover
Landscape	**Landscape Tree Cover**	% cover
	Landscape Shrub Cover	% cover
Aquatic Vegetation	**Underwater Vegetation**	% cover
	Emergent Vegetation	% cover
	Margin Vegetation	% cover
Substrate	**Stone**	% cover
	Rubble	% cover
	Gravel	% cover
	Sand	% cover
	Silt	% cover
	Leaf Litter	% cover

We also considered presence/absence data of the three species on the year 2000, referring to this data timeframe as "Past". This data was collected in stream pools using dipnet sweeps. Each pool was sampled at least three times (often four) during the year. Data regarding the 2021 sampling was referred to as "Present". Then, stream pools were categorized as follows:

According to the history of expansion of *P. clarkii* from 2000 to 2021 - variable "*P. clarkii* expansion":

0 - invaded stream pools in 2000 where the species continued to be present in 2021;

1 - pools where *P. clarkii* was not present in the past and that were now invaded by this species.

For the two studied amphibians, the categories were: species absence in a pool where it occurred in 2000 (0) and species presence in both timeframes (1). These variables were named "*A. cisternasii* maintenance" and "*S. salamandra* maintenance", respectively.

2.3.2 Species Distribution and Coexistence Probabilities Analysis

Utilizing the QGIS software (version 3.18.2) (QGIS Development Team, 2021), we constructed maps showing presence/absence data from the 2021 sampling for each species in each pool. The same methodology was applied for the 2000 data. A third map was built for each species (*P. clarkii*, *A. cisternasii* and *S. salamandra*) comparing their distribution in the study area between 2000 and 2021. Using the Mcnemar mid-P test, we inferred if the past and present distributions for each species are significantly different (Pembury Smith and Ruxton, 2020). Coexistence probabilities were obtained through the percentage of stream pools where species coexist divided by the total number of sampled stream pools.

2.3.3 Distance to the main watercourse

We used QGIS' distance measuring tool (QGIS Development Team, 2021) to estimate the distances (m) between each pool and the main permanent stream with a connection to that pool (Castelhanos or Cortilhões). The estimates regarding all points where each species was registered in 2021 were grouped. The same procedure was conducted for the points where each species was registered in 2000. We tested the normality for each group, as well as homoscedasticity between 5 group pairs: present/past for each species and between both amphibian species in each timeframe. For each of the previously mentioned pairs, we evaluated the equality of means using a Wilcoxon-Mann-Whitney test performed in R software (version 4.0.3) (R Core Team, 2020).

2.3.4 Environmental variables influencing species distribution

First, we analyzed if there were any correlations between all pairs of environmental variables for each dependent variable category using Spearman Correlation coefficients. All pairs with a coefficient > 0,7 were considered correlated and the variable with less ecological significance for the species under analysis was dropped. To infer on what environmental variables possibly influence the present distribution of *P. clarkii* in the Grândola mountain range, Generalized Linear Models (GLMs) with binomial distribution and logit link function were constructed, using presence (1)/absence (0) of this invasive species in each stream pool in 2021 as the dependent variable. The models composed of a single variable were analyzed based on their p-value. Those variables whose models showed significant effects were used in the next step. We built progressively more complex GLMs using the better fitted models from the previous step as a guide. With a reduced number of explanatory variables, we used the dredge function in the MuMin package (version 1.43.17) to model how every possible combination of these environmental factors influenced *P. clarkii*'s present distribution. We used AIC (Akaike's Information Criterion) to identify the most parsimonious model as well as the better fitted models. Particularly we used AICc, a form of AIC corrected for small sample sizes (Burnham and Anderson, 2002). Models with a ΔAIC < 2 were considered equally good (ΔAIC represents the difference between the model value and the most parsimonious model) (Burnham and Anderson, 2002). Lastly, the model average function of the MuMin package (version 1.43.17) was applied to the best models retrieved from the dredge analysis to produce one final model. Graphical analyses were used to clarify relevant interactions between significant factors.

We repeated this process for *P. clarkii* expansion, using the expansion (1) or maintenance (0) as dependent variable, in order to know what factors influenced the expansion of this invasive crayfish between 2000 and 2021. To evaluate what environmental factors influenced the maintenance (1) or disappearance (0) of *A. cisternasii* and *S. salamandra* in the same period, the same methodology was applied using the respective variables as dependent.

For all analyses, we considered p-values < 0,05 as statistically significant (to reject the null hypothesis). The R software (version 4.0.3.) was used to conduct all statistical analyses (R Core Team, 2020).

3. Results

A total of 407 *P. clarkii* individuals were sighted or captured, as well as 131 *A. cisternasii* tadpoles and 126 *S. salamandra* larvae. Average and standard deviation values for each explanatory variable can be consulted in Appendix 1. As a result of recording errors, data of some streampools were not recorded (see Appendix 2-8).

3.1 Species distribution and coexistence probabilities

The distribution of all species changed from 2000 to 2021 (Figures 3-5). *P. clarkii* was found in 40 stream pools in 2000, increasing to 51 locations in 2021. This is an increase from the 47% occupation in the past to 60% in 2021. Its past distribution consisted of the two main streams (Fig. 3). Stream pools to where this invasive species spread since 2000 are located in higher-order streams, namely SS1 and SS3, and upstream of previous records in Castelhanos (Fig. 3). *A. cisternasii* is present in 13 of the 85 sampled stream pools, having a 15% occupation. In 2000, this species occupied 57 stream pools, 67% of the sampled sites. In 2000, tadpoles were found in the main streams as well as in SS1, SS3 and SS4 (Fig. 4). This amphibian is now found in particular sections of the main watercourses (Castelhanos and Cortilhões streams), with just one specific pool located in a secondary stream (SS1) (Fig. 4). The pools where it is present in Castelhanos, are located between SS1 and SS2, and in a section between SS1 and Grândola (Fig. 4). In Cortilhões, it is present in a section about 1,5 km away from Grândola (Fig. 4). *S. salamandra* was recorded in 45 stream pools in 2000 and occupies 31 stream pools in 2021. This represents a shift in the occupation from 53% in the past to 36% in the present. In 2000, salamander larvae could be found in all secondary streams, in a single pool in Cortilhões and in a particular section of Castelhanos, between SS1 and SS2 (Fig. 5). Presently, it is the only species found in SS2 and SS4 (Fig. 5). It disappeared from the middle pools of SS3 and from the single pool in Cortilhões where it was found in 2000 (Fig. 5). In SS1, larvae are still present in the upstream section and in one particular pool, closer to the Castelhanos stream (Fig. 5). It also disappeared from several pools in Castelhanos, located between SS1 and SS2 (Fig. 5). These results are summarized in Table 2.

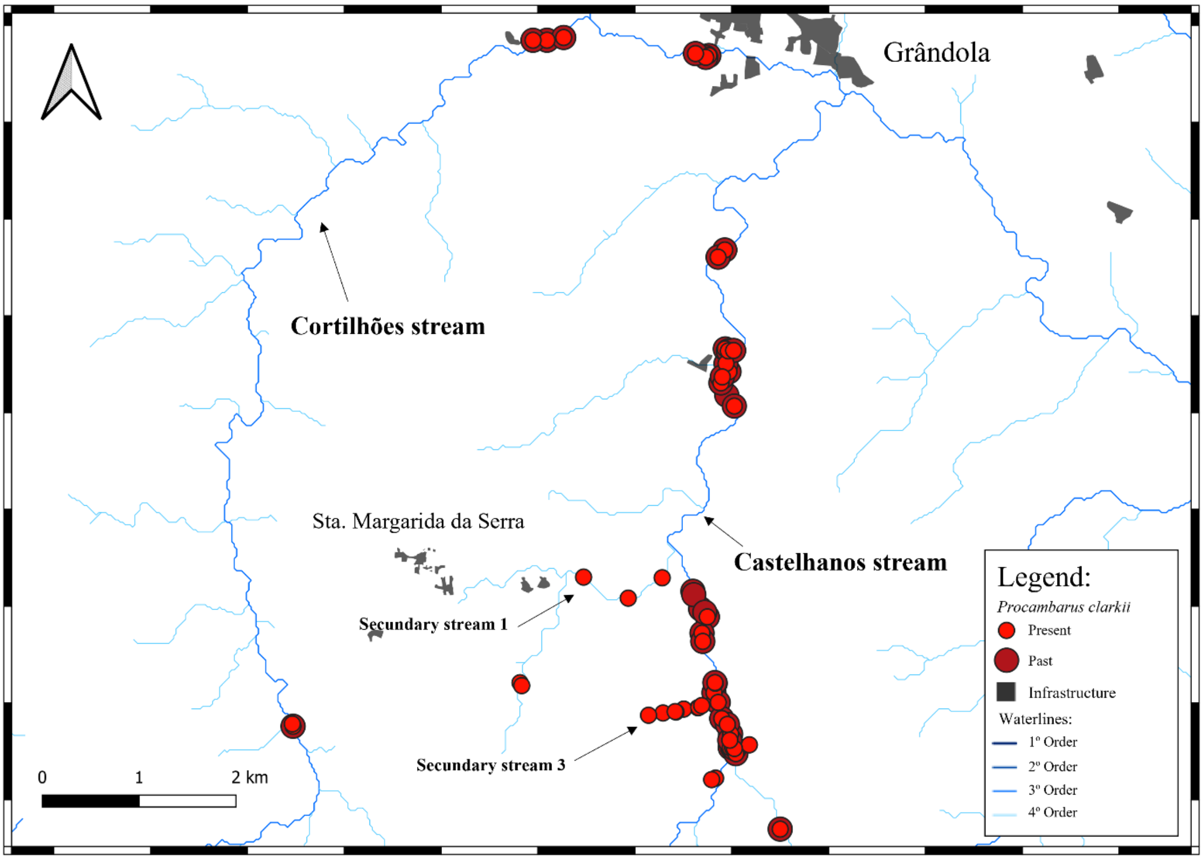

Figure 3 - *P. clarkii* distribution map comparing past (2000) and present (2021) distribution

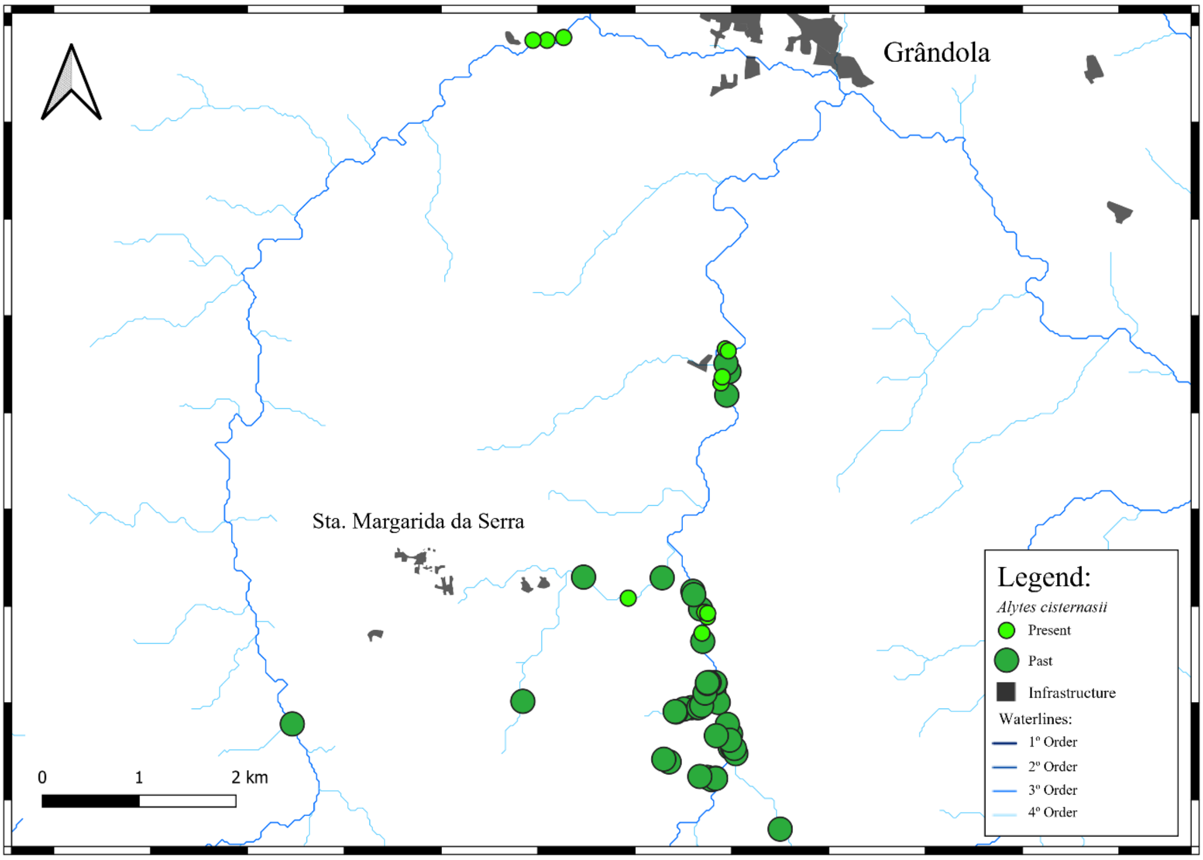

Figure 4 - *A. cisternasii* distribution map comparing past (2000) and present (2021) distribution

