UNIVERSIDADE DE LISBOA
FACULDADE DE CIÊNCIAS

Warm vegetarians? Heat wave effects on aquatic omnivorous ectotherms

Doutoramento em Biologia
Especialidade em Ecologia

Bruno Martins Carreira

Tese orientada por:
Professor Doutor Rui Rebelo e Professor Doutor Anssi Laurila

Documento especialmente elaborado para a obtenção do grau de doutor

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Nota Prévia

A presente tese apresenta resultados de trabalhos já publicados ou em preparação para publicação (capítulos 2 a 5), de acordo com o previsto no nº 2 do artigo 25º do regulamento de Estudos Pós-graduados da Universidade de Lisboa, publicado no Diário de República II série nº 57 de 23 de Março de 2015. Tendo os trabalhos sido realizados em colaboração, o candidato esclarece que participou integralmente na conceção dos trabalhos, obtenção dos dados, análise e discussão dos resultados, bem como na redação dos manuscritos.

Lisboa, Setembro de 2016

Bruno Martins Carreira

To the pirates

of the past, present and future

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Resumo

Algumas questões ecológicas clássicas voltaram a ganhar relevância devido às projecções futuras das alterações climáticas em curso e à perda de biodiversidade sem precedentes a que se assiste mundialmente. Entre estas questões destaca-se a influência da temperatura na aquisição de nutrientes em animais ectotérmicos, recentemente identificada como fundamental para a compreensão dos impactos das alterações globais e para uma previsão realista das alterações nos ecossistemas.

Os resultados de estudos experimentais recentes mostraram que a temperatura tem efeitos diferentes nas componentes anabólica e catabólica do metabolismo, que podem favorecer a assimilação de dietas vegetais (ricas em hidratos de carbono) a temperaturas mais elevadas. O efeito da temperatura nos processos catabólicos é mais forte do que nos processos anabólicos e, consequentemente, o maior aumento da respiração do que do crescimento à medida que a temperatura aumenta dá origem a uma maior necessidade de carbono do que de azoto. Além disso, visto que um aumento da temperatura promove um maior aumento da taxa de consumo do que da taxa de assimilação na parede do intestino, as temperaturas mais elevadas podem dificultar a assimilação de nutrientes de digestão lenta. Sendo, assim, favorecida a assimilação de nutrientes de menores dimensões e estruturalmente menos complexos, como os hidratos de carbono. Outros estudos sugerem ainda que, dado o maior teor em hidratos de carbono das dietas vegetais, o aumento do seu consumo pode ajudar os animais ectotérmicos a satisfazer as maiores necessidades energéticas impostas pelas temperaturas mais elevadas, porque os hidratos de carbono constituem fontes de energia de disponibilização rápida. E, de facto, alguns estudos recentes demonstraram um aumento da preferência por fontes de hidratos de carbono por ectotérmicos sujeitos a temperaturas elevadas.

As comunidades dulçaquícolas são particularmente vulneráveis às alterações climáticas, uma vez que são compostas maioritariamente por espécies ectotérmicas, cujo metabolismo é fortemente influenciado pela temperatura. Esta relação é particularmente preocupante, visto que as projecções climáticas indicam, para além do aquecimento global, um aumento da frequência e intensidade de eventos climáticos extremos, como por exemplo inundações, secas e ondas de calor. Embora negligenciados até recentemente, estes eventos climáticos discretos podem representar uma forte ameaça, uma vez que os organismos podem ser incapazes de responder de forma eficaz e imediata. Os modelos climáticos de circulação geral indicam um aumento da intensidade, frequência e duração das ondas de calor, especialmente nas áreas actualmente mais afectadas por estas anomalias climáticas, como é o caso da bacia do Mediterrâneo. Na Península Ibérica, o aumento da severidade das ondas de calor decorrerá sobretudo no mês de Junho, coincidindo com o período de seca dos charcos temporários mediterrânicos.

O objetivo principal desta tese foi investigar os efeitos da temperatura na aquisição de nutrientes em omnívoros ectotérmicos pertencentes a três Filos diferentes. Mais especificamente, procurou-se determinar: 1) se o desempenho com dietas animais diminuiu a temperaturas mais elevadas; 2) se o desempenho com dietas vegetais aumentou a temperaturas mais elevadas; 3) se a assimilação dos dois tipos de dieta variou com a temperatura; 4) se as alterações na assimilação melhoraram o desempenho dos indivíduos; e 5) se as alterações na assimilação foram devidas a mudanças nas preferências alimentares. Como modelos de estudo foram utilizadas cinco espécies dulçaquícolas, facilmente encontradas em charcos temporários mediterrânicos: larvas de três anuros que se reproduzem ao longo de um gradiente climático sazonal (*Discoglossus galganoi*, *Hyla arborea* e *Hyla meridionalis*), um gastrópode (*Radix balthtica*) e um decápode invasor (*Procambarus clarkii*). Os indivíduos destas espécies foram alimentados à base de dietas animais, vegetais ou mistas, e expostos a regimes térmicos semelhantes aos impostos pelas ondas de calor de curta duração que ocorrem actualmente no sudoeste de Portugal, ou a ondas de calor prolongadas, que deverão tornar-se mais frequentes no futuro. As respostas aos tratamentos experimentais foram avaliadas pela medição da sobrevivência dos indivíduos, de alterações nos parâmetros da história de vida e, adicionalmente no caso das larvas de anuro, de alterações comportamentais. Nos indivíduos alimentados com a dieta mista estimaram-se ainda as proporções de material animal e vegetal assimiladas utilizando isótopos estáveis. A quantificação do consumo é uma tarefa difícil em espécies de tamanho reduzido, especialmente quando são aquáticas, e a análise dos rácios de isótopos estáveis ($^{13}C/^{12}C$, $^{15}N/^{14}N$) permitiu a obtenção de estimativas integradas no tempo da dieta destas espécies.

A tese está organizada em 6 capítulos, correspondendo os capítulos 1 e 6, respectivamente, a uma introdução geral e a uma discussão onde é feita uma integração dos principais resultados. Os capítulos 2 a 5 correspondem a trabalhos já publicados ou submetidos para publicação em revistas com circulação internacional e sujeitas a revisão por pares.

No Capítulo 2 são apresentados os resultados das experiências realizadas com vertebrados (três espécies de anuros). Foi demonstrada a ocorrência de um gradiente na especialização das preferências alimentares das larvas das três espécies, que é concordante com o gradiente de temperatura mínima a que estas larvas estão naturalmente sujeitas – de uma maior carnivoria na espécie que se reproduz em águas mais frias a uma maior herbivoria na espécie que se reproduz nas mais quentes. Observou-se também uma alteração da qualidade das dietas com a temperatura, caracterizada por uma diminuição do desempenho com dietas animais e por um aumento do desempenho com dietas vegetais a temperaturas mais elevadas. Foram ainda detectadas alterações na assimilação de matéria vegetal em resposta às ondas de calor que, apesar de não terem sido uniformes em todas as espécies, foram sempre no sentido de uma maior assimilação da dieta vegetal. Embora adaptativa numa espécie e potencialmente adaptativa noutra, esta resposta revelou-se mal-adaptativa na espécie mais carnívora.

No Capítulo 3 são apresentadas as alterações comportamentais sofridas pelos girinos no decurso da experiência referente ao capítulo 2. Demonstrou-se que a alteração da qualidade das dietas experimentais induzida pela temperatura foi acompanhada por mudanças na actividade dos girinos de todas as espécies. Usando a redução no crescimento como uma indicação de stress, verificou-se que a variação nos níveis de actividade dos girinos em função do nível de stress a que estiveram sujeitos se adequou a uma típica curva de desempenho. Neste caso, a baixa actividade nos dois extremos da curva é explicada por ausência de stress (cauda à esquerda) e stress intenso (cauda à direita), enquanto que a actividade elevada no centro da distribuição é explicada por uma resposta a níveis moderados de stress, cuja eficácia depende da sua intensidade. Verificou-se que os girinos regularam a sua actividade de acordo com os níveis de stress a que foram sujeitos nas diferentes combinações de dieta e temperatura.

No Capítulo 4 foram estudadas as respostas de uma espécie invasora de decápode – o lagostim-vermelho-americano. O aumento da assimilação de material vegetal nas ondas de calor foi visível essencialmente nos indivíduos juvenis, permitindo-lhes a manutenção de uma taxa elevada de crescimento na onda de calor longa, o que é uma indicação que esta alteração na assimilação da dieta vegetal foi adaptativa. Surpreendentemente, embora esta espécie seja nativa de regiões com climas mais quentes do que o da Península Ibérica, os indivíduos de *P. clarkii*, juvenis e adultos, foram afectados negativamente pelas ondas de calor. No entanto, a assimilação diferencial da dieta vegetal pelos juvenis demonstrou a existência de um potencial para o aumento do impacto desta espécie por herbivoria com o aumento da temperatura, potencialmente agravando os seus impactos económicos nas plantações de arroz.

No Capítulo 5, onde são apresentados os resultados da experiência realizada com um gastrópode, verificou-se um aumento da assimilação da dieta vegetal em ambas as ondas de calor, o que na onda de calor de longa permitiu aos caracóis alimentados com a dieta mista alcançar uma taxa de crescimento superior, sem custos evidentes no número de ovos produzidos. Além disso, o aumento desproporcional na assimilação de material vegetal pelos caracóis na onda de calor curta, indicou que para além de um efeito da temperatura na assimilação desta dieta, pode ainda ter havido alteração das preferências alimentares e aumento da herbivoria.

De um modo geral, as respostas em termos de sobrevivência e da alteração dos parâmetros da história de vida suportam que a qualidade das dietas variou com a temperatura, e que o desempenho com dietas animais diminuiu a temperaturas mais elevadas, contrariamente ao desempenho com dietas vegetais, que aumentou. Embora o tipo de onda de calor responsável pelo aumento na assimilação da dieta vegetal tenha variado e as espécies tenham respondido com intensidades diferentes, em geral as ondas de calor aumentaram a assimilação de material vegetal, o que foi adaptativo em quase todas as espécies. Apesar de não terem sido medidos consumos reais (mas sim a proporção de material assimilado com diferentes origens), os resultados sugeriram ainda que, em alguns casos, a maior assimilação da dieta vegetal a temperaturas mais elevadas pode ter sido causada por um aumento da herbivoria, ou seja, alteração nas preferências alimentares e não apenas alterações nas taxas de assimilação de alimentos já ingeridos.

Em conclusão, a repetibilidade dos resultados em espécies de três Filos diferentes sugere fortemente que a temperatura tem um papel importante na modulação da aquisição de nutrientes – um efeito latente e generalizado em organismos ectotérmicos que era desconhecido até muito recentemente. A maior assimilação de material vegetal, ou mesmo da herbivoria, pode ajudar os ectotérmicos omnívoros a suportar o stress imposto por temperaturas mais altas. Contudo, uma redução generalizada do nível trófico destes organismos pode desencadear alterações no funcionamento e estrutura das comunidades de água doce e das suas teias alimentares. Ainda que os padrões aqui descritos revelem apenas uma vertente da influência da temperatura na aquisição de nutrientes, a consciência da existência deste efeito irá contribuir para uma melhor compreensão e reavaliação dos efeitos ecológicos das alterações climáticas em curso.

Abstract

Freshwater communities are particularly vulnerable to climate change, as they are mainly composed of ectotherm species, whose metabolism is strongly influenced by temperature. This relationship is particularly relevant, as projections show both a global warming and an increase in the frequency, intensity and duration of extreme climatic events such as heat waves. Measurements of imbalanced temperature effects on various metabolic aspects of ectotherms lead to the suggestion that these organisms may optimize energetic intake at higher temperatures by increasing herbivory and avoiding protein-rich animal diets.

In this thesis, I tested the effects of different types of heat waves on diet assimilation (and indirectly on diet choice) of five species of freshwater omnivorous ectotherms, belonging to three different phyla. As study models, I used species commonly found in Mediterranean temporary ponds: the tadpoles of three anuran species breeding along a seasonal climatic gradient (*Discoglossus galganoi*, *Hyla arborea* and *Hyla meridionalis*), the gastropod *Radix balthica* and the invasive crayfish *Procambarus clarkii*. Individuals were fed animal, plant or mixed diets and exposed to temperature treatments simulating the current short heat waves, as well as long heat waves expected to become more frequent in the future. Treatment responses were recorded in terms of survival and life history traits. In the individuals feeding on the mixed diet, the proportion of animal and plant material assimilated was estimated with stable isotopes.

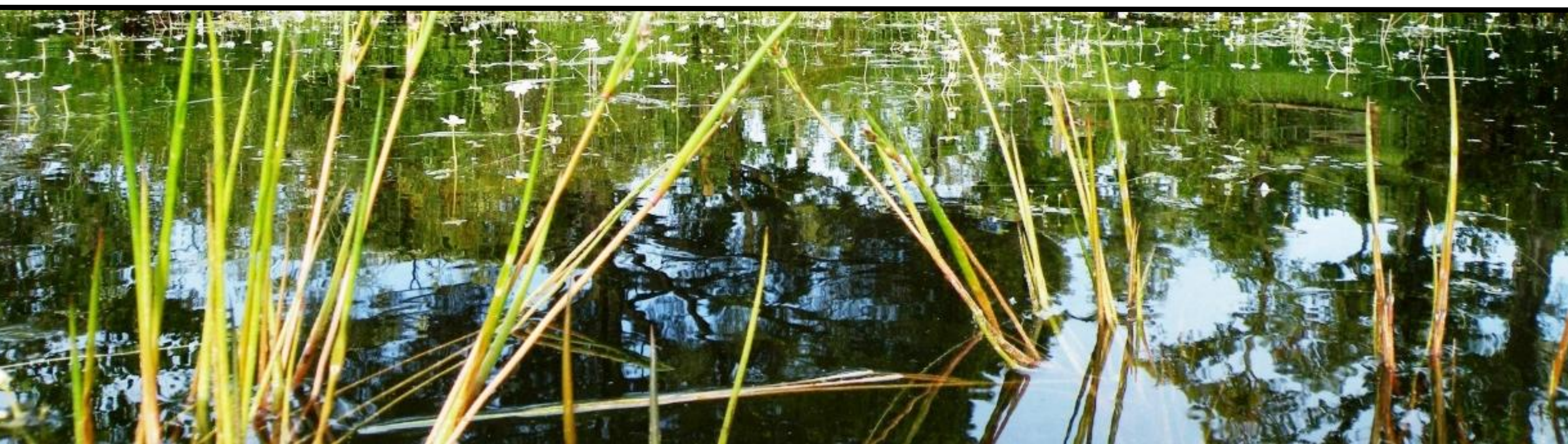
Overall, I found evidence that performance at higher temperatures decreased on animal diets and increased on plant diets, suggesting that diet quality changed with temperature. Although the type of heat wave inducing assimilation shifts varied and the species responded to different extents, heat waves generally increased the assimilation of plant material, and this was adaptive in almost all species. Furthermore, the seasonal

climatic gradient in the three anuran species was found to match a gradient in their feeding preferences, the degree of herbivory increasing with minimum temperature experienced in nature. Surprisingly, albeit native to climates warmer than the Iberian Peninsula, the invasive crayfish *P. clarkii* was negatively affected by the heat waves. However, the results of nutrient assimilation showed that warmer temperatures may increase this species' impact in freshwater communities through increased herbivory.

In conclusion, my results suggest that temperature plays an important role in modulating nutrient acquisition in omnivorous ectotherms. A greater assimilation of plant material or increased herbivory may help ectotherms to cope with the challenges imposed by higher temperatures. However, a reduction in the trophic positions of the omnivores may trigger changes in the functioning and structure of freshwater communities and food webs.

CHAPTER 1

General Introduction



CHAPTER 1

General Introduction

Metabolic ecology

Climate change unfolded many ecological questions that have gained particular relevance under the projections for future climates and the current unprecedented biodiversity loss. Among these topics, the influence of temperature on nutrient acquisition by organisms is now attracting a great deal of interest and recently became the focus of several studies. Indeed, better understanding of temperature and nutrient interactions across ecological organization levels was recently identified as critical for realistic predictions of ecological responses to global change drivers (Cross et al. 2015).

Several large-scale studies on marine and freshwater fish communities have found an association between latitude and the prevalence of herbivory, with higher relative abundance and richness of herbivorous species at lower latitudes (Floeter et al. 2005, Behrens & Lafferty 2007, Jeppesen et al. 2010). Furthermore, the extent of herbivory in omnivorous invertebrates was found to increase at lower latitudes (Pennings et al. 2009, Schemske et al. 2009, Gonzalez-Bergonzoni et al. 2012). Several factors could be driving these patterns (Floeter et al. 2005): (1) insufficient time for the evolution of herbivory or for the expansion of herbivorous species into temperate and cold waters; (2) a selection pressure against herbivory caused by the seasonal and prolonged shortage of algae in temperate habitats; (3) lower palatability of algae at higher latitudes; (4) an earlier evolution of herbivory among tropical fish communities and, consequently, a more efficient use of low energy food resources such as algae and sponges. However, discarding the previous hypotheses, the authors suggest (5) temperature-related physiological constraints to herbivory at higher latitudes, implying that some herbivorous fishes may be unable to meet their energetic demands in colder temperatures due to a thermal constraint on the digestion of algae. This may be only a partial explanation, as it is also possible that some carnivorous ectotherms are unable to meet their nutritional demands at higher temperatures.

Experimental evidence suggests that various components of metabolism of ectotherms may scale differently with temperature and that these imbalanced effects may favour herbivory at higher temperatures. Some studies show that metabolic processes may positively discriminate plant diets (carbohydrate-rich) at higher temperatures. By promoting a greater increase in feeding and gut passage rates than in assimilation (Myrick & Cech 2000, Seifert et al. 2014), higher temperatures passively favor the assimilation of smaller and structurally less complex nutrients, increasing the assimilation efficiency of carbohydrates. Likely linked to this, the protein to carbohydrate assimilation ratio of *Procambarus zonangulus* and *Procambarus clarkii*, two freshwater crayfishes, was found to shift to a greater assimilation of carbohydrates as temperature increases (Croll & Watts 2004). Another aspect in favor of greater herbivory at higher temperatures is the enhanced digestion of plant material aided by the more abundant and diverse gut microflora (Clements et al. 2009).

Alternatively, other studies highlight that increasing the consumption of plant diets (carbohydrate-rich) would help ectotherms to better cope with the greater energetic demands at higher temperatures. The temperature effect on catabolic processes, which takes place in all of the organism’s cells and are proportional to tissue volume, is stronger than on anabolic processes, which are limited by the passage of molecules trough bidimensional structures like the gut or cell walls. Consequently, as temperature rises it promotes a greater increase in respiration than in growth and increases demand for carbon over nitrogen (Karl & Fischer 2008, Forster et al. 2011). Furthermore, because temperature promotes a greater increase in metabolic than in feeding rates (Kingsolver & Woods 1997, Lemoine & Burkepile 2012), higher temperatures decrease digestion efficiency and the consumption of fast energy sources could compensate for this. Indeed, recent studies showed not only that higher temperatures increased caterpillar preference for carbohydrates (Lee et al. 2015), but also that locusts preferred higher temperatures when fed on carbohydrate-rich diets (Clissold et al. 2013). More recent studies showed a seasonal variation in the trophic level of marine copepods, lower in the summer, and an avoidance of animal diets (protein-rich) at higher temperatures (Boersma et al. 2016, Malzahn et al. 2016). The same studies also showed an increased performance on plant diets at higher temperatures, in opposition to a decreased performance on animal diets, leading the authors to suggest that increasing the assimilation of plant material at higher temperatures is an adaptive response.

The large-scale patterns in the abundance and relative richness of herbivorous and omnivorous fishes, as well as the imbalanced temperature effects on various metabolic aspects suggest that ectotherms may optimize energetic intake at higher temperatures by increasing herbivory and avoiding protein-rich diets. However, some studies found no evidence for temperature-induced changes in the feeding preferences of locusts (Miller et al. 2009) and freshwater snails (Zhang et al. 2016), but only increased consumption ratios driven by the accelerated metabolism at higher temperatures (Gillooly et al. 2001).

Food web implications

Temperature-induced changes in the feeding preferences that compensate for the changes in the relative quality of animal and plants diets are unlikely to affect all ectotherms equally. Over short time scales this response should be adopted by omnivorous species, which – given their necessarily higher trophic plasticity – should more easily shift their trophic preferences. The potential unidirectional shift toward greater herbivory induced in omnivorous ectotherms by the increasing temperatures may have a strong impact on freshwater food webs (Estes et al. 2011), since feeding behavior is particularly important in shaping their structure (Kortsch et al. 2015).

The trophic downgrading that results from temperature-induced increased herbivory in omnivores may affect food webs in two distinct ways. Firstly, omnivory buffers the effects of perturbations by promoting stable equilibria and reducing the probability of extinctions (Vandermeer 2006, Ingram et al. 2009). The greater array of omnivore trophic interactions increases food web resilience (Stouffer & Bascompte 2011), as perturbations are retained and diffused through the many trophic links, delaying or stopping propagation to other trophic levels (Krause et al. 2003, Bascompte et al. 2005, Stouffer & Bascompte 2011). Ultimately, omnivory reduces the likelihood of trophic cascades or strong population cycles (Thompson et al. 2007). Secondly, macrophytes are important to the maintenance of the “clear water state” in freshwaters and the greater herbivory associated with trophic downgrading may induce a switch from a clear to a turbid state, as phytoplankton replaces the macrophyte community (Rodríguez et al. 2003). This shift is usually accompanied by deep changes in food web structure, through cascading effects, driven by the loss of biodiversity during the transition from clear to turbid state (Rodríguez et al. 2003).