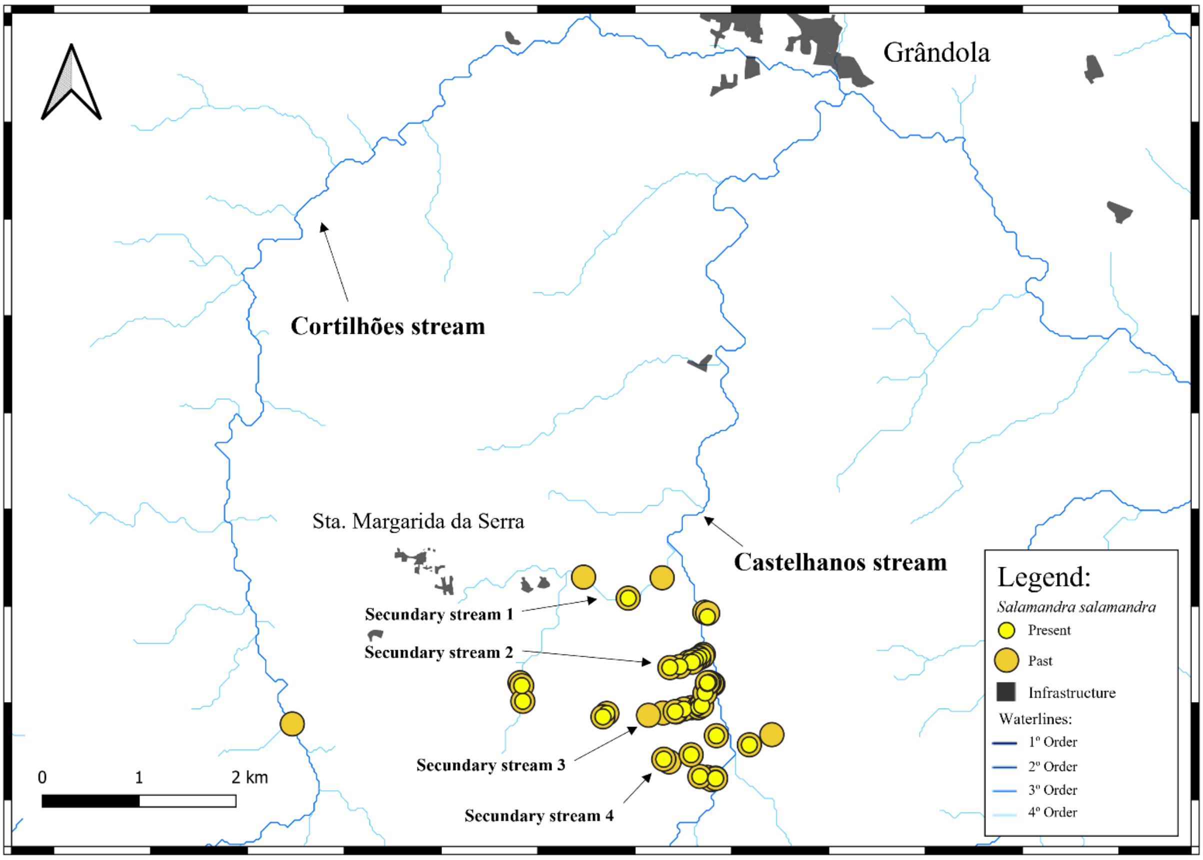

Figure 5 - *S. salamandra* distribution map comparing past (2000) and present (2021) distribution

The p-values from the Mcnemar mid-P tests comparing each species' past and present distributions are all below 0.05 (for *P. clarkii* p-value = 0.023; for *A. cisternasii* p-value = 1.137e^{-13}; for *S. salamandra* p-value = 2.747e^{-4}), rejecting the null hypothesis. This indicates that, for each species, the 2000 and 2021 distributions are significantly different.

According with the changes in each species distribution, there was also a change in their coexistence probabilities between 2000 and 2021 (Table 3). Presently, *P. clarkii* coexists with *A. cisternasii* and *S. salamandra* in 11 and 10 stream pools, respectively. In the past, it coexisted in 31 and 2 steam pools respectively. This represents a change in the coexistence probability of *P. clarkii* and *A. cisternasii* from approximately 32% in 2000 to approximately 13% in 2021. For *P. clarkii* and *S. salamandra*, the coexistence probability increased from approximately 2% in 2000 to approximately 12% in 2021. In the past the two amphibian species coexisted in 33% of the sampled sites, corresponding to 28 stream pools. In 2021 these species coexist in 2 stream pools, having a coexistence probability of ~ 2%. All three species coexisted in 2 stream pools in 2000 and 2021 but not the same ones (see Appendix 2).

Table 2 - Presence and occupation comparison between past (2000) and present (2021) data

	Stream pools (2000)	**Past Occupation (%)**	**Stream pools (2021)**	**Present Occupation (%)**
Procambarus clarkii	40	47.1	51	60.0
Alytes cisternasii	57	67.1	13	15.3
Salamandra salamandra	45	52.9	31	36.5

Table 3 - Species coexistence in the past (2000) and present (2021); P.c. – *Procambarus clarkii*; A.c. – *Alytes cisternasii*; S.s. – *Salamandra salamandra*

	Stream pools (2000)	%	Stream pools (2021)	%
P.c. + A.c.	31	36.5	11	12.9
P.c. + S.s.	2	2.4	10	11.8
A.c. + S.s.	28	32.9	2	2.4
A.c. + S.s. + P.c.	2	2.4	2	2.4

3.2 Distance to the main watercourse

The average distance to the main watercourse of the three species also changed (Figure 6). In 2000 the average distance of the stream pools occupied by *P. clarkii* to the main watercourse was 106.44 m ± 535.83 m. In 2021 this value is 281.77 m ± 648.60 m. *A. cisternasii* stream pools were located at an average of 278.58 ± 648.13 m of the main watercourse in 2000. In 2021 this distance is 59.19 ± 213.43 m. For *S. salamandra* stream pools, the average distance to the main watercourse was 609.92 m ± 883.81 m in 2000. In 2021 this distance increased to 637.86 m ± 949.48 m.

As the assumptions of normality and homoscedasticity were not met, we used the Wilcoxon-Mann-Whitney test to compare each pair mentioned above as well as to compare both amphibian species in each timeframe. The results are shown in Table 4 and Table 5. Only for *S. salamandra* there was no difference between the average distance to the main watercourse in the past and the present (p-value = 0.89); the other tests rejected the null hypothesis (*A.cisternasii* p-value = 0.02 and *P. clarkii* p-value = $1.97e^{-03}$). Comparisons between amphibian species in each timeframe both revealed significant differences (year 2000 p-value = $3.88e^{-05}$; year 2021 p-value = $9.66e^{-06}$).

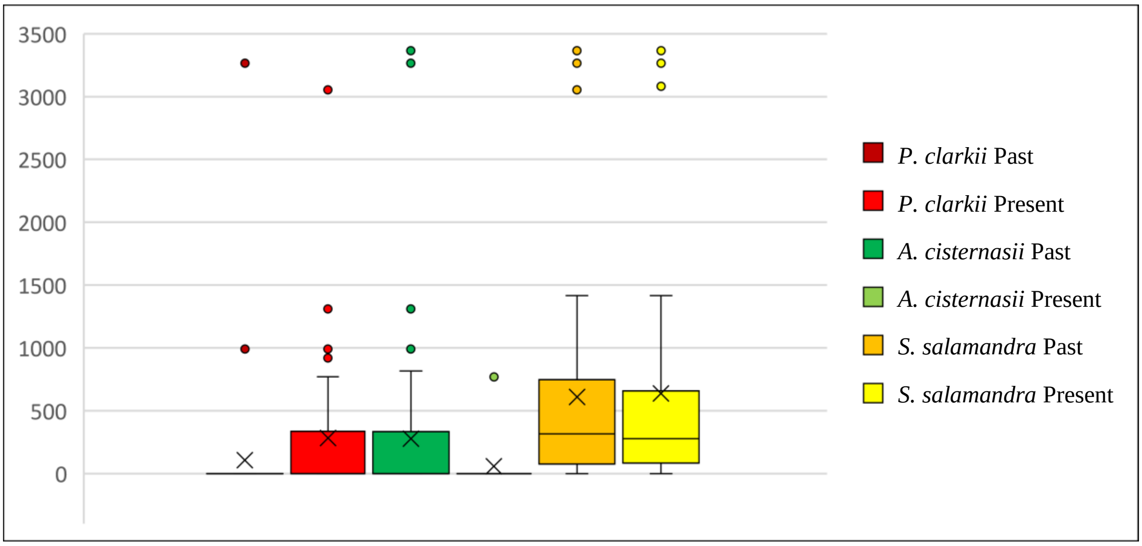

Figure 6 - Past-present comparison of the distance to the main watercourse for each species; × marks the mean value for each group while the line dividing each box is the median; each whisker (upper and lower) boundary is the standard deviation for that group; points outside each box are outliers

Table 4 - Wilcoxon-Mann-Whitney test results comparing past (2000) and present (2021) distance to the main watercourse for each species

Sample Pair	**W**	**p-value**
A. cisternasii - Past vs. Present	515.5	0.02
S.salamandra - Past vs. Present	676.5	0.89
P. clarkii - Past vs. Present	744.5	$1.97e^{-03}$

Table 5 - Wilcoxon-Mann-Whitney test results comparing both amphibian species' distance to the main watercourse in the past (2000) and present (2021)

Sample Pair	**W**	**p-value**
A.cisternasii Past vs. *S. salamandra* Past	1951	$3.88e^{-05}$
A.cisternasii Present vs. *S. salamandra* Present	360.5	$9.66e^{-06}$

3.3 Environmental variables influencing *P. clarkii* present distribution

Two equally good models (ΔAICc < 2) resulted from the final *dredge* analysing the variables influencing *P. clarkii*'s 2021 distribution; model weights were similar (Table 6). The factors that influence the distribution of *P. clarkii* in 2021 were stream pool width, margin tree cover and the percentage of stone that covers the bottom of the stream pool. All these factors have a positive influence on the response variable. The significant interactions are also presented in Table 6 and Table 7 and are depicted as graphs in Appendix 9-10: in narrower stream pools, both margin tree cover and stone cover have a positive influence on the dependent variable. The final model, resulting from the model average of the best models, indicates that stone is the most important factor (Pr = 0.01061), as well as its interaction with stream pool width (Pr = 0.01729).

Table 6 - Best models that describe the factors that influence the present distribution of *P. clarkii*

Model	**Width**	**Stone**	**Margin Tree Cover**	**Width x Stone**	**Width x Margin Tree Cover**	**df**	**ΔAICc**	**AICc**	**Weight**
1	0.94	0.26	0.05	-0.06	-	5	0.00	80.17	0.33
2	0.47	0.25	0.01	-0.06	0.02	6	0.07	80.24	0.31

Table 7 - Model resulting from the average of the best models that describe the factors influencing the presence of *P. clarkii* in 2021

	Estimate	**Standard Error**	**Adjusted Standard Error**	**z value**	**Pr(>|z|)**
Intercept	-3.61	1.22	1.23	2.93	0.00
Width	0.71	0.41	0.42	1.71	0.09
Stone	0.26	0.10	0.10	2.55	0.01
Margin Tree Cover	0.03	0.03	0.03	0.88	0.38
Width : Stone	-0.06	0.03	0.03	2.38	0.02
Width : Margin Tree Cover	0.01	0.01	0.01	0.70	0.48

3.4 Environmental variables influencing *P. clarkii* expansion

The four best models resulting from the dredge analysis are presented in Table 8. These models diverge in the inclusion of mean depth, as well as different interactions. Weights of each model vary between 0.074 and 0.186. The results produced by the best model explaining *P. clarkii* expansion (obtain through model averaging of the four best models) show that this was influenced by stream pool depth, stream pool length, margin shrub cover and leaf litter. All these factors have a negative influence on the dependent variable. Some interactions between the above-mentioned variables were also shown to significantly influence *P. clarkii* expansion. These results are summarized in Table 8 and Table 9 and depicted in Appendix 11-15: in shallower stream pools (20-30 cm) length has a positive influence on the dependent variable; in longer stream pools margin shrub cover influences expansion positively; in short stream pools leaf litter positively influences the dependent variable; finally, in stream pools with higher margin shrub cover (i.e. more than about 40% cover), mean depth negatively influences *P. clarkii* expansion while leaf litter has a positive influence on this variable in the same circumstances. Some three- variable- interactions were significant, namely between length, margin shrub cover and leaf litter, and between mean depth, length and margin shrub cover (Table 9). The Pr values are very similar and vary between 0.994 and 0.998, not highlighting any variable as particularly significant.