Freshwater communities

Freshwater habitats cover 0.8% of the Earth’s surface and contain only 0.01% of the world’s water, yet they are home to a high number of animal and plant species, with unique adaptations. Recent estimates indicate that freshwater habitats may harbor as much as 6% of the global biodiversity and one third of all vertebrate species (Dudgeon et al. 2006). This places freshwater biodiversity disproportionally at risk (Parmesan 2006, Perkins et al. 2010, Woodward et al. 2010), and raises great concern as biodiversity loss in freshwater habitats is far more pronounced than in the most endangered terrestrial ecosystems (Ricciardi & Rasmussen 1999). Often occurring isolated and fragmented within the terrestrial landscape (Perkins et al. 2010), freshwater habitats are particularly vulnerable to climate change (Hobday & Lough 2011). Furthermore, the effects of temperature change are expected to be particularly pervasive, as freshwaters are dominated by ectotherms, which compose approximately 99% of their communities (Parmesan & Yohe 2003, Woodward et al. 2010). Consequently, global warming is expected to have a strong impact on individual metabolism and by extension on the physiology, bioenergetics, behavior, abundance and distribution of ectotherms, which will affect all organizational levels of freshwater ecosystems (Ings et al. 2009).

Climate change

Over the last decades an increasing number of studies showed broad effects of climate change on natural systems (Chapman et al. 2014). Climate change effects at the species level has been reported in morphology (Sheridan & Bickford 2011, Goodman et al. 2012), abundance and distribution (Barry et al. 1995, Parmesan & Yohe 2003, Perry et al. 2005, Thomas 2010, Dietl & Flessa 2011), and phenology (Parmesan & Yohe 2003, Walther 2004, Parmesan 2006, Burgmer et al. 2007, Pau et al. 2011). At the community level, changes have been reported in species interactions (Voigt et al. 2003, Van der Putten et al. 2004, Schweiger et al. 2008, Tylianakis et al. 2008, Van der Putten et al. 2010) and community diversity (McKee et al. 2002, Daufresne et al. 2009); and at ecosystem level altered function and service provisioning (Traill et al. 2010, Lavergne et al. 2010).

Most of this research addressed changes in the mean climatic conditions. However, changes in mean values are long-term trend effects and organisms may acclimate through phenotypic plasticity, evolve genetic adaptations or migrate and follow the displacement of suitable habitats (Gunderson et al. 2010, Hoffmann & Sgrò 2011). By contrast, discrete weather events may pose a greater challenge, because organisms may be unable to display an immediate and effective response, even if they have the potential to evolve genetic adaptations (Reusch et al. 2005). Neglected until recently, short-term stress resulting from increased climatic variability and extreme climatic events is now recognized as an important component of climate change (Chapman et al. 2014, Vasseur et al. 2014). These events constitute large, infrequent, stochastic perturbations (e. g. floods, droughts, heat waves) that can severely affect species' fitness and life-history traits, and change ecological processes (Thibault & Brown 2008, Clusella-Trullas et al. 2011, Paaijmans et al. 2013, Kingsolver et al. 2013).

Varying in duration and intensity, heat waves are considered to occur when the daily maximum temperature exceeds the average maximum temperature by 5^{o}C for more than five consecutive days, the reference period being 1961–1990 (Frich et al. 2002). Recent studies showed that heat waves were responsible for episodes of mass mortality (Garrabou et al. 2009); decreased condition, immune function and storage reserves (Fischer et al. 2014); range shifts and loss of genetic diversity (Reusch et al. 2005); and changes in species interactions (Eggers et al. 2012), community structure (Ma et al. 2015) and ecosystem function (Wernberg et al. 2012).

Global climatic models project more intense heat waves, with positive anomalies greater than 3°C; more frequent heat waves, with up to a 31% increase in the frequency of occurrence; and longer lasting heat waves, with the average duration expected to increase up to 35% (Meehl & Tebaldi 2004). A severity increase expected to be more marked in the areas currently most susceptible to heat waves, such as the Mediterranean basin, one of the world’s two most prominent climate change “hot-spots” (Meehl & Tebaldi 2004, Giorgi 2006). In the Iberian Peninsula, greater severity of heat waves should be concentrated in the month of June (Dasari et al. 2014). Including increased temperature variability, current climatic models explain the occurrence of events statistically extremely unlikely such as the summer of 2003, for which heat wave conditions persisted over nearly 50 days (Schär et al. 2004, Dasari et al. 2014).

In the southwest of Portugal, heat waves occur frequently during spring, when important freshwater ecosystems such as Mediterranean temporary ponds are home to larvae and adults of many ectotherm species. Typically, this region is afflicted by a heat wave in the end of April that lasts on average 7.8 ± 1.3 days, which in some years is followed by a second heat wave in mid-May that lasts on average 7.5 ± 1.3 days (B. M. Carreira, unpubl. data 2002-2012; Fig. 1A). A five-year survey (2010-2014) carried out in 42 temporary ponds of Southwest Portugal (Fig. 1B) showed that during April and May the water temperature ranged from 17 to 25^{o}C, with an average temperature of 20^{o}C (R. Rebelo, unpubl. data).

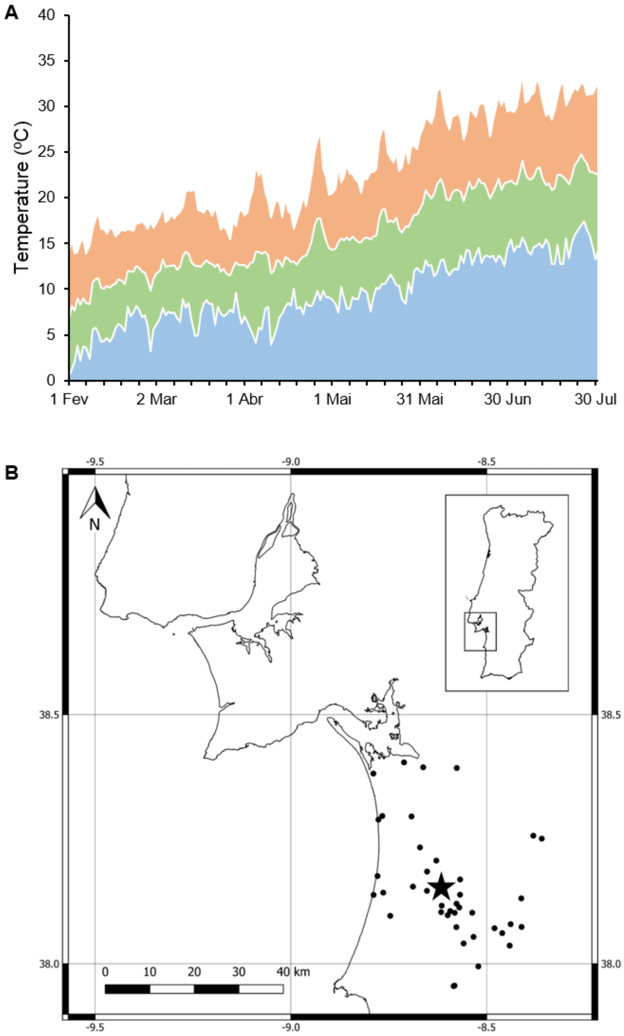


Figure 1. A) Average daily air temperatures at the field station in Grândola for the period of 2002-2012; minimum temperature in blue, average temperature in green and maximum temperature in orange. B) Spatial distribution of the 42 temporary ponds in the Southwest Portugal for which temperatures were surveyed for the period of 2010-2014; the dots mark temporary ponds and star marks the field station in Grândola (Fig. 1B).

Invasive alien species

Besides climate change, freshwater ecosystems suffer from the effects of other global change drivers that may interact with each other, further increasing damages to their communities (Vitousek et al. 1997, Pereira et al. 2010). For instance, freshwaters are also expected to be strongly afflicted by invasive alien species (IAS), especially in the Mediterranean (Sala et al. 2000, Theoharides & Dukes 2007). The high connectivity of freshwater environments facilitates spreading of IAS and the weakening or removal of geographic barriers by climate change may provide new suitable habitats, opening a window for the expansion of IAS (Dukes & Mooney 1999, Pyšek & Richardson 2010, Leishman & Gallagher 2015). In turn, the impacts of IAS at the invaded areas may increase ecological susceptibility to climate change and impose additional biotic stress upon freshwater food webs (Perkins et al. 2010).

Invasions are more likely to succeed when climatic conditions in the new environments are similar to those in the invasive species native distribution range, but often climatic conditions in the invasive and native ranges differ substantially (Walther et al. 2009, Davidson et al. 2011). For instance, many of the most abundant IAS in Europe are native to tropical and subtropical climates where they experience warmer conditions (Robinet & Roques 2010). Unlike tolerance to the heat, which is largely conserved across species, tolerance to the cold varies among and within species and shapes their global distribution (Araújo et al. 2013). As climate change proceeds, global warming may boost the populations of cold-limited IAS and increase habitat suitability by extending their growing and reproductive seasons, lessening the constraints imposed by cold temperatures (Easterling et al. 1997, Walther et al. 2009, Bradley et al. 2010, Walther et al. 2010). In addition, IAS may also benefit from the changes projected for extreme climatic events, as these strong perturbations often create resource pulses and reduce the communities' biotic resistance to invaders (Easterling et al. 2000, Parmesan et al. 2000, Chesson et al. 2004, Miriti et al. 2007, Thibault & Brown 2008).

Ranking among Europe's worst IAS, the red swamp crayfish *Procambarus clarkii* (Girard, 1852) is native to warm environments in the central south of the USA and the northeast of Mexico, where its optimum growth temperature ranges from 20 to 30ºC (Croll & Watts 2004). This crayfish was first introduced in the Iberian Peninsula in 1973 and rapidly spread through western Europe, being widespread and abundant in the south and sustaining scattered and isolated populations in the north (Chucholl 2011). In Europe, the colder temperatures prompted changes in the life-history of *P. clarkii*, causing a switch from multivoltine to uni- or bivoltine life cycles and the onset of the breeding period to vary latitudinally according to water temperature (Chucholl 2011; Peruzza et al. 2015). Although well-established in the Iberian Peninsula, the Iberian populations experience average minimum temperatures 2 to 5ºC lower than in the native range, with spring and summer temperatures being 7 to 8ºC lower. However, in this region winter minima are projected to rise 3ºC and heat waves are projected to become longer, more frequent and intense (Jerez et al. 2012, Dasari et al. 2014). These changes could alleviate or temporarily lift the constraints imposed by the cold temperatures and boost *P. clarkii* populations. Hence, climatic conditions closer to those in the native distribution range may intensify ecological impacts of invasive *P. clarkii* on biological communities.

Stable isotopes

Studying the diet of animals has always been one of the major challenges in Ecology. Traditional methods such as direct observations and analyses of gut contents or faeces only capture dietary choices over short time windows (Levey & Karasov 1994, Afik & Karasov 1995). Because accounting for the detectability, retention and digestibility of the different prey items and identifying undigested material may be challenging, these inferences are often biased (Kasai et al. 2005). Stable isotopes analysis introduced an expeditious alternative method that provides reliable and time-integrated estimates of the relative importance of the prey items (Szepanski et al. 1999).

Upon nutrient assimilation consumers incorporate the stable isotope ratios of the prey items in their tissues and by comparing the isotopic signatures it is possible to estimate the relative contribution of prey items (sources) and infer diet composition (Inger et al. 2006). Based on Euclidean distances, the earliest stable isotope mixing models allowed only for a restricted number of sources and the output simply reflected the range of possible solutions for the contribution of each prey item (Phillips & Gregg 2001, 2003). Nowadays, these models allow for multiple sources and use Bayesian inference to estimate the contribution of the different prey items to the isotopic signature of the consumers by generating potential dietary solutions as true probability distributions (Inger et al. 2010, Parnell 2010, Lemons et al. 2011). Furthermore, these models allow for concentration dependencies of the elements and incorporate uncertainty in the estimation of the isotopic signature of consumers and sources, corrected for each source trophic enrichment factors (TEF), the increase in consumer isotopic ratio compared to its diet (Hopkins & Fergusson 2012, Parnell et al. 2013). In this thesis, the estimation of the study organisms' feeding preferences was based on stable isotopes signatures. In the stable isotope mixing models the TEF for each of the isotopes, determined using single diet treatments, were specified per species, life stage and sex of the consumers, source (animal and plant diets) and temperature treatment.

Recent Bayesian stable-isotope mixing models are highly sensitive to variation in TEFs and their accurate characterization is of the utmost importance (Bond & Diamond 2011). Although the sources of TEF variation (e.g., taxa, site, tissue) have been reviewed recently by Caut et al. (2009), who developed regression-based methods to estimate TEFs, these estimates should be used only when no data are available. TEFs are calculated when the consumers' isotopic signature reaches isotopic equilibrium with their sources and stabilizes. However, the rate at which elemental isotopes of the diet are incorporated into consumer tissues, the isotopic turnover, depends on several factors (e. g. body size, growth rate, protein turnover), making it hard to predict the temporal window over which consumer tissues reflect their diet (Martínez del Rio et al. 2009). To relax this assumption, researchers often estimate TEF's when the half-life of an element in the tissue has been met, since due to the non-linearity of the turnover rate there is no significant change beyond this time point. Half-life values for ^{13}C and ^{15}N turnover rates were determined in non-experimental individuals maintained at 20ºC and fed on commercial fish food, and found to be in agreement with values obtained in a recent review (Thomas & Crowther 2015). In all cases experiment duration was set to comfortably exceed the half-life values for ^{13}C and ^{15}N turnover rates.

Goals

In this thesis, I addressed the temperature effects on nutrient acquisition in an array of omnivorous ectotherm species. Experimentally, I attempted to simulate the heat waves currently afflicting Mediterranean temporary ponds in the southwest of Portugal and study its effects on the species survival and life history traits. This allowed for a simplified assessment of the fitness effects of contrasting diets on the performance and activity of the study organisms under current short heat waves, with a parallelism to the potential effects of longer heat waves. Additionally, I assessed the potential for these heat waves to alleviate the constraints imposed by cold and facilitate a subtropical alien invasive species. The use of controlled diets facilitated the assessment of each species' feeding choices, estimated through the analysis of stable isotope ratios ($^{13}C/^{12}C$, $^{15}N/^{14}N$). By using these methods, I gained an improved insight of temperature effects on nutrient acquisition and obtained time-integrated estimates of the food assimilated by the study organisms, overcoming the obstacle imposed by their small size.

The study species used in this thesis are commonly found in Mediterranean temporary ponds – high-biodiversity freshwater habitats included in the European Union list of priority habitats for conservation, that contain a unique collection of fauna and flora (Oertli et al. 2005). For a wider taxonomic approach, I chose species representative of the most important taxonomic groups of omnivorous ectotherms inhabiting these ponds, both native (anurans and gastropods) or invasive (decapods) (Fig. 2). Although mostly experimental, this approach relied heavily on data collected in field stations in the southwest of Portugal to address realistic temperatures in nature. I opted to use heat waves to study temperature effects on nutrient acquisition by ectotherms because heat waves are common in the study area and, given the ephemeral character of the Mediterranean temporary ponds, the projected changes in their frequency, intensity and duration may have a profound impact in these ecosystems' communities.

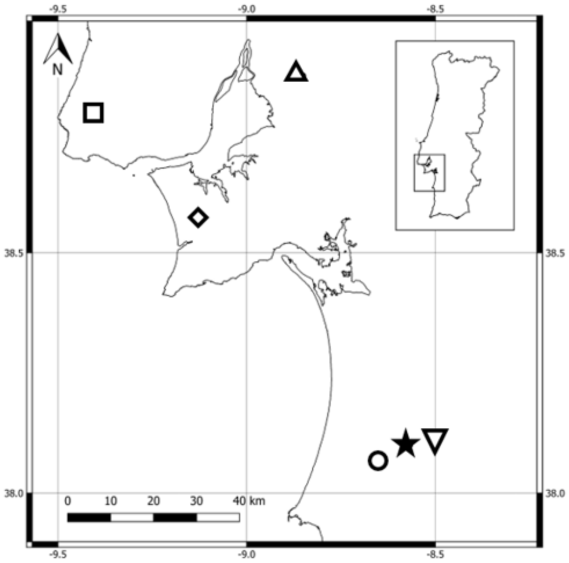


Figure 2. Collection sites of the five species used in the experiments. The star marks the location of the field station.

The main goal of this thesis was to study heat waves effects on omnivorous ectotherms from Mediterranean temporary ponds of the southwest of the Iberian Peninsula within the context of metabolic ecology. More specifically the objectives were to determine if:

1. performance on the animal diets decreased at higher temperatures;
2. performance on the plant diets increased at higher temperatures;
3. higher temperatures induced assimilation shifts;
4. assimilation shifts were adaptive;
5. assimilation shifts resulted from altered feeding preferences.

Papers presented

This thesis compiles a series of four papers (one accepted for publication and three manuscripts), each corresponding to a chapter (Chapters 2–5), in which the specific objectives are addressed along with other particularities. These chapters are preceded by a general introduction to the topics focused in the papers (Chapter 1) and followed by a general discussion where the most important findings are integrated and discussed (Chapter 6).

Chapter 2

Carreira BM, Segurado P, Orizaola G, Gonçalves N, Pinto V, Laurila A, Rebelo R (2016). Warm vegetarians? Heat waves and diet shifts in tadpoles. Ecology 97 (11): 2964–2974.

This chapter addressed all the specific objectives, investigating the role of temperature in determining the feeding preferences of omnivore ectotherms. This was achieved by using tadpoles of three anuran species breeding along a seasonal climatic gradient (*Discoglossus galganoi*, *Hyla arborea*, *Hyla meridionalis*), in which the tadpoles of the earliest species meet lower water temperatures than tadpoles of the later breeding species. Furthermore, this chapter investigated how short heat waves (that these species experience currently) and long heat waves (predicted to increase under climate change) affected tadpole diet assimilation as well as their survival and larval life history.

Chapter 3

Pinto V & Gonçalves N, **Carreira BM**, Laurila A, Rebelo R. Disentangling stress: complex response of tadpole activity level to diet and temperature. *Submitted*

This chapter addresses the first two specific objectives, investigating how the changes in food quality induced by temperature may affect tadpole foraging and how tadpole activity may follow the general shape of a performance curve in function of stress. This was achieved by assessing the isolated and combined effects of diet and temperature on the activity levels of the tadpoles from the three anuran species investigated in the previous chapter, using growth reduction as an indicator and a measure of stress.

Activity levels are easily measured and often used to assess the effects of multiple stressors in aquatic organisms such as tadpoles. Examples include chemical agents (Bridges 1997, Hatch & Blaustein 2000, Fraker & Smith 2004, Smith & Burgett 2005), predation (Werner & Anholt 1993, Bridges 2002, Davis et al. 2012, Nunes et al. 2013), amount and nutritive quality of food (Werner 1992, Anholt & Werner 1995, Semlitsch et al. 1995, Nicieza & Metcalfe 1997, Laurila et al. 1998, Richardson 2001, Barry 2014), and temperature (Finkler & Claussen 1999, Laurila et al. 2008, Katzenberger et al. 2014). However, there are no studies on the combined effects of diet and temperature on tadpole activity levels and, since food quality changes with temperature, addressing them together may bring new developments in the interpretation of tadpole activity in function of stress.

Chapter 4

Carreira BM, Segurado P, Laurila A, Rebelo R. Heat waves in temperate climate: diet shifts and increased performance of a subtropical invasive crayfish? *Submitted*

This chapter addressed all the specific objectives, investigating how current and future heat waves affect a cold-limited invasive species, *Procambarus clarkii*. As this species undergoes an ontogenetic shift in its feeding preferences from mainly carnivorous juveniles to mainly herbivorous adults, heat wave effects on diet assimilation and the subsequent effects on survival and life history traits were addressed separately for juvenile and adult crayfish. Furthermore, this chapter also addressed the potential for climate change to induce a shift in the trophic niche of this keystone species, potentially aggravating its impacts on aquatic vegetation, as well as its economic damage to crops.

Chapter 5

Carreira BM, Segurado P, Laurila A, Rebelo R. Heatwaves increase herbivory in a freshwater snail. *Submitted*

This chapter focused on the three last specific objectives, investigating shifts in the assimilation of the experimental diets induced by short and long heat waves on a freshwater snail, *Radix balthica*. In addition, this chapter addressed the adaptive value of the shifts by assessing heat wave effects on life history traits. Importantly, however, the main focus of this chapter was to discuss the reason for the greater assimilation of plant material at higher temperatures and bring some clarification into the controversy on the temperature effects on ectotherm feeding preferences. More specifically, it focused on the nature of underlying mechanism responsible for assimilation shifts and whether these reflected altered feeding preferences or resulted from a passive positive discrimination of plant material during assimilation.

References

Afik, D., and W. H. Karasov. 1995. The trade-offs between digestion rate and efficiency in warblers and their ecological implications. Ecology 76:2247–2257.

Anholt, B. R., and E. E. Werner. 1995. Interaction between food availability and predation mortality mediated by adaptive behavior. Ecology 76:2230–2234.

Araújo, M. B., F. Ferri-Yáñez, F. Bozinovic, P. A. Marquet, F. Valladares, and S. L. Chown. 2013. Heat freezes niche evolution. Ecology Letters 16:1206–1219.

Barry, J. P., C. H. Baxter, R. D. Sagarin, and S. E. Gilman. 1995. Climate-related, long-term faunal changes in a California rocky intertidal community. Science 267:672–675.

Barry, M. J. 2014. The energetic cost of foraging explains growth anomalies in tadpoles exposed to predators. Physiological and Biochemical Zoology 87:829–836.

Bascompte, J., C. J. Melián, and E. Sala. 2005. Interaction strength combinations and the overfishing. Proceedings of the National Academy of Sciences of the United States of America 102:5443–5447.

Behrens, M. D., and K. D. Lafferty. 2007. Temperature and diet effects on omnivorous fish performance: implications for the latitudinal diversity gradient in herbivorous fishes. Canadian Journal of Fisheries and Aquatic Sciences 64:867–873.

Boersma, M., K. A. Mathew, B. Niehoff, K. L. Schoo, R. M. Franco Santos, and C. L. Meunier. 2016. Temperature driven changes in the diet preference of omnivorous copepods: no more meat when it’s hot? Ecology Letters 19:45–53.

Bond, A. L., and A. W. Diamond. 2011. Recent Bayesian stable-isotope mixing models are highly sensitive to variation in discrimination factors. Ecological applications : a publication of the Ecological Society of America 21:1017–1023.

Bradley, B. A., D. S. Wilcove, and M. Oppenheimer. 2010. Climate change increases risk of plant invasion in the Eastern United States. Biological Invasions 12:1855–1872.