Table 8 - Best models that describe the factors that influence *P. clarkii* expansion

Model	**Length**	**Leaf Litter**	**Mean Depth**	**Margin Shrub Cover**	**Length x Leaf Litter**	**Length x Mean Depth**	**Length x Margin Shrub Cover**	**Leaf Litter x Margin Shrub Cover**	**Mean Depth x Margin Shrub Cover**	**Length x Leaf Litter x Margin Shrub Cover**	**Length x Mean Depth x Margin Shrub Cover**	**df**	**ΔAICc**	**AICc**	**Weight**
1	-3584	-185.5	-294.5	-273.4	40.81	88.01	72.22	6.05	6.34	-0.92	-1.79	12	0.00	32.43	0.19
2	-3.08	-0.21	-	-0.29	-	-	0.06	0.01	-	-	-	6	0.43	32.87	0.15
3	-2.60	-0.22	-0.07	-0.26	-	-	0.06	0.01	-	-	-	7	1.66	34.09	0.08
4	-6.14	-1.12	-	-0.58	0.19	-	0.13	0.04	-	-0.00	-	8	1.85	34.28	0.07

Table 9 - Model resulting from the average of the best models that describe the factors influencing *P. clarkii* expansion

	Estimate	**Standard Error**	**Adjusted Standard Error**	**z value**	**Pr(>|z|)**
Intercept	4465	1332000	1376000	0.00	1
Length	-1361	523600	540800	0.00	1
Leaf Litter	-70.62	8584	8866	0.01	0.99
Mean Depth	-111.7	33740	34840	0.00	1
Margin Shrub Cover	-103.9	15970	16500	0.01	1
Length : Leaf Litter	15.50	3968	4099	0.00	1
Length : Mean Depth	33.37	11690	12070	0.00	1
Length : Margin Shrub Cover	27.43	7073	7305	0.00	1
Leaf Litter : Margin Shrub Cover	2.31	470.9	486.4	0.00	1
Mean Depth : Margin Shrub Cover	2.41	450.6	465.4	0.00	1
Length : Leaf Litter : Margin Shrub Cover	-0.35	51.22	52.90	0.01	1
Length : Mean Depth : Margin Shrub Cover	-0.68	163.7	169.1	0.00	1

3.5 Environmental variables influencing *A.cisternasii* maintenance

There were 9 equally good models explaining *A. cisternasii* maintenance (Table 10). These mainly diverge in the inclusion of several different interactions. Weights ranged between 0.084 and 0.031, the lowest values presented in this work. The final model results from the model average of these 9 models and is summarized in Table 11. The best-fitted model that explains the persistence of *A.cisternasii* include stream pool width, margin herb cover, margin shrub cover and emergent vegetation as significant variables. Margin shrub cover showed a negative influence on the dependent variable while the other factors positively influenced the persistence of this species. Some interactions between these variables were also significant and are depicted in Appendix 16-19. These results are summarized in Table 10 and Table 11: regarding intermediate values of stream pool width, both margin herb cover and emergent vegetation had a positive influence; contrarily, for higher width values margin shrub cover had a negative influence; for lower margin shrub cover values, margin herb cover positively influences this factor. Pr values for each factor range from 0.253 to 0.878, not showing any particularly significant influence.

Table 10 - Best models that describe the factors that influence *A. cisternasii* persistence

Model	**Width**	**Margin Shrub Cover**	**Margin Herb Cover**	**Emergent Vegetation**	**Width x Margin Herb Cover**	**Width x Emergent Vegetation**	**Margin Shrub Cover x Margin Herb Cover**	**Width x Margin Shrub Cover**	**df**	**ΔAICc**	**AICc**	**Weight**
1	0.79	-0.06	0.05	0.03	-	-	-	-	5	0.00	46.07	0.08
2	0.68	-0.07	-	0.04	-	-	-	-	4	0.23	46.29	0.08
3	1.10	-0.06	-	0.09	-	-0.01	-	-	5	1.53	47.60	0.04
4	0.83	-0.05	0.06	-	-	-	-	-	4	1.58	47.64	0.04
5	0.84	-0.25	-0.02	0.03	-	-	0.00	-	6	1.63	47.69	0.04
6	1.20	-0.03	-	0.04	-	-	-	-0.01	5	1.84	47.90	0.03
7	1.12	-0.06	0.05	0.08	-	-0.01	-	-	6	1.87	47.94	0.03
8	1.22	-0.02	0.05	0.03	-	-	-	-0.01	6	1.94	48.00	0.03
9	-0.21	-0.07	-0.00	0.03	0.01	-	-	-	6	1.99	48.06	0.03

Table 11 - Model resulting from the average of the best models that describe the factors influencing *A. cisternasii* persistence

	Estimate	**Standard Error**	**Adjusted Standard Error**	**z value**	**Pr(>|z|)**
Intercept	-4.27	4.50	4.56	0.93	0.35
Margin Herb Cover	0.02	0.05	0.05	0.44	0.66
Width	0.82	0.71	0.72	1.14	0.25
Margin Shrub Cover	-0.07	0.10	0.10	0.75	0.45
Emergent Vegetation	0.04	0.04	0.04	1.05	0.30
Width : Emergent Vegetation	-0.00	0.01	0.01	0.30	0.77
Margin Herb Cover : Margin Shrub Cover	0.00	0.00	0.00	0.20	0.84
Width : Margin Shrub Cover	-0.00	0.01	0.01	0.26	0.80
Margin Herb Cover : Width	0.00	0.01	0.01	0.15	0.88

3.6 Environmental variables influencing *S. salamandra* maintenance

Four equally good models best explain *S. Salamandra* maintenance, differing in the inclusion of length and of the interaction between two substrate related variables (Table 12). Weights varied between 0.232 and 0.089. According to the best model, *S. salamandra* maintenance is influenced by stream pool length, stone cover and rubble cover. All these factors have a negative influence on the dependent variable. These results and significant interactions are summarized in Table 12 and Table 13, with interactions depicted in Appendix 20: in stream pools with a higher stone cover, the rubble cover has a positive effect until it reaches intermediate values. Rubble apparently had a more important influence than the other factors (Pr = 0.02286).

Table 12 - Best models that describe the factors that influence *S. salamandra* persistence

Model	Rubble	Length	Stone	Rubble x Stone	df	ΔAICc	AICc	Weight
1	-0.04	-0.15	-0.08	-	4	0.00	50.49	0.23
2	-0.04	-	-0.07	-	3	0.88	51.36	0.15
3	-0.06	-	-0.20	0.00	4	1.13	51.62	0.13
4	-0.05	-0.12	-0.16	0.00	5	1.92	52.40	0.09

Table 13 - Model resulting from the average of the best models that describe the factors influencing *S. salamandra* persistence

	Estimate	Standard Error	Adjusted Standard Error	z value	Pr(>|z|)
Intercept	3.06	1.07	1.10	2.78	0.01
Rubble	-0.05	0.02	0.02	2.28	0.02
Length	-0.08	0.10	0.10	0.78	0.44
Stone	-0.11	0.09	0.09	1.26	0.21
Rubble : Stone	0.00	0.00	0.00	0.50	0.62

4. Discussion

With this work, we show that the distribution of both native amphibians, as well as of the invasive crayfish, changed from 2000 to 2021. We confirmed the expansion of *P. clarkii* in the Castelhanos and Cortilhões basins, away from the main watercourses and into shallower and smaller pools with little leaf litter and riverbank shrub cover, located in higher order streams. These environmental characteristics are generally consistent with less preferred conditions by this invasive species, and our results might indicate a generalist invasive behavior. The midwife toad suffered a massive population decline, with tadpoles persisting in a few pools in the main streams. These stream pools are wide, with high cover by emergent vegetation and open riverbanks with no shrubs and covered by grasses. These conditions promote predator avoidance by tadpoles, as well as favor clutch deposition by proximity to favorable adult habitats. The fire salamander suffered a localized and smaller, although important, decline and is the only species present in the two most temporary streams. This urodele tends to persist in smaller stream pools with fine stream bed granulometry, characteristics matching those of intermediate pools of secondary streams in the study area.

4.1 Distribution changes

Examining the maps comparing each species 2000 (past) and 2021 (present) distributions, there was a clear change in both *P. clarkii* and *A. cisternasii*. The invasive crayfish past distribution is maintained and expanded to secondary streams. This is consistent with other works that identify this species as a generalist invader that prefers more lentic conditions (large and deep watercourses with water year-round), but can also thrive in more temporary streams (Alcorlo et al., 2009; Ficetola et al., 2012a; Gherardi, 2006; Loureiro et al., 2015). This invasive species has been found in lakes, marshes, rice fields, temporary streams and ponds (Alcorlo et al., 2009; Chucholl, 2011; Cruz and Rebelo, 2007; Ficetola et al., 2012a; Gherardi et al., 2002; Grey and Jackson, 2012). Grey & Jackson (2012) even found individuals living in flooded hippopotamus footprints on the coast of invaded Lake Naivasha, Kenya. This illustrates this species's adaptation capability and phenotypic plasticity, allowing it to colonize a wide range of habitats. Despite this ability, we did not record an expansion to two secondary streams. These have a shorter hydroperiod, drying out faster and keeping less water (Rui Rebelo personal communication), which might hinder the capacity for crayfish to permanently establish in these watercourses. Based on Gutiérrez-Yurrita & Montes (1999), Rebelo & Cruz (2005) suggest that *P. clarkii* is unable to permanently colonize stream pools with a hydroperiod smaller than 4 months, which can explain the crayfish absence in these streams. Other works found evidence of the inability of *P. clarkii* to colonize headwaters and low order streams (Bernardo et al., 2011; Gil-Sánchez and Alba-Tercedor, 2002; Mota-Ferreira and Beja, 2020). Results concerning distance to the main watercourse (further discussed below) are coherent with the upstream expansion of *P. clarkii*.

Contrasting with the invasive crayfish, *A. cisternasii* disappeared from about three-quarters of its previous distribution. We think that this is probably a result of *P. clarkii*'s predation pressure after the establishment of individuals in new watercourse sections, and population growth in the main streams (Ficetola et al., 2012b). The Castelhanos and Cortilhões streams had already been invaded in 2000 but, based on the invasion time, *P. clarkii* was probably in the early stages of invasion. This is supported by Cruz & Rebelo (2007) that refer *P. clarkii* to be restricted to the main watercourses between 2002 and 2004. Ribeiro (2009) studied stream pools in SS3 and found no invasive crayfish in 2009 and high densities of both *A. cisternasii* and *S. salamandra*. That stream is presently invaded by *P. clarkii* and *A. cisternasii* tadpoles are absent while *S. salamandra* larvae are still present. So, probably during the last 12 years, and concurrently with *P. clarkii* expansion into secondary streams, *A. cisternasii* tadpoles were eliminated from most stream pools, with the exception of those where stream pool conditions allow for coexistence (see below) and/or efficient anti-predator strategies could be developed. In Paul do

Boquilobo Nature Reserve, *P. clarkii* heavily impacted the amphibian community, leading to the drastic reduction of the populations of several amphibian species in less than eight years (Cruz et al., 2008). This indicates that it can take some time for *P. clarkii* to have noticeable impacts in native ecosystems and that this time may vary between distinct locations with distinct environmental conditions. However, *A. cisternasii* also disappeared from SS4, a stream with yet no record of crayfish. This hints that another factors may have caused this distribution change.

Like the other species, *S. salamandra* distribution changed significantly - it disappeared from the main watercourses and the lower section of SS1 (except for two pools where all three species coexist, one in SS1 and another in Castelhanos). It is worth noting that *S. salamandra* disappeared from the only Cortilhões pool where it was found in 2000, now colonised by *P. clarkii*. This, with *P. clarkii* expansion matching the decline, suggests that the invader colonization caused the urodele's disappearance, but, for Cortilhões, that conclusion should not be drawn based on a single pool. Another hypothesis is that *S. salamandra* has a higher ability to coexist with *P. clarkii*, as is still present in most newly invaded stream pools (see below). Additionally, with invasive population establishment and growth, *S. salamandra* larvae in newly invaded pools will be subject to increased predation pressure (Ficetola et al., 2012b). Consequently, we predict that this will lead to increasing impacts and possible larvae restriction to stream pools with ideal conditions for survival in the higher sections of secondary streams. *S. salamandra* seems to be the only studied species that presently reproduces in the SS2 and SS4 stream pools. Although these are the most temporary streams, fire salamander larvae are still able to colonize them, probably due to life history adaptation to short hydroperiods, like faster metamorphosis (Ribeiro, 2009).

Our results show that over 20 years there was a ~67% decrease in the coexistence of invasive crayfish and *A. cisternasii* in the same stream pools; and a more than 400% increase in coexistence of *P. clarkii* and *S. salamandra*. The increase in coexistence between crayfish and *S. salamandra* corroborates the invader's expansion upstream to smaller streams, the salamander's preferred reproductive habitat (Manenti et al., 2009b). The decrease in coexistence between *P. clarkii* and *A. cisternasii* is due to the major decline of the Iberian midwife toad.