Bridges, C. M. 1997. Tadpole swimming performance and activity affected by acute exposure to sublethal levels of Carbaryl. Environmental Toxicology and Chemistry 16:1935–1939.

Bridges, C. M. 2002. Tadpoles Balance Foraging and Predator Avoidance: Effects of Predation, Pond Drying, and Hunger. Journal of Herpetology 36:627–634.

Burgmer, T., H. Hillebrand, and M. Pfenninger. 2007. Effects of climate-driven temperature changes on the diversity of freshwater macroinvertebrates. Oecologia 151:93–103.

Caut, S., E. Angulo, and F. Courchamp. 2009. Variation in discrimination factors (Δ 15 N and Δ 13 C): the effect of diet isotopic values and applications for diet reconstruction. Journal of Applied Ecology 46:443–453.

Chapman, S., K. Mustin, A. R. Renwick, D. B. Segan, D. G. Hole, R. G. Pearson, and J. E. M. Watson. 2014. Publishing trends on climate change vulnerability in the conservation literature reveal a predominant focus on direct impacts and long time-scales. Diversity and Distributions 20:1221–1228.

Chesson, P., R. L. E. Gebauer, S. Schwinning, N. Huntly, K. Wiegand, M. S. K. Ernest, A. Sher, A. Novoplansky, and J. F. Weltzin. 2004. Resource pulses, species interactions, and diversity maintenance in arid and semi-arid environments. Oecologia 141:236–253.

Chucholl, C. 2011. Population ecology of an alien "warm water" crayfish (*Procambarus clarkii*) in a new cold habitat. Knowledge and Management of Aquatic Ecosystems:29.

Clements, K. D., D. Raubenheimer, and J. H. Choat. 2009. Nutritional ecology of marine herbivorous fishes: ten years on. Functional Ecology 23:79–92.

Clissold, F. J., N. Coggan, and S. J. Simpson. 2013. Insect herbivores can choose microclimates to achieve nutritional homeostasis. The Journal of experimental biology 216:2089–2096.

Clusella-Trullas, S., T. M. Blackburn, and S. L. Chown. 2011. Climatic predictors of temperature performance curve parameters in ectotherms imply complex responses to climate change. The American Naturalist 177:738–751.

Croll, S. L., and S. A. Watts. 2004. The effect of temperature on feed consumption and nutrient absorption in *Procambarus clarkii* and *Procambarus zonangulus*. Journal of the World Aquaculture Society 35:478–488.

Cross, W. F., J. M. Hood, J. P. Benstead, A. D. Huryn, and D. Nelson. 2015. Interactions between temperature and nutrients across levels of ecological organization. Global Change Biology 21:1025–1040.

Dasari, H. P., I. Pozo, F. Ferri-yáñez, and M. B. Araújo. 2014. A regional climate study of heat waves over the Iberian Peninsula. Atmospheric and Climate Sciences 4:841–853

Daufresne, M., K. Lengfellner, and U. Sommer. 2009. Global warming benefits the small in aquatic ecosystems. Proceedings of the National Academy of Sciences of the United States of America 106:12788–12793.

Davidson, A. M., M. Jennions, and A. B. Nicotra. 2011. Do invasive species show higher phenotypic plasticity than native species and, if so, is it adaptive? A meta-analysis. Ecology Letters 14:419–431.

Davis, M. J., J. L. Purrenhage, and M. D. Boone. 2012. Elucidating predator-prey interactions using aquatic microcosms: complex effects of a crayfish predator, vegetation, and atrazine on tadpole survival and behavior. Journal of Herpetology 46:527–534.

Dietl, G. P., and K. W. Flessa. 2011. Conservation paleobiology: putting the dead to work. Trends in Ecology and Evolution 26:30–37.

Dudgeon, D., A. H. Arthington, M. O. Gessner, Z.-I. Kawabata, D. J. Knowler, C. Lévêque, R. J. Naiman, A.-H. Prieur-Richard, D. Soto, M. L. J. Stiassny, and C. a Sullivan. 2006. Freshwater biodiversity: importance, threats, status and conservation challenges. Biological reviews of the Cambridge Philosophical Society 81:163–182.

Dukes, J. S., and H. a. Mooney. 1999. Does global change increase the success of biological invaders? Trends in Ecology and Evolution 14:135–139.

Easterling, D. R., B. Horton, P. D. Jones, T. C. Peterson, T. R. Karl, D. E. Parker, M. J. Salinger, V. Razuvayev, N. Plummer, P. Jamason, and C. K. Folland. 1997. Trends for the Globe Emm. Science 277:364–366.

Easterling, D. R., G. A. Meehl, C. Parmesan, S. A. Changnon, T. R. Karl, and L. O. Mearns. 2000. Climate Extremes: observations, modeling, and impacts. Science 289:2068–2074.

Eggers, S. L., B. K. Eriksson, and B. Matthiessen. 2012. A heat wave and dispersal cause dominance shift and decrease biomass in experimental metacommunities. Oikos 121:721–733.

Estes, J. A, J. Terborgh, J. S. Brashares, M. E. Power, J. Berger, W. J. Bond, S. R. Carpenter, T. E. Essington, R. D. Holt, J. B. C. Jackson, R. J. Marquis, L. Oksanen, T. Oksanen, R. T. Paine, E. K. Pikitch, W. J. Ripple, S. a Sandin, M. Scheffer, T. W. Schoener, J. B. Shurin, A. R. E. Sinclair, M. E. Soulé, R. Virtanen, and D. a Wardle. 2011. Trophic downgrading of planet Earth. Science (New York, N.Y.) 333:301–306.

Finkler, M. S., and D. L. Claussen. 1999. Influence of Temperature, Body Size, and Inter-Individual Variation on Forced and Voluntary Swimming and Crawling Speeds in Nerodia sipedon and Regina septemvittata. Journal of Herpetology 33:62–71.

Fischer, K., M. Klockmann, and E. Reim. 2014. Strong negative effects of simulated heat waves in a tropical butterfly. Journal of Experimental Biology 217:2892–2898.

Floeter, S. R., M. D. Behrens, C. E. L. Ferreira, M. J. Paddack, and M. H. Horn. 2005. Geographical gradients of marine herbivorous fishes: Patterns and processes. Marine Biology 147:1435–1447.

Forster, J., A. G. Hirst, and G. Woodward. 2011. Growth and development rates have different thermal responses. American Naturalist 178:668–678.

Fraker, S. L., and G. R. Smith. 2004. Direct and interactive effects of ecologically relevant concentrations of organic wastewater contaminants on *Rana pipiens* tadpoles. Environmental Toxicology 19:250–256.

Frich, P., L. V. Alexander, P. Della-Marta, B. Gleason, M. Haylock, a. M. G. Tank Klein, and T. Peterson. 2002. Observed coherent changes in climatic extremes during the second half of the twentieth century. Climate Research 19:193–212.

Garrabou, J., R. Coma, N. Bensoussan, M. Bally, P. Chevaldonné, M. Cigliano, D. Diaz, J. G. Harmelin, M. C. Gambi, D. K. Kersting, J. B. Ledoux, C. Lejeusne, C. Linares, C. Marschal, T. Pérez, M. Ribes, J. C. Romano, E. Serrano, N. Teixido, O. Torrents, M. Zabala, F. Zuberer, and C. Cerrano. 2009. Mass mortality in Northwestern Mediterranean rocky benthic communities: Effects of the 2003 heat wave. Global Change Biology 15:1090–1103.

Gillooly, J. F., J. H. Brown, G. B. West, V. M. Savage, and E. L. Charnov. 2001. Effects of size and temperature on metabolic rate. Science (New York, N.Y.) 293:2248–2251.

Giorgi, F. 2006. Climate change hot-spots. Geophysical Research Letters 33:1–4.

González-Bergonzoni, I., M. Meerhoff, T. A. Davidson, F. Teixeira-de Mello, A. Baattrup-Pedersen, and E. Jeppesen. 2012. Meta-analysis shows a consistent and strong latitudinal pattern in fish omnivory across ecosystems. Ecosystems 15:492–503.

Goodman, R. E., G. Lebuhn, N. E. Seavy, T. Gardali, and J. D. Bluso-Demers. 2012. Avian body size changes and climate change: Warming or increasing variability? Global Change Biology 18:63–73.

Gunderson, C. A., K. H. O'hara, C. M. Campion, A. V. Walker, and N. T. Edwards. 2010. Thermal plasticity of photosynthesis: The role of acclimation in forest responses to a warming climate. Global Change Biology 16:2272–2286.

Hatch, A. C., and A. R. Blaustein. 2000. Combined effects of UV-B, nitrate, and low pH reduce the survival and activity level of larval cascades frogs (*Rana cascadae*). Archives of Environmental Contamination and Toxicology 39:494–499.

Hobday, A. J., and J. M. Lough. 2011. Projected climate change in Australian marine and freshwater environments. Marine and Freshwater Research 62:1000–1014.

Hoffmann, A. A, and C. M. Sgrò. 2011. Climate change and evolutionary adaptation. Nature 470:479–485.

Hopkins III, J. B., and J. M. Ferguson. 2012. Estimating the diets of animals using stable isotopes and a comprehensive Bayesian mixing model. PloS one 7:e28478.

Inger, R., G. D. Ruxton, J. Newton, K. Colhoun, J. A. Robinson, A. L. Jackson, and S. Bearhop. 2006. Temporal and intrapopulation variation in prey choice of wintering geese determined by stable isotope analysis. Journal of Animal Ecology 75:1190–1200.

Inger, R., R. A. McDonald, D. Rogowski, A. L. Jackson, A. Parnell, J. S. Preston, C. Harrod, C. Goodwin, D. Griffiths, J. T. A. Dick, R. W. Elwood, J. Newton, and S. Bearhop. 2010. Do non-native invasive fish support elevated lamprey populations? Journal of Applied Ecology 47:121–129.

Ingram, T., L. Harmon, and J. Shurin. 2009. Niche evolution, trophic structure, and species turnover in model food webs. The American Naturalist 174:56–67.

Ings, T. C., J. M. Montoya, J. Bascompte, N. Blüthgen, L. Brown, C. F. Dormann, F. Edwards, D. Figueroa, U. Jacob, J. I. Jones, R. B. Lauridsen, M. E. Ledger, H. M. Lewis, J. M. Olesen, F. J. F. Van Veen, P. H. Warren, and G. Woodward. 2009. Ecological networks – beyond food webs. Journal of Animal Ecology 78:253–269.

Jeppesen, E., M. Meerhoff, K. Holmgren, I. González-Bergonzoni, F. T. Mello, S. A. J. Declerck, L. Meester, M. Søndergaard, T. L. Lauridsen, R. Bjerring, J. M. Conde-Porcuna, N. Mazzeo, C. Iglesias, M. Reizenstein, H. J. Malmquist, Z. Liu, D. Balayla, and X. Lazzaro. 2010. Impacts of climate warming on lake fish community structure and potential effects on ecosystem function. Hydrobiologia 646:73–90.

Jerez, S., J. P. Montavez, J. J. Goméz-Navarro, P. A. Jimenez, P. Jimenez-Guerrero, R. Lorente, and J. F. Gonzalez-Rouco. 2012. The role of the land-surface model for climate change projections over the Iberian Peninsula. Journal of Geophysical Research 117:D01109.