Mota-Ferreira & Beja (2020) predicted that amphibian distributions will suffer major contractions to lower order streams and headwaters with further crayfish expansion in the Northeast of Portugal. The authors highlight the importance of these strongholds as refuge from the impacts of crayfish invasion. In our study, this was not the case for *A. cisternasii* as it was restricted to some pools in the main watercourse. This change might be due to the habitat preference of this species, that chooses more permanent streams for egg depositions or, most likely, the favourable conditions for coexistence between tadpoles and crayfish. For *S. salamandra* the preference for higher stream pools in our study area indeed decreases the impacts of *P. clarkii* invasion due to spatial avoidance between the two. Still, it is plausible that fire salamander will continue to suffer a contraction to upper stream pools, those where avoidance of invasive crayfish is possible.

4.2 Coexistence probabilities

Prior to the invasion of *P. clarkii* or in its early stages, midwife toad tadpoles and fire salamander larvae coexisted in many pools throughout the region. In fact, Ribeiro (2009) found both species in all but one pool in SS3 in 2009. Although *S. salamandra* predates on tadpoles of other species (Blaustein et al., 1996), it can only prey on recently hatched tadpoles of *A. cisternasii* due to mouth-size constraints as tadpoles of this species grow very large. This may be a response to this and other native mouth-size dependent predators. This relation must have aided in the coexistence between the two species in high densities. Ribeiro (2009) also reported that leaf litter and pool depth influenced the survival probability of *A. cisternasii*, while *S. Salamandra* survival was influenced by shade and aquatic vegetation. These characteristics highlight the importance of native predator avoidance (one of which is salamander larvae) and food availability as major factors for survival in the temporary pools. Our results (discussed further) show a shift to pools with characteristics that promote the avoidance of crayfish predation, leading to non-coexistence between the midwife toad and the salamander because of distinct responses and adaptations to avoid the invasive predator. This is consistent with Mota-Ferreira & Beja (2020) that found that invasive crayfish distribution was the most significant factor predicting the distribution of both species.

In 2021, these amphibians only coexist in two invaded pools: one in the Castelhanos stream (BP65), near SS1, and another on the lower section of this secondary stream (BP67). This hints at specific conditions that allow for coexistence of the three species in these two locations. Conditions of these pools are particularly favourable for *A. cisternasii* maintenance (further discussed below). This makes sense since *A. cisternasii* seems to be less generalist on the choice of reproductive habitat (Alcorlo et al., 2009; Baumgartner et al., 1999; Cogliati et al., 2022; Egea-Serrano et al., 2006; Rebelo and Crespo, 1999; Ribeiro and Rebelo, 2011; Weitere et al., 2004). As previously mentioned, *P. clarkii* can reproduce in a wide variety of conditions, from permanent ponds and lakes to summer-flooded rice fields and winter-flooded temporary ponds, demonstrating an enormous phenotypic plasticity (Alcorlo et al., 2009). *S. salamandra* is known for its reproductive variability. In addition to reproducing in different environments, including deciduous woodland streams (Baumgartner et al., 1999), temporary streams and pools (Burgstaller et al., 2021; Ribeiro, 2009), and even underground (Manenti et al., 2009a), this species has ovoviviparous and viviparous populations (Buckley et al., 2007), demonstrating high levels of adaptability to its environmental conditions and phenotypic plasticity. The midwife toads seems to be able to reproduce in temporary streams that turn into separate pools along spring and summer, as well as in main permanent streams (Rebelo and Crespo, 1999; Ribeiro and Rebelo, 2011). Being an endemic anuran, it is adapted to more limited conditions where it evolved and has a more restrict niche than another species of its genus (Rodríguez-Rodríguez et al., 2020), being probably the less plastic of the three species.

Assuming that the native amphibians will respond behaviorally to the invader, the conditions of the stream pools where coexistence still occurs may promote it. In this case, this is synonymous with conditions that promote survival and in particular predator avoidance. The two pools where the toad, the salamander and the crayfish coexist are characterized by intermediate depths and high cover of the stream floor by rubble, features that can facilitate predator avoidance and increase refuge availability. These pools are also surrounded by favorable *A. cisternasii* adult habitat (discussed below). *A. cisternasii* tadpoles are agile swimmers and have a high capacity to escape predators. They also reach significant sizes that hinder predation efforts by many native aquatic predators. Tadpoles of this species have the capacity to induce a faster growth rate, taking less time to reach metamorphosis, in response to chemical cues given by conspecifics eaten by *P. clarkii* (Nunes et al., 2014). They can also alter their behavior by promoting predator avoidance, in response to cues from the invasive crayfish (Gonçalves et al., 2011; Nunes et al., 2014, 2013; Rebelo and Cruz, 2005). In contrast, *S. salamandra* larvae seem to have less evasion capacity. However, they are sit and wait predators (Manenti et al., 2013) with a powerful startle response and can alter their behavior, favoring predator avoidance, in response to *P. clarkii* (Rebelo and Cruz, 2005). Additionally, fire salamanders tend to take less time to metamorphize than the tadpoles of *A. cisternasii* (Ribeiro, 2009). These traits might help this species to avoid predation by crayfish. Both amphibians lack an egg stage, which is beneficial because *P. clarkii* easily predates on amphibian eggs (Rebelo and Cruz, 2005). Although both species seem to possess traits favorable for coexistence with the invasive predator in comparison with other native species, we propose that *A. cisternasii* faces higher predation pressure due to major spatial compatibility leading to exclusion from reproductive habitats. With *P. clarkii*'s main invasion pathway being the larger and more permanent streams (Cruz and Rebelo, 2007), *A. cisternasii* tadpoles, already in the year 2000 had a superior spatial overlap with the invasive species, with 36,5% coexistence against *S. salamandra*'s 2,4%.

4.3 Distance to the main watercourse

Distance to the main watercourse was identified as the main factor influencing *P. clarkii* distribution in the Sado River Basin (Cruz and Rebelo, 2007). The dispersion path of this invasive crayfish proceeds from the source population in the main, more stable watercourses, to smaller and more temporary streams with less preferred conditions (Treguier et al., 2011). Dispersion upstream highlights *P. clarkii* ability to disperse not only by water but overland (Cruz and Rebelo, 2007; Kerby et al., 2005) due to its tolerance to desiccation and ability to move on terrestrial habitats (Banha and Anastácio, 2014). Other works reported that physical barriers and increased water flow downstream are factors that hinder *P. clarkii* dispersion (Banha and Anastácio, 2014; Bernardo et al., 2011; Kerby et al., 2005). As invaded secondary streams have no significant aquatic or land barriers, crayfish dispersion was not limited in our study area.

Because *A. cisternasii* present distribution is now restricted to thirteen stream pools essentially located in the main streams, its average distance to the main watercourse decreased. *S. salamandra* was the only species that did not significantly change its positions in relation to the average distance to the main watercourse. This is consistent with its small and relatively dispersed decline. If crayfish upstream dispersion proceeds, this measure will continue to increase, with further separation between the Iberian midwife toad in sections of the main streams and fire salamander in the higher and more temporary streams.

4.4 Pool features that favoured *P. clarkii* distribution in 2021 and expansion

Our results imply that the 2021 distribution of the invasive crayfish was influenced by pool width, margin tree cover and stream bed stone cover. Furthermore, in narrower pools, both stream bank tree cover and cover by stone increase crayfish's presence probability. Wide pools are mainly located in the main watercourses and their bottom tends to have a higher stone cover than those in secondary streams due to the lower water flow. Wide streams can also support a higher density of individuals (Ficetola et al., 2012b). Tall riparian forests provide shade to freshwater ecosystems and tend to indicate more stable conditions. Shade acts as a thermal regulator for water temperature, avoiding water temperature peaks (Broadmeadow et al., 2011). This might allow crayfish to better thermoregulate in order to achieve optimal growth rates and activity. Stone acts as refuge for *P. clarkii*, providing protection from predators (Aquiloni et al., 2005), and sheltering from higher velocity waterflow conditions (Bernardo et al., 2011). Like shade, this type of refuge is also important for avoiding temperature extremes (Ilhéu et al., 2003). This variable seems to be the most significant factor highlighted by this study and corroborates the importance of refuge for this species, particularly in narrower pools. Refuge is essential for *P.clarkii* especially when burrowing is not possible, normally due to substrates dominated by large boulders (Aquiloni et al., 2005; Barbaresi et al., 2004b; Gherardi et al., 2002).

Crayfish expansion in the study area in the last 21 years was significantly influenced by several factors. Newly invaded stream pools tend to be smaller, shallower and with less leaflitter. All these conditions are unfavorable for *P.clarkii*. Larger and deeper stream pools support more individuals while leaflitter acts both as refuge and food source (Carvalho et al., 2016; Fidalgo et al., 2013). Recently invaded stream pools also tend to have less bank shrub cover. This indicates a higher tree cover that provides shade, which might suggest that *P. clarkii* is expanding to the more developed riparian galleries in intermediate stream pools. There were several significant interactions between the variables that explain the expansion. For example, in shallower pools, higher pool lengths allow for more space and are therefore more favorable for the crayfish. Additionally, leaf litter increases the probability of expansion into smaller pools probably through the higher provision of refuges and food. But, although these variables and interactions were indicated as significant, the interpretation of these results requires caution. The lack of variables with statistically significant relationships (Pr < 0,05) with the dependent variable in our final model, as well as the selection of many interactions that contribute to a lower AICc, indicate that it is poorly adjusted. This suggests that the invader does not prefer waterbodies with specific environmental conditions and probably attempts to invade every available location in the study area. As previously mentioned, *P. clarkii* is a generalist invader, capable of surviving in a wide range of freshwater habitats by adapting its behavior to environmental restrictions (Alcorlo et al., 2009; Ficetola et al., 2012a).

4.5 Pool features that favored *A. citsernasii* and *S. salamandra* maintenance in 2021

Our results show a steep decline in *A. cisternasii* distribution. In order to design and apply conservation actions, it is of utmost importance to understand what stream pool characteristics contribute to the maintenance of this endemic anuran. According to this study, tadpoles of this species are resisting in wide stream pools with high cover of emergent vegetation and margins with high herb cover and low shrub cover. This is biologically sound because wider pools promote predator spatial avoidance, reducing predation pressure and promoting survival (Oertli et al., 2002). High values of emergent vegetation provide high availability of refuge, beneficial to survival, and it is an indication of more permanent abiotic conditions (Aquiloni et al., 2005; Ribeiro, 2009). Open herb-dominated margins are also an important factor in pools where *A. cisternasii* males still deposit the egg masses. Adults are semi-fossorial and prefer warm and open habitats with low shrub density (Almeida et al., 2001; IUCN SSC Amphibian Specialist Group, 2020; Maravalhas and Soares, 2017). Open margins are suitable and may be preferred habitats for adults that benefit from the smaller travel distance needed for egg deposition in the reproductive season. It is also important to highlight that several interactions between these variables also influence *A. cisternasii* maintenance significantly. Most of these interactions suggest the higher importance of favourable adult habitats in the vicinity of suitable stream pools to maintain this species.

Just like for the midwife toad, it is important to know what pool conditions are favorable for *S. salamandra* maintenance in the study area, so that adequate conservation measures can be applied. This urodele is highly adaptable to local conditions with different factors influencing its presence in distinct parts of its distribution (Baumgartner et al., 1999; Egea-Serrano et al., 2006; Manenti et al., 2013, 2009b; Ribeiro, 2009; Wagner et al., 2020). In general, *S. salamandra* prefers higher order stream pools with high food availability inserted into a forest landscape (favorable adult habitat) (Baumgartner et al., 1999; Ficetola et al., 2009; IUCN SSC Amphibian Specialist Group, 2022; Manenti et al., 2009b; Mota-Ferreira and Beja, 2020; Wagner et al., 2020). The best model predicting the influencing variables on *S. salamandra* maintenance includes length, and stone and rubble cover, all with a negative influence. To our knowledge, none of these pool characteristics are known to be avoided by the species. In fact, salamander larvae can also use stone and rubble as refuge (Manenti et al., 2013). However, these conditions are consistent with higher order stream pools and are considered unfavorable for *P. clarkii.* Furthermore, we observed that invasive crayfish were often found under and in between stones and rubble (personal observation), acting as a refuge (Aquiloni et al., 2005). This may suggest that although these microhabitats are beneficial for the urodele, they are avoided because of their use by predatory crayfish.

4.6 Possible errors

This work was subject to some sources of error, like uneven sampling (e.g. some stream pools were visited just once while others were visited two or three times in 2021), different sampling methods (electrofishing vs dipnet sweeps) or seasons (*S. salamandra* larvae tend to have short larval periods). Uneven sampling was particularly relevant in the last sampling season of 2021 as some amphibian tadpoles or larvae might have already completed metamorphosis and became terrestrial.

The major difference between the sampling strategies in 2000 and 2021 was the use of electrofishing in 2021. This is a more effective sampling method than regular dipnet sweeps which suggests an uneven detectability between the two timeframes, in favor of the 2021 sampling (Alonso, 2001). Since that, in the past sampling, each amphibian species' larvae were found in more pools using a less effective method, these species may have indeed suffered stronger declines than those detected in this work. In contrast, and although unlikely due to the effective sampling method, false negatives could have occurred because some larvae disappeared from specific pools surrounded by sites where they were still present. For instance, in 2021, *A. cisternasii* was found alone in just two pools located next to a *P. clarkii*-invaded section (BP77 and BP79). Although these two pools might have characteristics preventing invasion, it is likely that *P. clarkii* also occurs, even if transiently, due to the proximity to the source population.

4.6 Other possible causes for distribution change

Although our results suggest *P. clarkii* expansion as the main factor influencing these native amphibians' distribution changes and declines, we cannot rule out the possibility of other factors playing a part in the changes in the distribution of the native species. Amphibian populations can have high interannual and intrannual variability because of their high dependence on environmental conditions that change along and between years (Dubos et al., 2020; Gómez-Rodríguez et al., 2010; Green, 2003). Still, amphibians breeding in small streams tend to have more stable populations than those breeding in ponds (Green, 2003). We believe the decline detected for *A. cisternasii* is real, due to its scale and geographical pattern. However, although unlikely, the detection of a false decline is more plausible for *S. salamandra* because it was minor and localized, based on a small number of pools. More long-term studies with yearly sampling are necessary to completely discard this hypothesis and better understand interannual distribution changes. For *S. salamandra,* future research should aim to confirm the impact of *P. clarkii* in its distribution.

Follow-up works shall also test the impact of climate change on the distribution of these species with a special focus on its interaction with the invasive crayfish. Climate change is having a significant impact on amphibian reproduction and recruitment. The frequency of drought years has increased, leading to poor reproductive seasons for amphibians and, for instance, the disappearance of the fall breeding episode in both species (Ribeiro, 2009). With less rainfall, amphibians can lose reproductive habitat as temporary watercourses may have a shorter hydroperiod or don't accumulate water at all (Gould et al., 2022). Drought also affects *P. clarkii,* as it reduces hydroperiod and water levels, hindering expansion. Increased extreme phenomena are also a consequence of climate change. In this case, increased rainfall in shorter timeframes promotes higher water flow that negatively affects crayfish expansion (Kerby et al., 2005). Heatwaves might also affect diet preferences in *P. clarkii*, promoting herbivory, particularly in juveniles, otherwise the most carnivorous life stage (Carreira et al., 2017). These changes in diet will inevitably change the impact of this invader on native ecosystems, probably decreasing predation pressure in amphibian larval stages.

Another factor not tested in this study that might influence amphibian communities is the presence of pathogens like *Batrachochytrium dendrobatidis* or *B. salamandrivorans*. These pathogens are responsible for several mortality episodes in different amphibian species, causing great harm to ecological communities through trophic cascades (Cheng et al., 2011; Crawford et al., 2010; Lötters et al., 2020; Rosa et al., 2013). There is evidence that Bd is not restricted to high-altitude mountain ranges and can survive below its experimental temperature interval (Walker et al., 2010). Also, there is evidence that suggests that *Alytidae* frogs, like *A. cisternasii*, are more susceptible to Bd (Baláž et al., 2014). This emphasizes the need to monitor these populations and take measures to prevent outbreaks. Bsal is spreading throughout Europe and has been detected in nearby Spain (Ribas et al., 2022). This fungus is reported to have very severe impacts on *S. salamandra* populations, causing massive declines (Bosch et al., 2021; Lötters et al., 2020). We advise further studies to continuously check for the presence, distribution and incidence of amphibian pathogens in native freshwater communities. Special attention should be given to *P. clarkii* as it can be a vector and reservoir of these particularly impactful organisms, namely Bd (Oficialdegui et al., 2019b).

It is also possible that a combination of several of these factors might influence this distribution changes. Particularly, climate change may promote the incidence and spread of amphibian pathogens through debilitating amphibian immune systems due to stress or allowing better grow conditions for the pathogens (Cohen et al., 2019a, 2019b; Pounds et al., 2006).

5. Conclusions

In conclusion, we confirm the expansion of the red swamp crayfish away from the main permanent streams into less preferred habitat and the decline of the two native amphibian species, with the restriction of Iberian midwife toad tadpoles to a few favourable sections of the invaded main watercourses; and the maintenance of fire salamander larvae in more temporary stream pools. Newly invaded pools have less than ideal conditions and we believe this is another indication that this species' invasion in our study area depends on the distance to the population source. The midwife toad *A. cisternasii* seems to prefer the main streams for larval development while *S. salamandra* tends to use smaller and higher order stream pools in the study area and the model results reflect that. Our study also adds evidence to show the impacts of *P. clarkii* can have on native communities. These declines may have cascading effects in native trophic webs and alter ecosystem processes and interactions (Carreira et al., 2014; Cruz et al., 2008; Zipkin et al., 2020).

This work reveals a need for yearly population monitoring to best comprehend the impact of invasive species on native communities and their interannual development (Blaustein et al., 2011; Stuart et al., 2004). Preferably, monitoring should encompass seasonal sampling in order to analyze fluctuations along the year and be extended to include other amphibian species. Further studies should assess how other non-considered factors interact and relate to the recorded declines. Other variables like hydroperiod (Ficetola et al., 2012a), shade (Ribeiro, 2009) or biotic factors (Manenti et al., 2009b) might be relevant for the distribution of these species. In the future, it is important to also measure tadpole survival to evaluate if tadpoles of the now rare *A. cisternasii* complete metamorphosis (Ribeiro and Rebelo, 2011).

We also highlight the importance of controlling invasive populations so that declines like those we recorded can be prevented. As eradication of this invader is unrealistic (Gherardi et al., 2011; Loureiro et al., 2018; Souty-Grosset et al., 2016), long-term management is advised to keep crayfish numbers down. In this case, we urge that measures be taken to combat the further spread of *P. clarkii* and mitigate its effects through yearly intensive removal campaigns. As this species might have a negative feedback mechanism for dealing with population decrease, intensive removal of individuals should be more effective in keeping crayfish numbers down than more extensive approaches (Loureiro et al., 2018). These removal campaigns should specially target stream pools where *A. cisternasii* reproduction still occurs and surrounding stream sections in order to protect the populations of this endemic anuran on the long term. River bank habitat should also be protected to safeguard reproductive adult individuals. Focus should also be in preventing invasion of higher stream pools that are important to *S. salamandra* reproduction. As stream pools are key reproductive habitats for most amphibian species occurring in the southwest of the Iberian peninsula (De Vries and Marco, 2017), which elevates the importance of conserving these ecosystems, measures shall also be taken to impede on new invasions that may threaten their balance.

6. References

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7. Appendices

Appendix 13 - Average and standard deviation values of the stream pool variables

Variable	Average	Standard Deviation
Length (m)	5,82	3,87
Width (m)	2,56	1,36
Mean Depth (cm)	31,74	13,26
Mean Depth Standard Deviation (cm)	6,43	3,67
Water Flow (m/s)	0,07	0,08
Temperature (°C)	14,11	1,98
Margin Tree Cover (% cover)	37,56	24,46
Margin Shrub Cover (% cover)	46,95	22,98
Margin Herb Cover (% cover)	72,56	23,39
Margin Slope (0-2)	1,01	0,98
Landscape Tree Cover (% cover)	39,24	14,86
Landscape Shrub Cover (% cover)	46,48	23,50
Underwater Vegetation (% cover)	18,92	23,64
Emergent Vegetation (% cover)	18,82	24,85
Margin Vegetation (% cover)	65,82	32,97
Stone (% cover)	10,87	19,02
Rubble (% cover)	34,74	25,63
Gravel (% cover)	16,36	18,09
Sand (% cover)	9,10	14,57
Silt (% cover)	9,74	18,60
Leaf Litter (% cover)	20,01	28,57

Appendix 14 - Table with presence/absence data for each species in each timeframe (2000 and 2021) and *P. clarkii* expansion (*P.clarkii*Exp), *A. cisternasii* maintenance (*A.cisternasii*M) and *S. salamandra* maintenance (*S.salamandra*M) for each pool; For *P.clarkii*Exp: expansion (1) and maintenance (0); For *A.cisternansii*M and *S.salamandra*M: maintenance (1) and disappearance (0)

Pool	***P.clarkii*** **2000**	***P.clarkii*** **2021**	***P.clarkii*** **Exp**	***A.cisternasii*** **2000**	***A.cisternasii*** **2021**	***A.cisternasii*** **M**	***S.salamandra*** **2000**	***S.salamandra*** **2021**	***S.salamandra*** **M**
BP33	0	1	1	1	0	0	1	1	1
BP17	0	0	-	0	0	-	1	1	1
BP14	0	0	-	1	0	0	1	1	1
BP35	0	1	1	1	0	0	1	1	1
BP36	0	1	1	1	0	0	1	0	0
BP37	0	1	1	1	0	0	1	1	1
BP3	0	1	1	0	0	-	1	0	0
BP39	0	1	1	0	0	-	1	0	0
BP41	0	1	1	1	0	0	1	1	1
BP4	0	0	-	1	0	0	1	1	1
BP46	0	0	-	1	0	0	1	0	0
BP45	0	0	-	1	0	0	1	0	0
BP47	0	0	-	0	0	-	1	1	1
BP48	0	0	-	0	0	-	1	1	1
BP49	0	0	-	0	0	-	1	1	1
BP50	0	0	-	0	0	-	1	1	1
BP23	0	0	-	0	0	-	1	1	1
BP2	1	1	0	1	0	0	0	0	-
BP51	1	1	0	1	0	0	0	0	-
BP52	1	1	0	1	0	0	0	0	-
BP42	0	1	1	1	0	0	1	1	1
BP10-A	0	0	-	1	0	0	1	1	1
BP10-B	0	1	1	1	0	0	1	1	1
BP53	0	1	1	1	0	0	1	0	0
BP54	0	0	-	1	0	0	1	0	0
BP11	0	0	-	1	0	0	1	1	1
BP55	1	0	-	1	0	0	1	1	1

BP25	0	0	-	1	0	0	1	0	0
BP56	0	0	-	1	0	0	1	1	1
BP58	0	0	-	1	0	0	1	1	1
BP59	0	0	-	1	0	0	1	1	1
BP62	0	1	1	0	0	-	1	1	1
BP9	0	0	-	0	0	-	1	1	1
BP63	0	0	-	0	0	-	1	1	1
BP64	0	0	-	0	0	-	1	1	1
BP65	1	1	0	1	1	1	0	1	-
BP66	0	1	1	1	0	0	1	0	0
BP67	0	1	1	1	1	1	1	1	1
BP68	0	0	-	1	0	0	1	1	1
BP29	0	1	1	0	0	-	1	0	0
BP27	0	0	-	1	0	0	1	1	1
BP69	0	1	1	0	0	-	1	1	1
BP70	0	0	-	0	0	-	1	1	1
BP60	0	0	-	0	0	-	1	1	1
BP71	1	1	0	1	0	0	0	0	-
BP0	1	1	0	1	0	0	0	0	-
BP72	1	1	0	1	0	0	0	0	-
BP74	1	1	0	1	0	0	0	0	-
BP75	1	1	0	0	0	-	0	0	-
BP76	0	1	1	1	0	0	1	0	0
BP87	1	1	0	1	1	1	0	0	-
BP88	1	1	0	1	1	1	0	0	-
BP89	1	1	0	0	0	-	0	0	-
BP90	1	1	0	0	0	-	0	0	-
BP91	1	1	0	1	1	1	0	0	-
BP77	0	0	-	1	1	1	1	0	0
BP79	1	0	-	1	1	1	1	0	0
BP6	1	0	-	1	0	0	0	0	-

BP92	1	0	-	1	0	0	0	0	-
BP83	1	0	-	1	0	0	0	0	-
BP93	0	1	1	1	0	0	1	0	0
BP94	1	1	0	0	0	-	0	0	-
BP95	1	1	0	0	0	-	0	0	-
BP96	1	1	0	1	1	1	0	0	-
BP97	1	1	0	1	1	1	0	0	-
BP99	1	1	0	1	0	0	0	0	-
BP100	1	1	0	1	0	0	0	0	-
BP102	1	0	-	1	0	0	0	0	-
BP103	1	1	0	0	0	-	0	0	-
BP104	1	1	0	1	0	0	0	0	-
BP105	1	1	0	1	0	0	0	0	-
BP106	1	1	0	0	0	-	0	0	-
BP107	1	1	0	0	0	-	0	0	-
BP108	1	1	0	1	1	1	0	0	-
BP109	1	1	0	1	1	1	0	0	-
BP85	1	1	0	1	1	1	0	0	-
BP86	1	1	0	1	1	1	0	0	-
BP110	0	0	-	0	0	-	1	0	0
BP1	1	1	0	1	0	0	0	0	-
BP111	1	1	0	1	0	0	0	0	-
BP112	1	1	0	0	0	-	0	0	-
BP113	1	1	0	1	0	0	0	0	-
BP73	1	1	0	1	0	0	0	0	-
BP115	-	0	-	0	-	-	0	-	-