Karl, I., and K. Fischer. 2008. Why get big in the cold? Towards a solution to a life-history puzzle. Oecologia 155:215–225.

Kasai, A., S. Yano, and A. Takafuji. 2005. Prey-predator mutualism in a tritrophic system on a camphor tree. Ecological Research 20:163–166.

Katzenberger, M., J. Hammond, H. Duarte, M. Tejedo, C. Calabuig, and R. A. Relyea. 2014. Swimming with predators and pesticides: How environmental stressors affect the thermal physiology of tadpoles. PLoS ONE 9:1–11.

Kingsolver, J. G., and H. a Woods. 1997. Thermal sensitivity of growth and feeding in *Manduca sexta* caterpillars. Physiological Zoology 70:631–638.

Kingsolver, J. G., S. E. Diamond, and L. B. Buckley. 2013. Heat stress and the fitness consequences of climate change for terrestrial ectotherms. Functional Ecology 27:1415–1423.

Kortsch, S., R. Primicerio, M. Fossheim, A. V. Dolgov, M. Aschan, and S. Kortsch. 2015. Climate change alters the structure of arctic marine food webs due to poleward shifts of boreal generalists. Proceedings of the Royal Society B 282:1–9.

Krause, A. E., K. a Frank, D. M. Mason, R. E. Ulanowicz, and W. W. Taylor. 2003. Compartments revealed in food-web structure. Nature 426:282–285.

Laurila, A., J. Kujasalo, and E. Ranta. 1998. Different antipredator behaviour in two anuran tadpoles: Effects of predator diet. Behavioral Ecology and Sociobiology 83:307–317.

Laurila, A., B. Lindgren, and A. T. Laugen. 2008. Antipredator defenses along a latitudinal gradient in Rana temporaria. Ecology 89:1399–1413.

Lavergne, S., N. Mouquet, W. Thuiller, and O. Ronce. 2010. Biodiversity and climate change: integrating evolutionary and ecological responses of species and communities. Annual Review of Ecology, Evolution, and Systematics, Vol 41 41:321–350.

Lee, K. P., T. Jang, N. Ravzanaadii, and M. S. Rho. 2015. Macronutrient balance modulates the temperature-size rule in an ectotherm. The American Naturalist 186:212–222.

Leishman, M. R., and R. V. Gallagher. 2015. Will there be a shift to alien-dominated vegetation assemblages under climate change? Diversity and Distributions 21:848–852.

Lemoine, N. P., and D. E. Burkepile. 2012. Temperature-induced mismatches between consumption and metabolism reduce consumer fitness. Ecology 93:2483–2489.

Lemons, G., R. Lewison, L. Komoroske, A. Gaos, C.-T. Lai, P. Dutton, T. Eguchi, R. LeRoux, and J. A. Seminoff. 2011. Trophic ecology of green sea turtles in a highly urbanized bay: insights from stable isotopes and mixing models. Journal of Experimental Marine Biology and Ecology 405:25–32.

Levey, D., and W. Karasov. 2012. Gut passage of insects by European starlings and comparison with other species. American Ornothologists' Union 111:478–481.

Ma, G., V. H. W. Rudolf, and C. Sen Ma. 2015. Extreme temperature events alter demographic rates, relative fitness, and community structure. Global Change Biology 21:1794–1808.

Malzahn, A. M., D. Doerfler, and M. Boersma. 2016. Junk food gets healthier when it' s warm. Limnology and Oceanography, *in press.*

Martínez del Rio, C., N. Wolf, S. A. Carleton, and L. Z. Gannes. 2009. Isotopic ecology ten years after a call for more laboratory experiments. Biological Reviews 84:91–111.

McKee, D., D. Atkinson, S. Collings, J. Eaton, I. Harvey, T. Heyes, K. Hatton, D. Wilson, and B. Moss. 2002. Macro-zooplankter responses to simulated climate warming in experimental freshwater microcosms. Freshwater Biology 47:1557–1570.

Meehl, G. A, and C. Tebaldi. 2004. More intense, more frequent, and longer lasting heat waves in the 21st century. Science (New York, N.Y.) 305:994–997.

Miller, G. A., F. J. Clissold, D. Mayntz, and S. J. Simpson. 2009. Speed over efficiency: locusts select body temperatures that favour growth rate over efficient nutrient utilization. Proceedings of the Royal Society B: Biological Sciences 276:3581–3589.

Miriti, M. N., S. Rodriguez-Buritica, S. J. Wright, H. F. Howe, S. Rodríguez-Buriticá, S. J. Wright, and H. F. Howe. 2007. Episodic death across species of desert shrubs. Ecology 88:32–36.

Myrick, C. A., and J. J. Cech. 2000. Temperature influences on California rainbow trout physiological performance. Fish Physiology and Biochemistry 22:245–254.

Nicieza, A. G., and N. B. Metcalfe. 1997. Growth compensation in juvenile Atlantic salmon: responses to depressed temperature and food availability. Ecology 78:2385–2400.

Nunes, A. L., A. Richter-Boix, A. Laurila, and R. Rebelo. 2013. Do anuran larvae respond behaviourally to chemical cues from an invasive crayfish predator? A community-wide study. Oecologia 171:115–127.

Oertli, B., J. Biggs, R. Céréghino, P. Grillas, P. Joly, and J.-B. Lachavanne. 2005. Conservation and monitoring of pond biodiversity: introduction. Aquatic Conservation: Marine and Freshwater Ecosystems 15:535–540.

Paaijmans, K. P., R. L. Heinig, R. a. Seliga, J. I. Blanford, S. Blanford, C. C. Murdock, and M. B. Thomas. 2013. Temperature variation makes ectotherms more sensitive to climate change. Global Change Biology 19:2373–2380.

Parmesan, C., T. Root, and M. R. Willig. 2000. Impacts of extreme weather and climate on terrestrial biota. Bulletin of the American Meteorological Society 81:1–10.

Parmesan, C., and G. Yohe. 2003. A globally coherent fingerprint of climate change impacts across natural systems. Nature 421:37–42.

Parmesan, C. 2006. Ecological and evolutionary responses to recent climate change. Annual of Ecology, Evolution and Systematics 37:637–669.

Parnell, A. C., R. Inger, S. Bearhop, and A. L. Jackson. 2010. Source partitioning using stable isotopes: coping with too much variation. PloS one 5:e9672.

Parnell, A. C., D. L. Phillips, S. Bearhop, B. X. Semmens, E. J. Ward, J. W. Moore, A. L. Jackson, J. Grey, D. J. Kelly, and R. Inger. 2013. Bayesian stable isotope mixing models. Environmetrics 24:387–399.

Pau, S., E. M. Wolkovich, B. I. Cook, T. J. Davies, N. J. B. Kraft, K. Bolmgren, J. L. Betancourt, and E. E. Cleland. 2011. Predicting phenology by integrating ecology, evolution and climate science. Global Change Biology 17:3633–3643.

Pennings, S. C., C. K. Ho, C. S. Salgado, K. Wieski, N. Davé, A. E. Kunza, and E. L. Wason. 2009. Latitudinal variation in herbivore pressure in Atlantic Coast salt marshes. Ecology 90:183–195.

Pereira, H. M., P. W. Leadley, V. Proença, R. Alkemade, J. P. W. Scharlemann, J. F. Fernandez-Manjarrés, M. B. Araújo, P. Balvanera, R. Biggs, W. W. L. Cheung, L. Chini, H. D. Cooper, E. L. Gilman, S. Guénette, G. C. Hurtt, H. P. Huntington, G. M. Mace, T. Oberdorff, C. Revenga, P. Rodrigues, R. J. Scholes, U. R. Sumaila, and M. Walpole. 2010. Scenarios for global biodiversity in the 21st century. Science (New York, N.Y.) 330:1496–1501.

Perkins, D. M., J. Reiss, G. Yvon-Durocher, and G. Woodward. 2010. Global change and food webs in running waters. Hydrobiologia 657:181–198.

Perry, A. L., P. J. Low, J. R. Ellis, and J. D. Reynolds. 2005. Climate change and distribution shifts in marine fishes. Science 308:1912–1915.

Peruzza, L., F. Piazza, C. Manfrin, L. C. Bonzi, S. Battistella, and P. G. Giulianini. 2015. Reproductive plasticity of a *Procambarus clarkii* population living 10ºC below its thermal optimum. Aquatic Invasions 10:199–208.

Phillips, D. L., and J. W. Gregg. 2001. Uncertainty in source partitioning using stable isotopes. Oecologia 127:171–179.

Phillips, D. L., and J. W. Gregg. 2003. Source partitioning using stable isotopes: coping with too many sources. Oecologia 136:261–269.

Pyšek, P., and D. M. Richardson. 2010. Invasive species, environmental change and management, and health. The Annual Review of Environment and Resources 35:25–55.

Reusch, T. B. H., A. Ehlers, A. Hämmerli, and B. Worm. 2005. Ecosystem recovery after climatic extremes enhanced by genotypic diversity. Proceedings of the National Academy of Sciences of the United States of America 102:2826–2831.

Ricciardi, A., and J. B. Rasmussen. 1999. Extinction rates of North American freshwater fauna. Conservation Biology 13:1220–1222.

Richardson, J. M. L. 2001. A comparative study of activity levels in larval anurans and response to the presence of different predators. Behavioral Ecology 12:51–58.

Robinet, C., and A. Roques. 2010. Direct impacts of recent climate warming on insect populations. Integrative Zoology 5:132–142.

Rodríguez, C. F., E. Bécares, and M. Fernández-Aláez. 2003. Shift from clear to turbid phase in Lake Chozas (NW Spain) due to the introduction of American red swamp crayfish (*Procambarus clarkii*). Hydrobiologia:421–426.

Sala, O. E., F. S. Chapin, J. J. Armesto, E. Berlow, J. Bloomfield, R. Dirzo, E. Huber-Sanwald, L. F. Huenneke, R. B. Jackson, A. Kinzig, R. Leemans, D. M. Lodge, H.

A. Mooney, M. Oesterheld, N. L. Poff, M. T. Sykes, B. H. Walker, M. Walker, and D. H. Wall. 2000. Global biodiversity scenarios for the year 2100. Science 287:1770–1774.

Schär, C., P. L. Vidale, D. Lüthi, C. Frei, C. Häberli, M. a Liniger, and C. Appenzeller. 2004. The role of increasing temperature variability in European summer heatwaves. Nature 427:332–336.

Schemske, D. W., G. G. Mittelbach, H. V Cornell, J. M. Sobel, and K. Roy. 2009. Is there a latitudinal gradient in the importance of biotic interactions? Annual Review of Ecology Evolution and Systematics 40:245–269.

Schweiger, O., J. Settele, O. Kudrna, S. Klotz, and I. Kühn. 2008. Climate change can cause spatial mismatch of trophically interacting species. Ecology 89:3472–3479.

Seifert, L. I., F. De Castro, A. Marquart, U. Gaedke, G. Weithoff, and M. Vos. 2014. Heated relations: temperature-mediated shifts in consumption across trophic levels. PLoS ONE 9:e95046.

Semlitsch, R. D., M. Foglia, A. Mueller, I. Steiner, E. Fioramonti, and K. Fent. 1995. Short term exposure to triphenyltin affects the swimming and feeding behavior of tadpoles. Environmental Toxicology and Chemistry 14:1419–1423.

Sheridan, J. A., and D. Bickford. 2011. Shrinking body size as an ecological response to climate change. Nature Climate Change 1:401–406.

Smith, G. R., and A. A. Burgett. 2005. Effects of three organic wastewater contaminants on American toad, *Bufo americanus*, tadpoles. Ecotoxicology 14:477–482.

Stouffer, D. B., and J. Bascompte. 2011. Compartmentalization increases food-web persistence. Proceedings of the National Academy of Sciences of the United States of America 108:3648–52.

Szepanski, M. M., M. Ben-David, and V. Van Ballenberghe. 1999. Assessment of anadromous salmon resources in the diet of the Alexander Archipelago wolf using stable isotope analysis. Oecologia 120:327–335.

Theoharides, K. A., and J. S. Dukes. 2007. Plant invasion across space and time: factors affectig nonindigenous species success during four stages of invasion. New Phytologist 176:256–273.

Thibault, K. M., and J. H. Brown. 2008. Impact of an extreme climatic event on community assembly. Proceedings of the National Academy of Sciences of the United States of America 105:3410–3415.

Thomas, C. D. 2010. Climate, climate change and range boundaries. Diversity and Distributions 16:488–495.

Thomas, S. M., and T. W. Crowther. 2015. Predicting rates of isotopic turnover across the animal kingdom: a synthesis of existing data. Journal of Animal Ecology 84:861–870.

Thompson, R. M., M. Hemberg, B. M. Starzomski and J. B. Shurin. 2007. Trophic levels and trophic tangles: the prevalence of omnivory in real food webs. Ecology 88:612–617.

Traill, L. W., M. L. M. Lim, N. S. Sodhi, and C. J. A. Bradshaw. 2010. Mechanisms driving change: Altered species interactions and ecosystem function through global warming. Journal of Animal Ecology 79:937–947.

Tylianakis, J. M., R. K. Didham, J. Bascompte, and D. A. Wardle. 2008. Global change and species interactions in terrestrial ecosystems. Ecology Letters 11:1351–1363.

Vandermeer, J. 2006. Omnivory and stability of food webs. Ecological Complexity 238:497–504.

Van Der Putten, W. H., P. C. De Ruiter, T. M. Bezemer, J. A. Harvey, M. Wassen, and V. Wolters. 2004. Trophic interactions in a changing world. Basic and Applied Ecology 5:487–494.

Van der Putten, W. H., M. Macel, and M. E. Visser. 2010. Predicting species distribution and abundance responses to climate change: why it is essential to include biotic interactions across trophic levels. Philosophical transactions of the Royal Society of London. Series B, Biological sciences 365:2025–2034.

Vasseur, D. A., J. P. DeLong, B. Gilbert, H. S. Greig, C. D. G. Harley, K. S. McCann, V. Savage, T. D. Tunney, and M. I. O’ Connor. 2014. Increased temperature variation poses a greater risk to species than climate warming. Proceedings of the Royal Society B 281:20132612.

Vitousek, P. M. 1997. Human domination of Earth ecosystems. Science 277:494–499.

Voigt, W., J. Perner, A. J. Davis, T. Eggers, J. Schumacher, R. Bährmann, B. Fabian, W. Heinrich, G. Köhler, D. Lichter, R. Marstaller, and F. W. Sander. 2003. Trophic levels are differentially sensitive to climate. Ecology 84:2444–2453.

Walther, G.-R. 2004. Plants in a warmer world. Perspectives in Plant Ecology Evolution and Systematics 6:169–185.

Walther, G.-R., A. Roques, P. E. Hulme, M. T. Sykes, P. Pyšek, I. Kühn, M. Zobel, S. Bacher, Z. Botta-Dukát, and H. Bugmann. 2009. Alien species in a warmer world: risks and opportunities. Trends in Ecology & Evolution 24:686–693.

Walther, G.-R. 2010. Community and ecosystem responses to recent climate change. Philosophical transactions of the Royal Society of London. Series B, Biological sciences 365:2019–2024.

Wernberg, T., D. A. Smale, F. Tuya, M. S. Thomsen, T. J. Langlois, T. de Bettignies, S. Bennett, and C. S. Rousseaux. 2012. An extreme climatic event alters marine ecosystem structure in a global biodiversity hotspot. Nature Climate Change 3:78–82.

Werner, E. E. 1992. Competitive interactions between Wood Frog and Northern Leopard Frog larvae – the influence of size and activity. Copeia 1992:26–35.

Werner, E. E., and B. R. Anholt. 1993. Ecological consequences of the trade-off between growth and mortality rates mediated by foraging activity. The American Naturalist 142:242–272.

Woodward, G., D. M. Perkins, and L. E. Brown. 2010. Climate change and freshwater ecosystems: impacts across multiple levels of organization. Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences

365:2093–2106.

Zhang, P., B. A. Blonk, R. F. van den Berg, and E. S. Bakker. 2016. The effect of temperature on herbivory by the omnivorous ectotherm snail *Lymnaea stagnalis*. Hydrobiologia, *in press*.

CHAPTER 2

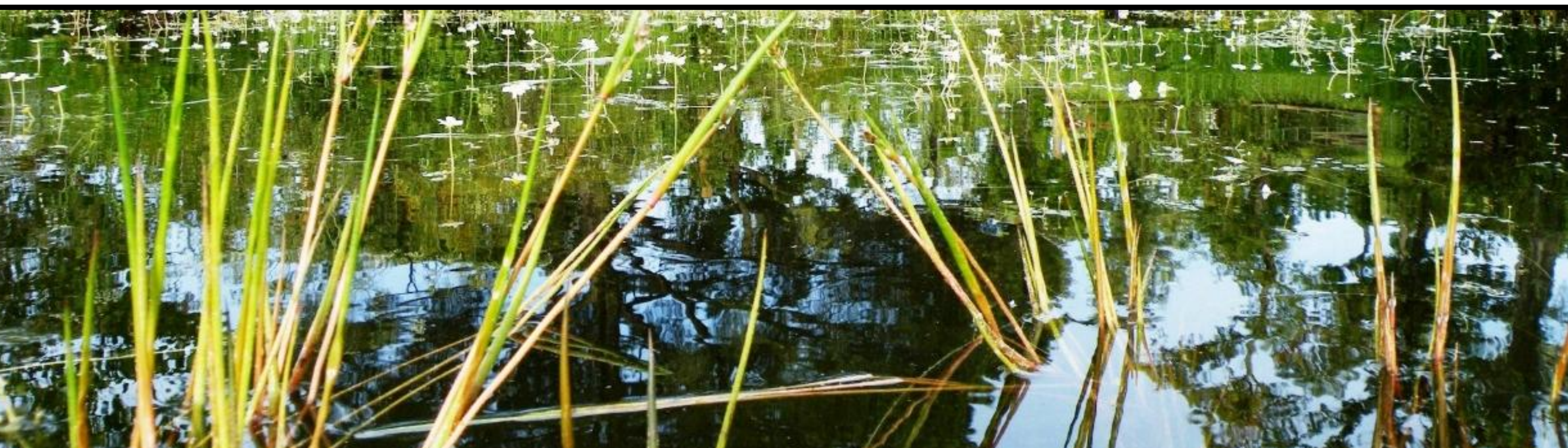
Warm vegetarians? Heat waves and diet shifts in tadpoles

B. M. Carreira, P. Segurado, G. Orizaola, N. Gonçalves, V. Pinto, A. Laurila and R. Rebelo

Discoglossus galganoi

Hyla arborea

Hyla meridionalis



CHAPTER 2

Warm vegetarians? Heat waves and diet shifts in tadpoles

Abstract

Temperature can play an important role in determining the feeding preferences of ectotherms. In light of the warmer temperatures arising with the current climatic changes omnivorous ectotherms may perform diet shifts towards higher herbivory to optimize energetic intake. Such diet shifts may also occur during heat waves, which are projected to become more frequent, intense and longer lasting in the future. Here, we investigated how heat waves of different duration affect feeding preferences in omnivorous anuran tadpoles and how these choices affect larval life history. In laboratory experiments, we fed tadpoles of three species on animal, plant or mixed diet and exposed them to short heat waves (similar to the heat waves these species experience currently) or long heat waves (predicted to increase under climate change). We estimated the dietary choices of tadpoles fed on the mixed diet using stable isotopes and recorded tadpole survival and growth, larval period and mass at metamorphosis. Tadpole feeding preferences were associated with their thermal background, herbivory increasing with breeding temperature in nature. Patterns in survival, growth and development generally support decreased efficiency of carnivorous diets and increased efficiency or higher relative quality of herbivorous diets at higher temperatures. All three species increased herbivory in at least one of the heat wave treatments, but the responses varied among species. Diet shifts towards higher herbivory were maladaptive in one species, but beneficial in the other two. Higher herbivory in omnivorous ectotherms under warmer temperatures may impact species differently and further contribute to changes in the structure and function of freshwater environments.

Key words: amphibian, carnivory, climate change, diet regulation, *Discoglossus galganoi*, herbivory, *Hyla arborea*, *Hyla meridionalis*, omnivory, stable isotope mixing models

Introduction

Studies on the influence of temperature on nutrient acquisition by organisms are now in the forefront of climate change biology (Cross et al. 2015). In ectotherms, higher temperatures promote a greater increase in feeding and gut passage rates than in assimilation rates (Myrick and Cech 2000, Seifert et al. 2014). Consequently, the overall assimilation efficiency of nutrients with contrasting size and complexity may vary with temperature. For instance in crayfish, omnivorous ectotherms, temperature changes the protein to carbohydrate assimilation ratio, which shifts to a greater assimilation of carbohydrates at higher temperatures (Croll and Watts 2004). Furthermore, higher temperatures have a stronger effect on catabolic than on anabolic processes, promoting a greater increase in respiration than in growth (Karl and Fischer 2008, Forster et al. 2011), and therefore increasing the demand for carbon over nitrogen. These imbalanced effects of temperature on different aspects of metabolism, together with the enhanced digestion of plant material at high temperatures (Floeter et al. 2005), suggest that in order to maximize energy intake and sustain higher metabolic rates omnivorous ectotherms should perform diet shifts towards increased herbivory. In support of this hypothesis, a recent study found a seasonal decrease in the trophic level from winter to summer in copepods, and showed experimentally that herbivory increased at higher temperatures (Boersma et al. 2016). The higher prevalence of herbivory found in fish communities of warmer waters at lower latitudes (Floeter et al. 2005, Behrens and Lafferty 2007, Jeppesen et al. 2010, Gonzalez-Bergonzoni et al. 2012) also suggests that optimization of energy intake at warmer temperatures may result in avoidance of protein-rich diets.