Appendix 15 - Physical variables' data for each pool

Pool	Length (m)	Width (m)	MeanDepth (cm)	MeanDepth Standard Deviation (cm)	Waterflow (m/s)	Temp (ºC)
BP33	12	1	21,4	9,12	0,04	13,86
BP17	1,1	2	23,46	7,17	-0,1	14,22
BP14	2,1	1	9,3	4,04	0,01	12,97
BP35	8	2	10	5,2	-	12,42
BP36	11	7	31,5	8,2	0,05	12,55
BP37	19	3,2	19	6,2	0,06	12,3
BP3	17	2,3	22,2	6,96	0,03	12,8
BP39	20	2	15,7	6,04	0,05	13,89
BP41	12	2	20,2	8,24	0,2	13,13
BP4	11	4	50,6	14,08	0,06	11,4
BP46	10,5	2	32,8	9,44	0,34	11,89
BP45	9	2	27,9	6,28	0,35	12,09
BP47	4,5	2	56,8	9,84	0,05	12,5
BP48	7	2,2	17	4,8	0,17	12,39
BP49	3	1,1	27,1	2,88	0,071	12,33
BP50	2,1	2	29,3	7,04	0,09	12,79
BP23	3	1	29,7	4,64	0,06	12,76
BP2	2,5	2,5	24,8	4,56	0,23	13,42
BP51	3,5	2	29	6	0,23	13,59
BP52	16	3	37	7,2	0,2	13,9
BP42	3	1,6	25,4	7,68	0,06	12,72
BP10-A	3	1,2	31,4	3,52	0,01	12,51
BP10-B	2,5	1	29	5,6	0,05	12,78
BP53	3,5	1,2	28,2	6,96	0,03	12,73
BP54	4	1,6	29	4	0,03	14
BP11	3,5	1,2	26	4	0,01	14,53
BP55	12	5	15,4	1,52	-	-
BP25	7	1	29,2	7,76	0,17	12,19
BP56	5	2	12,6	4,32	0,26	12,4
BP58	3,5	0,8	33	10,4	0,06	12,85
BP59	10	1	22	3,5	0,11	13,7
BP62	3,1	2,5	11,6	3,44	0,07	12,49
BP9	2,1	1	23,6	2,48	0	13,31
BP63	4	0,8	27,2	9,84	0,06	12,25
BP64	2,5	1	35,6	8,88	0,02	12,62
BP65	5	4	32,8	6,24	0,2	14,33
BP66	8	1,7	26,6	4,72	0,17	14,32
BP67	8	4	29	4,4	0,1	13,8
BP68	7	4,5	36,6	6,32	0	15,9
BP29	4,5	2,2	52	6,4	0,02	12,92
BP27	1,1	1,1	17,6	4,88	0,01	-
BP69	6	3	18,8	1,84	0,01	-
BP70	5,3	1,5	18,8	4,56	0	-
BP60	3,5	0,7	11,8	4,64	0	-

BP71	4,5	3,5	33,6	4,08	0,01	-
BP0	6	2	18,8	8,08	0,07	-
BP72	6,2	4	25,8	4,24	0,05	-
BP74	7,5	1,5	15	4,4	-	10,67
BP75	8	4	45	22	0,03	11,58
BP76	8	4	30,8	5,36	0,09	11,58
BP87	3,5	2,5	29	6,8	0,11	10,87
BP88	6,5	6,5	33,6	12,08	0,06	11,18
BP89	4,5	4	25,6	4,72	0,04	11,67
BP90	5,5	2,5	29,2	5,36	0,08	12,81
BP91	3	5	26,2	5,84	0,06	15,3
BP77	8	1,2	37	4,4	0,03	15,26
BP79	9,5	3	33,2	10,96	0,03	15,62
BP6	4,2	4,5	69,2	17,36	0,03	15,78
BP92	2,7	3,5	34,4	3,52	0,06	15,44
BP83	5	2	40,6	4,88	0,05	15,08
BP93	2,5	1,5	25	2	0,03	16,76
BP94	2,5	2,5	53,6	5,12	0,03	15,23
BP95	3,5	2	72,2	13,84	0,03	17,74
BP96	3,5	4,2	67,4	3,44	0,04	15,7
BP97	3	3,75	48	4,4	0,01	16,15
BP99	3	2,6	25,6	2,72	0,01	15,62
BP100	5	2,5	47,2	16,96	0,06	15,68
BP102	3,5	2	46,8	10,24	0,03	15,54
BP103	7,5	3,5	40	3,6	0,04	15,34
BP104	3,2	3,2	41	4,8	0,14	15,79
BP105	6	2	45,2	7,84	0,03	15,97
BP106	7	3	40,4	7,28	0,11	16,15
BP107	8	3	56,6	7,12	0,04	16,67
BP108	4,7	3	35	1,2	0,04	20,38
BP109	3	7	34,2	2,16	0,11	18,82
BP85	4	3	31,6	10,48	0,07	15,52
BP86	5	3	51,8	7,44	0,03	16,52
BP110	5	2,5	36,8	10,56	0,13	17,18
BP1	2	3	22	4,4	0,11	16,14
BP111	4,7	2,5	17,8	1,76	0,13	16,08
BP112	3	2,2	41,6	3,28	0,12	16,28
BP113	3,5	2	38,2	6,56	0,11	16,43
BP73	-	-	-	-	-	-
BP115	-	-	-	-	-	-

Appendix 16 - Margin variables' data for each pool

Pool	MarginTree (%)	MarginShrub (%)	MarginHerb (%)	MarginSlope
BP33	80	95	50	1
BP17	30	50	70	2
BP14	20	70	30	0
BP35	20	60	40	3
BP36	30	50	40	1
BP37	30	50	40	1
BP3	50	90	10	2
BP39	20	60	25	1
BP41	80	95	50	1
BP4	10	30	70	2
BP46	5	10	70	0
BP45	10	20	70	1
BP47	5	40	70	0
BP48	10	50	60	1
BP49	40	40	20	1
BP50	40	60	20	1
BP23	40	60	20	1
BP2	10	20	80	0
BP51	70	40	30	0
BP52	20	20	70	1
BP42	50	70	30	1
BP10-A	5	60	80	1
BP10-B	5	60	80	1
BP53	20	80	60	1
BP54	5	70	60	2
BP11	5	80	90	2
BP55	5	80	90	2
BP25	40	70	70	0
BP56	50	80	70	0
BP58	10	40	90	2
BP59	10	40	90	2
BP62	40	45	90	0
BP9	45	80	80	0
BP63	40	50	90	1
BP64	80	90	50	0
BP65	40	10	85	1
BP66	30	65	70	1
BP67	35	40	80	0
BP68	30	40	80	0
BP29	70	50	90	1
BP27	40	30	40	1
BP69	60	40	100	1
BP70	10	10	80	0
BP60	30	60	90	1
BP71	30	70	10	0

BP0	30	30	60	0
BP72	50	20	70	0
BP74	60	10	95	0
BP75	60	50	70	0
BP76	100	95	80	0
BP87	40	30	95	1
BP88	80	50	85	3
BP89	80	40	90	1
BP90	75	35	80	2
BP91	60	15	70	1
BP77	5	10	85	0
BP79	15	40	100	0
BP6	25	30	100	1
BP92	30	35	100	0
BP83	60	10	100	0
BP93	30	20	90	1
BP94	50	30	60	1
BP95	80	60	90	2
BP96	30	10	100	0
BP97	10	45	90	1
BP99	50	60	70	0
BP100	50	30	90	1
BP102	25	30	85	1
BP103	15	45	90	2
BP104	70	50	90	3
BP105	50	80	90	4
BP106	40	25	90	4
BP107	80	60	95	0
BP108	5	20	80	0
BP109	5	15	80	0
BP85	45	35	85	3
BP86	35	45	80	1
BP110	45	60	75	2
BP1	35	50	90	2
BP111	10	70	90	2
BP112	70	30	90	1
BP113	75	60	90	2
BP73	-	-	-	-
BP115	-	-	-	-

Appendix 17 - Landscape variables' data for each pool

Pool	LandscapeTree(%)	LandscapeShrub(%)
BP33	40	55
BP17	50	85
BP14	40	85
BP35	25	50
BP36	50	85
BP37	50	85
BP3	55	80
BP39	50	85
BP41	40	55
BP4	35	50
BP46	30	35
BP45	35	35
BP47	7,5	15
BP48	35	60
BP49	35	60
BP50	40	55
BP23	45	55
BP2	65	22,5
BP51	25	32,5
BP52	35	22,5
BP42	55	55
BP10-A	20	80
BP10-B	20	80
BP53	30	75
BP54	25	85
BP11	30	80
BP55	17,5	90
BP25	50	75
BP56	55	80
BP58	60	65
BP59	60	65
BP62	32,5	52,5
BP9	45	87,5
BP63	60	55
BP64	50	80
BP65	40	22,5
BP66	32,5	50
BP67	40	42,5
BP68	40	40
BP29	40	50
BP27	50	20
BP69	55	27,5
BP70	50	60
BP60	65	55
BP71	35	25

BP0	30	15
BP72	32,5	35
BP74	50	25
BP75	60	22,5
BP76	40	35
BP87	20	10
BP88	17,5	7,5
BP89	40	25
BP90	22,5	17,5
BP91	80	60
BP77	35	30
BP79	42,5	55
BP6	30	60
BP92	45	35
BP83	50	30
BP93	50	32,5
BP94	30	5,5
BP95	40	25
BP96	12,5	45
BP97	17,5	35
BP99	25	20
BP100	12,5	5,5
BP102	8	5
BP103	15	20
BP104	40	55
BP105	52,5	65
BP106	42,5	52,5
BP107	62,5	60
BP108	55	37,5
BP109	65	22,5
BP85	47,5	42,5
BP86	47,5	42,5
BP110	30	35
BP1	32,5	45
BP111	40	35
BP112	17,5	17,5
BP113	35	40
BP73	-	-
BP115	-	-

Appendix 18 - Aquatic vegetation variables' data for each pool

Pool	UnderwaterVeg(%)	EmergentVeg(%)	MarginVeg(%)
BP33	9,33	4	0
BP17	34	16	7,5
BP14	26	38	0
BP35	0	0	10
BP36	0	30	70
BP37	22	44	70
BP3	0	0	10
BP39	21,33	9,33	10
BP41	0	0	0
BP4	61,33	13,33	80
BP46	0	8	50
BP45	16	10	90
BP47	0	0	0
BP48	61,33	9,33	30
BP49	0	8	10
BP50	70	20	100
BP23	40	20	100
BP2	0	0	10
BP51	0	0	10
BP52	0	0	20
BP42	0	0	20
BP10-A	20	10	40
BP10-B	30	10	90
BP53	80	40	60
BP54	50	10	70
BP11	30	10	60
BP55	70	50	60
BP25	0	0	90
BP56	60	30	80
BP58	10	5	100
BP59	25	5	90
BP62	40	75	80
BP9	70	80	100
BP63	30	15	100
BP64	0	0	10
BP65	60	15	60
BP66	10	1	90
BP67	80	80	70
BP68	40	80	70
BP29	10	0	100
BP27	0	20	60
BP69	10	10	100
BP70	0	10	30
BP60	0	0	60
BP71	5	0	40

BP0	30	10	70
BP72	0	0	60
BP74	20	50	100
BP75	30	15	80
BP76	0	0	50
BP87	20	80	100
BP88	0	20	100
BP89	10	80	100
BP90	0	15	70
BP91	0	20	50
BP77	10	25	90
BP79	5	25	100
BP6	10	15	70
BP92	10	45	100
BP83	10	15	80
BP93	0	0	70
BP94	0	0	10
BP95	0	0	50
BP96	30	0	50
BP97	75	90	100
BP99	0	0	70
BP100	0	0	95
BP102	20	60	100
BP103	20	70	100
BP104	50	0	100
BP105	60	0	100
BP106	35	0	55
BP107	0	0	90
BP108	1	1	100
BP109	5	20	70
BP85	1	20	100
BP86	5	60	90
BP110	1	1	100
BP1	1	0	70
BP111	1	20	100
BP112	0	0	70
BP113	0	0	80
BP73	-	-	-
BP115	-	-	-

Appendix 19 - Substrate variables' data for each pool

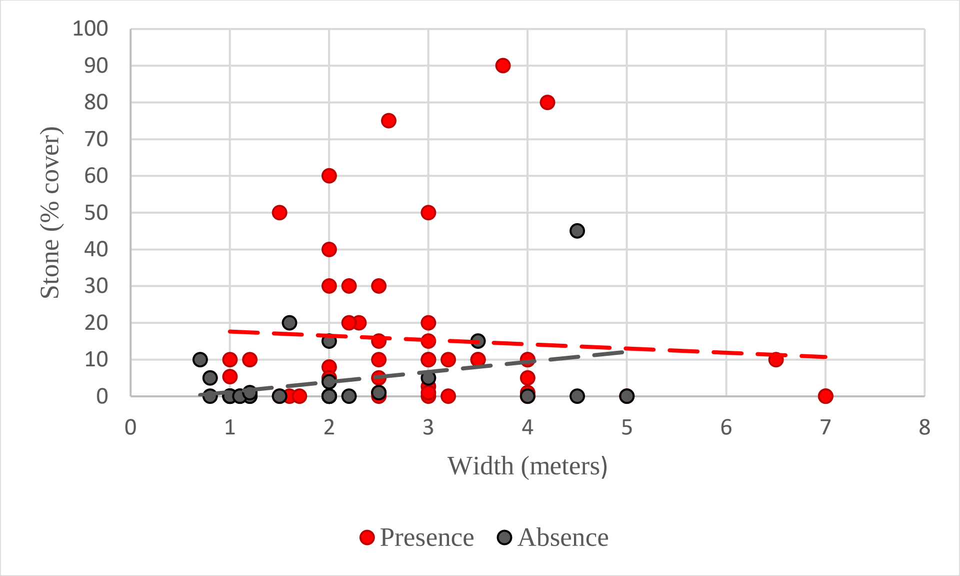
Pool	Stone(%)	Rubble(%)	Gravel(%)	Sand(%)	Silt(%)	LeafLitter(%)
BP33	5,33	9,33	20	65,33	0	0
BP17	0	40	42	4	4	22
BP14	0	0	2	0	60,5	37,5
BP35	40	25	5	0	0	30
BP36	0	0	0	0	10	90
BP37	0	10	16	0	10	79
BP3	20	5,33	20	8	3,333	43,33
BP39	0	57,33	36	0	0	6,67
BP41	0	60	10	30	0	0
BP4	0	33,33	26,667	26,67	0	13,33
BP46	0	60	22,667	9,33	8	0
BP45	0	34	18	46	0	2
BP47	4	60	36	0	0	0
BP48	0	30,67	9,333	0	0	60
BP49	0	8	0	28	0	40
BP50	0	0	0	0	0	100
BP23	0	10	0	0	0	90
BP2	10	85	0	0	0	5
BP51	8	54,67	4	26,67	0	6,67
BP52	2,67	93,33	0	0	0	4
BP42	0	16	0	72	0	12
BP10-A	0	20	0	0	10	70
BP10-B	10	20	0	0	10	60
BP53	10	10	20	0	0	60
BP54	20	30	10	10	0	30
BP11	0	10	0	20	10	60
BP55	0	0	10	0	30	60
BP25	0	60	10	20	10	0
BP56	0	0	85	0	10	5
BP58	5	45	20	30	0	0
BP59	0	55	30	10	0	5
BP62	0	0	0	0	10	90
BP9	0	0	0	0	0	100
BP63	0	30	10	5	15	40
BP64	0	0	0	0	10	90
BP65	10	50	10	10	20	0
BP66	0	80	10	10	0	0
BP67	0	50	10	0	10	30
BP68	0	0	0	0	60	40
BP29	30	30	30	0	0	10
BP27	0	0	0	0	80	20
BP69	0	0	0	0	70	30
BP70	0	0	0	0	70	30
BP60	10	60	0	20	30	0
BP71	10	30	20	20	10	10

BP0	0	10	40	40	0	10
BP72	0	30	20	10	10	30
BP74	0	20	0	0	70	10
BP75	5	60	5	15	0	15
BP76	10	70	10	10	0	0
BP87	1	60	29	10	0	0
BP88	10	80	0	10	0	0
BP89	1	70	10	10	9	0
BP90	1	70	15	5	4	4
BP91	0	40	40	10	5	5
BP77	1	49	40	5	50	0
BP79	5	40	55	0	0	0
BP6	45	35	20	0	0	0
BP92	15	45	30	5	5	0
BP83	15	65	20	0	0	0
BP93	50	30	15	0	5	0
BP94	5	60	35	0	0	0
BP95	5	40	40	10	0	5
BP96	80	10	10	0	0	0
BP97	90	0	0	0	10	0
BP99	75	20	0	0	0	5
BP100	15	60	20	0	0	5
BP102	0	15	75	5	5	0
BP103	10	40	40	5	5	0
BP104	10	30	30	30	0	0
BP105	30	50	10	5	5	0
BP106	20	50	20	5	0	5
BP107	15	70	10	0	5	0
BP108	1	94	0	0	0	5
BP109	0	10	10	40	0	40
BP85	10	40	20	30	0	0
BP86	10	10	75	0	0	5
BP110	1	54	10	10	20	5
BP1	50	40	10	0	0	0
BP111	30	40	20	5	5	0
BP112	20	40	10	0	20	10
BP113	60	30	5	0	5	0
BP73	-	-	-	-	-	-
BP115	-	-	-	-	-	-

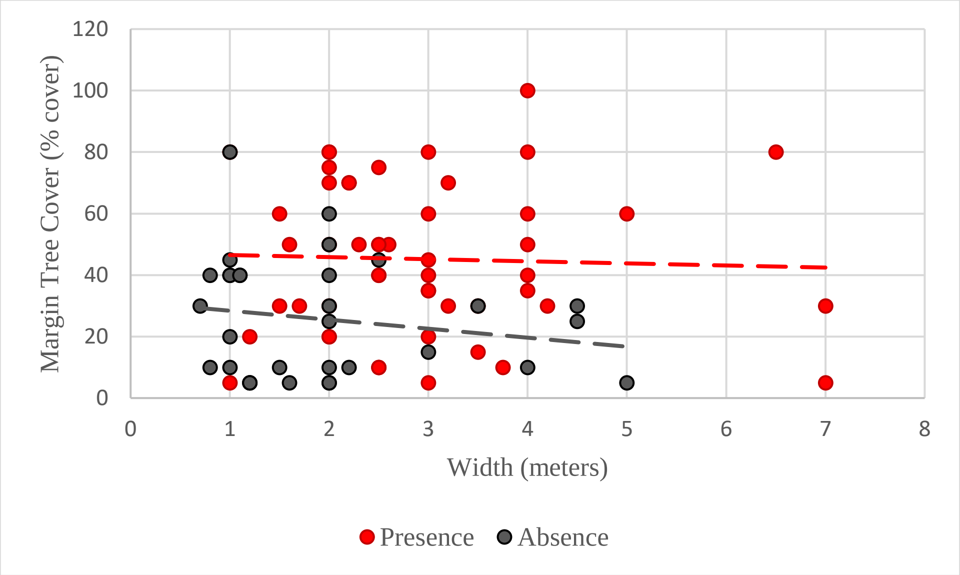
Appendix 20 - Distance to the main watercourse for each pool

Pool	Distance(m)
BP33	375,642
BP17	509,035
BP14	815,664
BP35	520,974
BP36	619,36
BP37	622,323
BP3	743,949
BP39	918,889
BP41	361,164
BP4	61,655
BP46	28,76
BP45	13,57
BP47	40,046
BP48	72,023
BP49	109,309
BP50	147,104
BP23	182,137
BP2	0
BP51	0
BP52	0
BP42	331,356
BP10-A	71,576
BP10-B	62,983
BP53	122,5
BP54	170,568
BP11	254,269
BP55	3266,148
BP25	421,629
BP56	196,236
BP58	84,495
BP59	78,115
BP62	150,164
BP9	419,8
BP63	302,722
BP64	405,757
BP65	0
BP66	336,182
BP67	769,529
BP68	143,489
BP29	3053,5
BP27	3366,476
BP69	3081,352
BP70	1415,914
BP60	1338,142
BP71	0

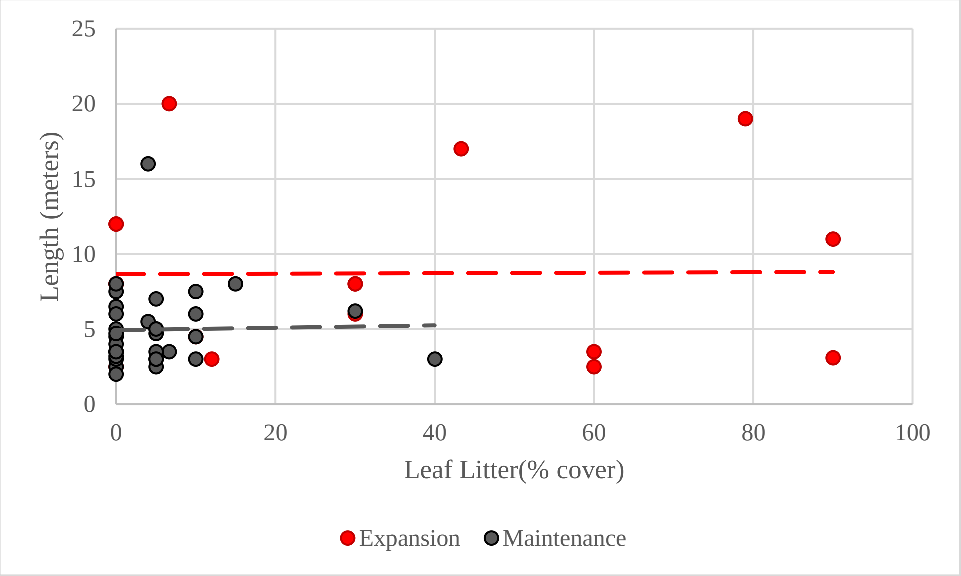
BP0	0
BP72	0
BP74	991,312
BP75	0
BP76	0
BP87	0
BP88	0
BP89	0
BP90	0
BP91	0
BP77	0
BP79	0
BP6	0
BP92	0
BP83	0
BP93	1309,062
BP94	0
BP95	0
BP96	0
BP97	0
BP99	0
BP100	0
BP102	0
BP103	0
BP104	0
BP105	0
BP106	0
BP107	0
BP108	0
BP109	0
BP85	0
BP86	0
BP110	0
BP1	0
BP111	0
BP112	0
BP113	0
BP73	0
BP115	-



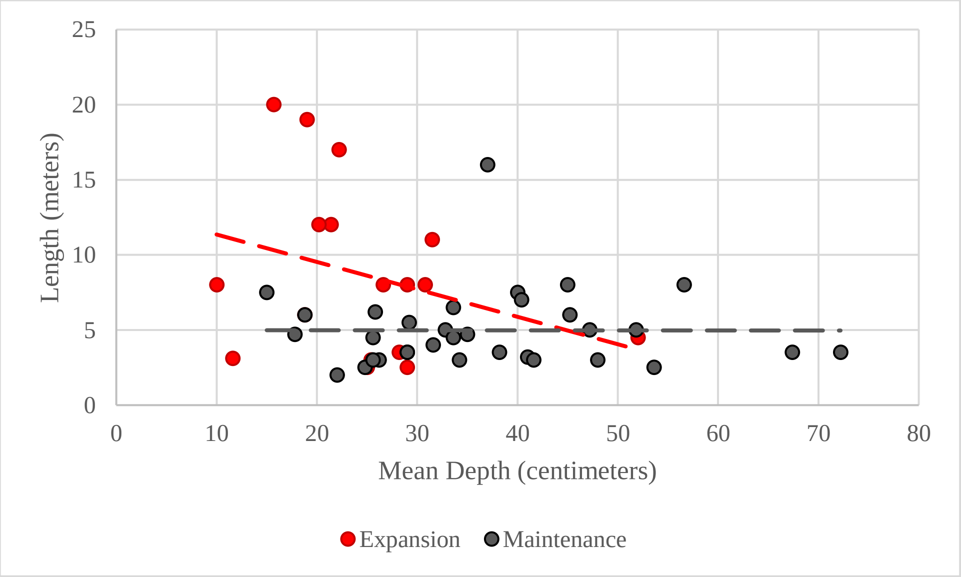
Appendix 21 - Interaction between pool width and stone cover for *P.clarkii* distribution in 2021



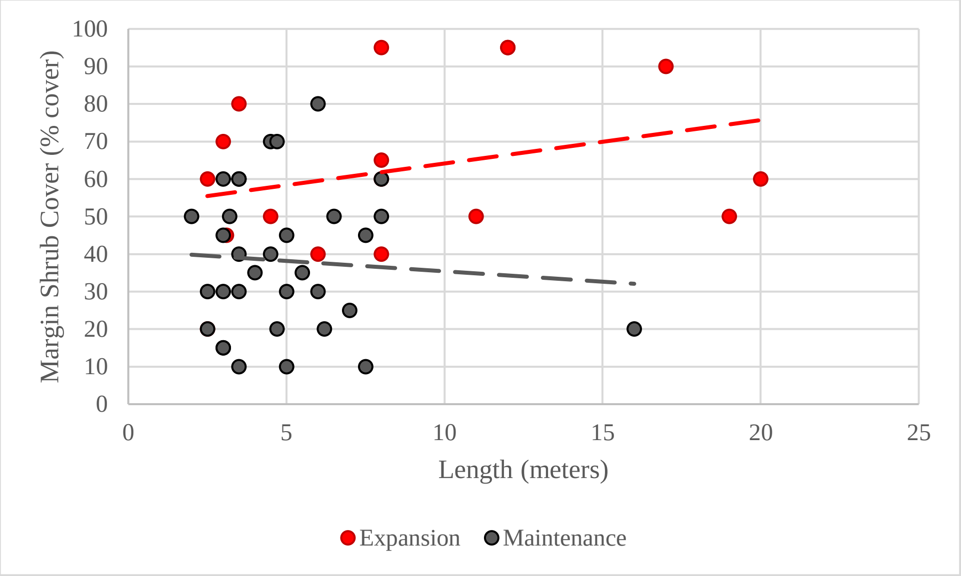
Appendix 22 - Interaction between pool width and margin tree cover for *P.clarkii* distribution in 2021



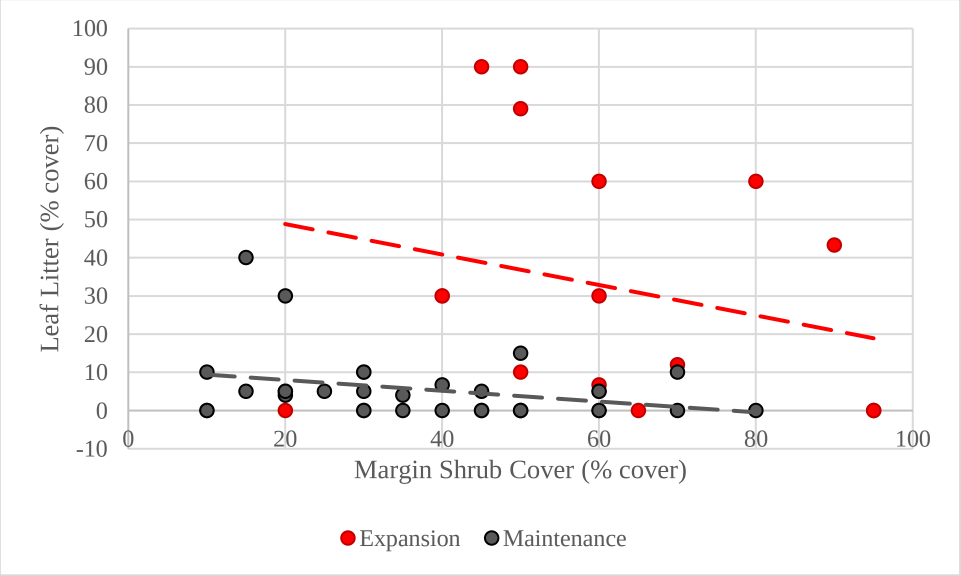
Appendix 23 - Interaction between leaf litter cover and pool length for *P.clarkii* expansion



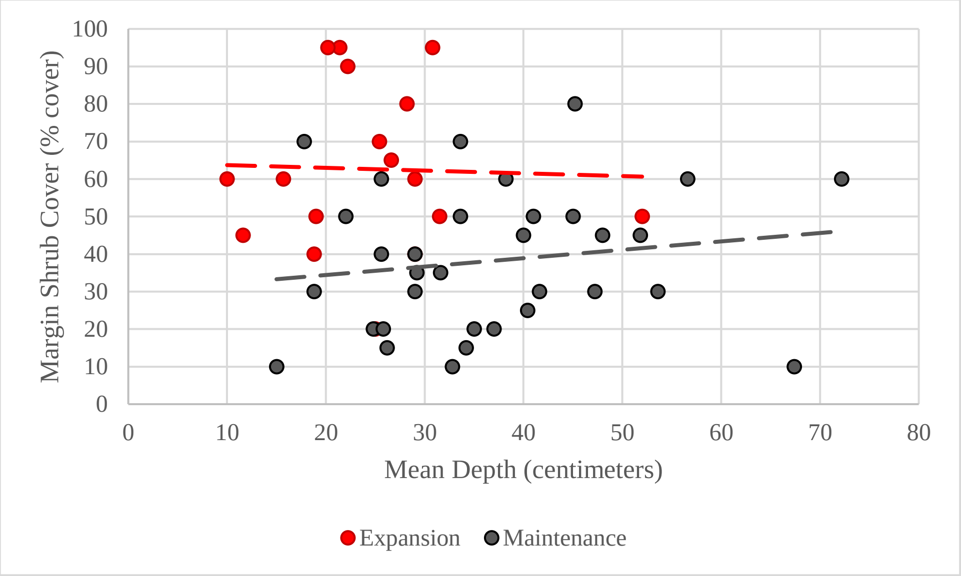
Appendix 24 - Interaction between pool mean depth and length for *P.clarkii* expansion



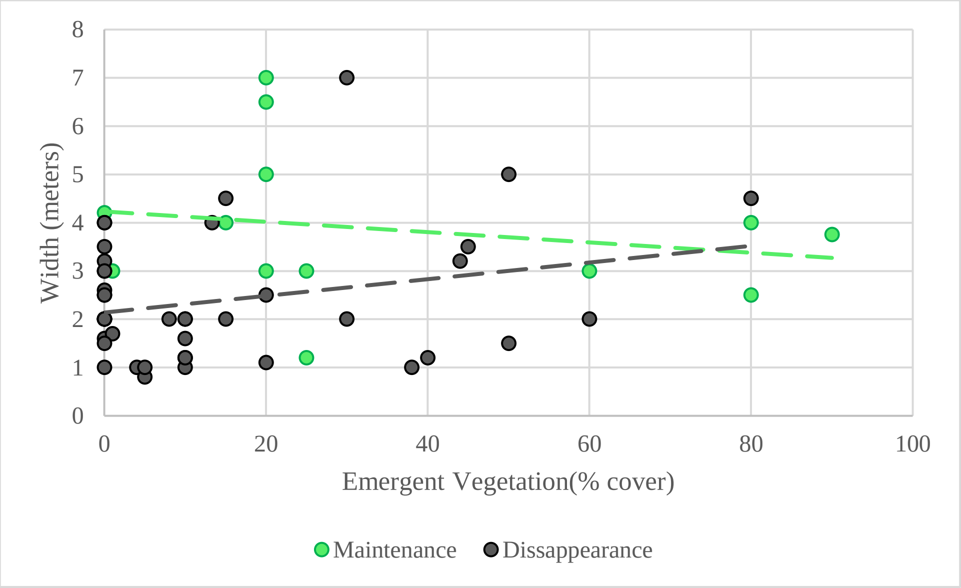
Appendix 25 - Interaction between pool length and margin shrub cover for *P.clarkii* expansion



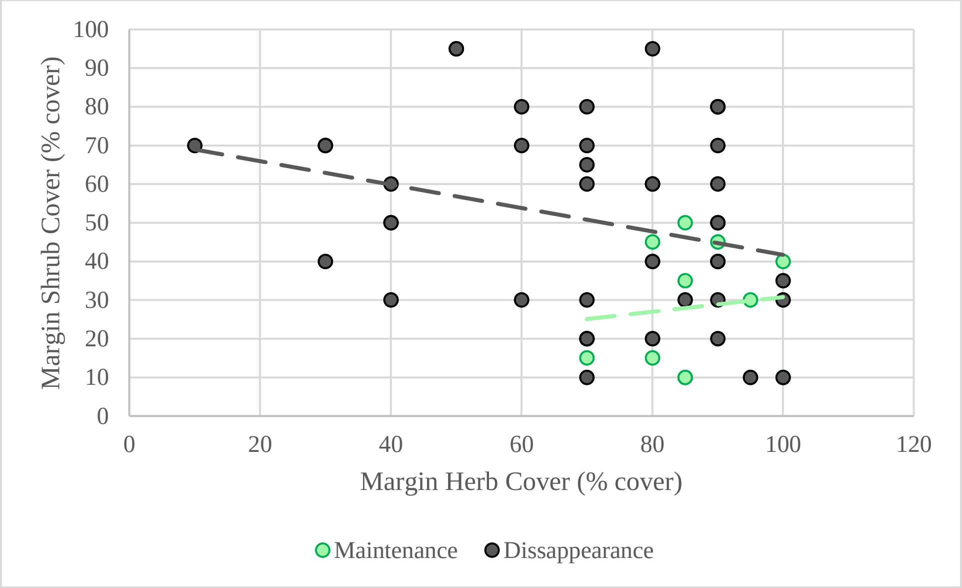
Appendix 26 - Interaction between margin shrub cover and leaf litter cover for *P.clarkii* expansion



Appendix 27 - Interaction between pool mean depth and margin shrub cover for *P.clarkii* expansion

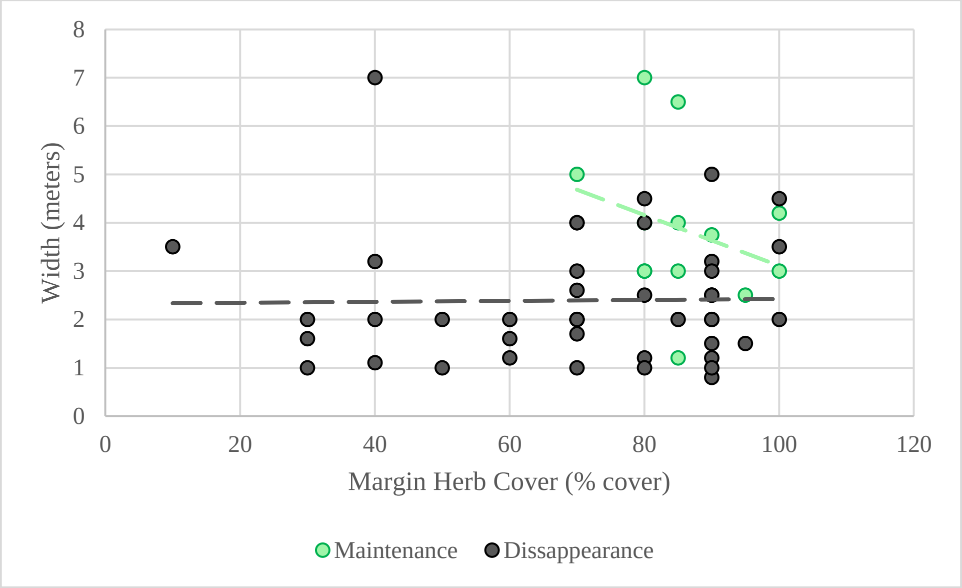


Appendix 28 - Interaction between emergent vegetation cover and pool width for *A. cisternasii* maintenance

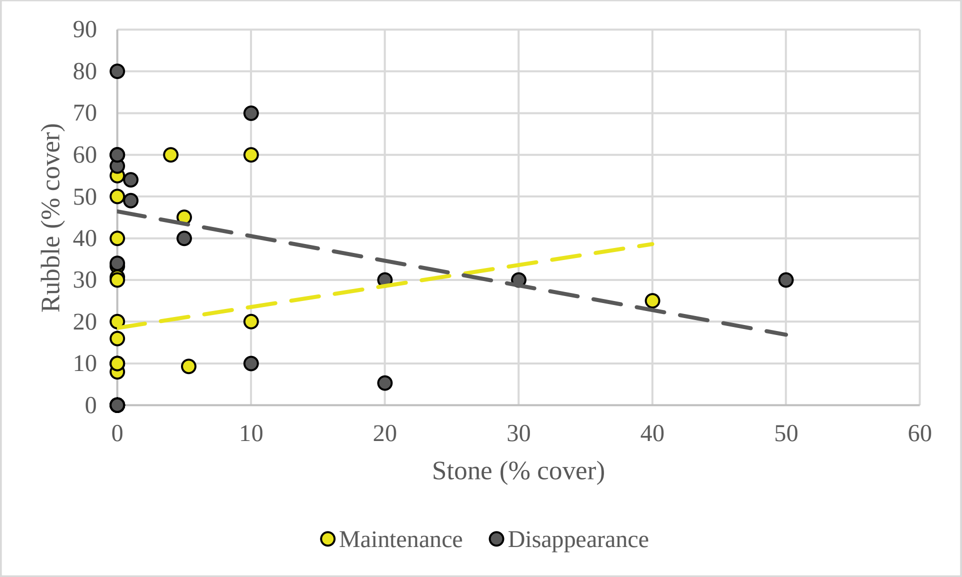


Appendix 29 - Interaction between margin herb cover and margin shrub cover for *A. cisternasii* maintenance

Appendix 30 - Interaction between pool width and margin shrub cover for *A. cisternasii* maintenance



Appendix 31 - Interaction between margin herb cover and pool width for *A. cisternasii* maintenance



Appendix 32 - Interaction between stone and rubble cover for *S. salamandra* maintenance