Freshwater ecosystems are particularly sensitive to climate change (Thompson et al. 2013), as approximately 99% of the species composing freshwater communities are ectotherms (Dudgeon et al. 2006). Yet, despite the strong influence of temperature in the physiological processes of ectotherms, there is only one recent study addressing the role of temperature in determining their feeding preferences (Boersma et al. 2016). Amphibian larvae are key components of freshwater communities and strongly affect nutrient recycling, zooplankton diversity and macrophyte biomass (Arribas et al. 2015). Most anuran tadpoles feed on multiple resources – detritus, periphyton, phytoplankton, zooplankton, macrophytes and macroinvertebrates (Petranka and Kennedy 1999, Altig et al. 2007, Arribas et al. 2015). Trophic plasticity can help amphibian larvae to mitigate the negative effects of different stressors, including higher temperatures, which – while accelerating tadpole growth and differentiation – sometimes have costs in survival, size at metamorphosis and post-metamorphic fitness (Wilbur and Collins 1973, Tejedo et al. 2010, but see Earl and Whiteman 2015). Tadpoles can shift diet in response to e.g. intraspecific competition and pond drying (Pfenning 1990, Caut et al. 2013), but no studies have examined diet shifts in response to changes in temperature.

While climate change can have severe effects on individual fitness, organisms may acclimate through phenotypic plasticity or evolve genetic adaptations (Hoffmann and Sgrò 2011). However, the increased climatic variability poses a more serious challenge, as species may be unable to show an immediate response, even if they have the potential for genetic adaptation (Reusch et al. 2005). Until recently, most experimental research neglected the importance of discrete weather events as components of climate change

(Chapman et al. 2014, Vasseur et al. 2014). However, short-term climatic stress – comprising increased climate variability and extreme climatic events – is predicted to severely affect species' fitness and life-history traits (Clusella-Trullas et al. 2011, Paaijmans et al. 2013, Kingsolver et al. 2013). Specifically, extreme events such as heat waves may cause mass mortality (Garrabou et al. 2009), decrease in condition, immune function and storage reserves of the organisms (Fischer et al. 2014), loss of genetic diversity and range shifts (Reusch et al. 2005), changes in species interactions (Eggers et al. 2012) and community structure (Ma et al. 2015), and affect ecosystem function (Wernberg et al. 2013).

In this study, we investigated feeding preferences and diet shifts in tadpoles of three anuran species breeding along a seasonal climatic gradient. The tadpoles were fed animal-based, plant-based or mixed diets and exposed to temperatures simulating either a currently commonly occurring short heat wave or a long heat wave expected to become more frequent in the near future (Meehl and Tebaldi 2004, Schär et al. 2004). These responses were compared to those of tadpoles reared under present spring conditions, or under an exceptionally cold spring (see Methods). We reconstructed the dietary choices of the mixed diet tadpoles with stable isotope mixing models to investigate diet shifts, and recorded treatment responses in terms of survival, growth rate, duration of the larval period and mass at metamorphosis. We predicted that (1) the proportion of the animal diet should decrease from the earliest breeding species to the latest breeding species, following the seasonal climatic gradient. In the heat wave treatments we predicted that (2) tadpole performance on the animal diet should decrease; (3) tadpole performance on the plant diet should improve; (4) tadpoles on the mixed diet should increase herbivory; (5) diet shifts should improve tadpole performance.

Materials and Methods

Study area and species

The Iberian Peninsula, Western Europe, is a climate change “hot-spot” that has experienced temperature increase during the latter half of 20th century, especially in early summer (Giorgi 2006, Dasari et al. 2014). In the 21st century the mean temperature of this region is expected to increase up to 6°C and heat waves are expected to become longer, more frequent and intense (Jerez et al. 2013). Air temperatures in our study area in southwestern Portugal (Grândola, 38º 05’ N, 8º 33’ W) are monitored since 2002, and we used a 10-year data set (2002-2012) to characterize the most common spring heat waves occurring in the area. We followed the heat wave definition of Frich et al. (2002): a heat wave occurs when the daily maximum temperature exceeds the average maximum temperature by 5°C for more than five consecutive days, the reference period being 1961–1990. We found that heat waves occurred frequently in the end of April and lasted on average 7.8 ± 1.3 days. In some years they were followed by a second heat wave in mid-May lasting on average 7.5 ± 1.3 days (B. M. Carreira, *unpubl. data*). According to a five-year survey carried out in 42 temporary ponds located in the study area during April and May (2010-2014), water temperatures ranged from 17 to 25°C with an average temperature of 20°C (R. Rebelo, *unpubl. data*).

We focused our study on three common anuran species with larval development during spring. These species have similar thermal performance limits (Katzenberger 2015), but differ in the timing of breeding, so that tadpoles of the earliest species meet lower water temperatures than tadpoles of the later breeding species. The Iberian painted frog *Discoglossus galganoi* (Capula, Nascetti, Lanza, Bullini & Crespo, 1985) is a Mediterranean species, which reproduces in temporary and ephemeral ponds between October and July (Rebelo and Crespo 1999). Its optimal thermal performance is between

20 and 34ºC (Katzenberger 2015), and tadpoles face an average temperature of 10.3 ± 1.5ºC during the coldest month of their development (R. Rebelo, *unpubl. data*). The European tree frog *Hyla* arborea (Linnaeus, 1758) is a Eurasian species, which in southern Portugal reproduces in temporary and semi-permanent ponds along the coast (Atlantic climate) between March and June (Rebelo and Crespo 1999). Its optimal thermal performance occurs between 23 and 36ºC (Katzenberger 2015), and tadpoles face an average temperature of 12.6 ± 0.7ºC in the coldest month of their development (R. Rebelo, *unpubl. data*). The Mediterranean tree frog *Hyla meridionalis* (Boettger, 1874) breeds in inland temporary ponds (Mediterranean climate) between April and June (Rebelo and Crespo 1999). Its optimal thermal performance is between 20 and 37ºC (Katzenberger 2015), and tadpoles face an average temperature of 13.9 ± 1.6ºC in the coldest month of their development (R. Rebelo, *unpubl. data*).

Experimental setup and procedures

We collected clutches of *D. galganoi* and *H. arborea* and brought them to the facilities at the University of Lisbon, where the eggs were kept at 20ºC in 1.5L aquaria (ca. 30 eggs per aquarium) under 12L:12D photoperiod until hatching (see Table 1). Because of the near threatened conservation status of *D. galganoi*, we collected only three large clutches (ca. 600 eggs/clucth), which were later equally divided in the experimental treatments. In *H. arborea*, we collected 60 small clutches (ca. 10 eggs/clucth), pooled the hatchlings and randomized tadpoles across the treatments. We found no egg clutches from *H. meridionalis* and collected newly-hatched tadpoles (Gosner stages 21-24) from different parts of a pond with a large population with hundreds of adult frogs (see Table 1 for details).

Table 1. Species origin and experimental procedures details. Number of clutches collected for the study species, their collection site (type of water body); coordinates and dates of collection; dates of the start and end of each experiment; and experimental day of start and end of each heat wave.

Species	# Clutches collected	Collection site	Site coordinates	Date of collection	Start of experiment	End of experiment	Early Spring Heat Wave (day)	Late Spring Heat Wave (day)
Discoglossus galganoi	3	Grândola (Rain pool)	38°06’N, 8°34’W	6 January 2014	4 February 2014	17 July 2014	28-35	-
Hyla arborea	60	Verdizela (Temporary pond)	38°34'N, 9°08'W	20 March to 20 May 2014	25 June 2014	16 December 2014	21-28	42-49
Hyla meridionalis	-	Grândola (Temporary pond)	38°06’N, 8°34’W	26 April 2012	20 June 2012	9 October 2012	21-28	42-49

At the start of the experiments room temperature was lowered to 17ºC to achieve the lowest experimental temperature, while temperatures in the treatments where warming was applied were obtained with aquarium heaters placed in the water baths. Tadpoles (stage 25; Gosner 1960) were photographed to determine their initial body size (using ImageJ software) and transferred to individual 0.5L plastic vials placed in water baths. In each of the three species, we followed a fully factorial experimental design with diet (three levels) and temperature (four or five levels, see below) as factors. In *D. galganoi* and *H. arborea*, we used 28 randomly assigned tadpoles individually-reared per treatment combination, while in *H. meridionalis* we used 14 individually-reared tadpoles per treatment combination. Tadpoles were fed *ad libitum* every other day along with water renewal, and the position of each vial in the water bath was randomized. Water bath temperatures were checked daily and adjusted when necessary. At the end of the early spring heat wave (see *Temperature* below) half of the tadpoles in each treatment were weighed to determine growth rate at a fixed time and removed from the experiment for another study. The remaining tadpoles were allowed to complete metamorphosis. After the first tadpole reached Gosner stage 42 (emergence of forelimbs), all vials were checked daily for metamorphs and the length of each individual's larval period was recorded. The metamorphs were transferred to plastic containers (12.5 × 12.5 × 9.0 cm) with moist paper towel in the bottom and allowed to absorb their tails (Gosner stage 46) at 20ºC. After that, froglets were euthanized, photographed and weighed (fresh mass). We euthanized froglets by rapid freezing at -18ºC to prevent contamination of their isotopic signature with euthanizing agents.

Diet

The three diet treatments were: Animal diet (A) – defrosted insect larvae collected in the study area or obtained commercially (see below); Plant diet (P) – defrosted macrophyte stalks collected in the same ponds as tadpoles; and Mixed diet (M) – both food items in diets A and P offered *ad libitum* in similar proportions of fresh mass. The experimental diets were selected based on the results of preliminary experiments with different macrophyte and invertebrate species abundant in temporary ponds. As the plant diet, we selected the floating macrophyte *Ranunculus peltatus* for both hylids, and the emergent macrophyte *Juncus heterophyllus* for *D. galganoi*, because the latter did not consume *R. peltatus.* As the animal diet for *H. meridionalis*, we selected Ephemeroptera (mayfly) larvae, collected in the same pond as the tadpoles and killed by rapid freezing. Ephemeropterans occur at high densities in temporary ponds, but capturing the amount necessary to maintain tadpoles on this diet was extremely challenging. Consequently, we used commercially obtained frozen chironomid larvae for *D. galganoi* and *H. arborea*, as chironomids are also common in temporary ponds. The diet treatments complied with the basic assumptions that the animal diet was protein-rich, the plant diet was protein-poor, and the diets had very distinct C:N ratios (Table 2). Because of the use of different invertebrates and macrophytes in the diet treatments, we do not conduct life history comparisons among the tadpoles of the three species.

Table 2. Isotopic and elemental composition of the food sources. Isotopic signatures and C:N ratios of the food items provided to the tadpoles of each of the study species (average ± standard deviation).

	D. galganoi			*H. arborea*			*H. meridionalis*		
	$\delta\ ^{13}C$	$\delta\ ^{15}N$	C:N	$\delta\ ^{13}C$	$\delta\ ^{15}N$	C:N	$\delta\ ^{13}C$	$\delta\ ^{15}N$	C:N
Animal diet	-24.61 ± 0.18	5.50 ± 0.81	3.76 ± 0.04	-24.61 ± 0.13	5.09 ± 0.92	3.79 ± 0.05	-29.30 ± 1.08	14.54 ± 1.02	7.84 ± 2.01
Plant diet	-28.63 ± 0.19	4.41 ± 0.44	23.69 ± 11.52	-28.09 ± 0.05	3.36 ± 0.12	27.11 ± 0.05	-28.09 ± 0.05	3.36 ± 0.12	27.11 ± 0.05

Temperature

The experiment included four temperature treatments (Fig. 1): Cold Spring (CS) – constant temperature of 17°C; Normal Spring (NS) – temperature was gradually increased from 17 to 25°C at the rate of 1°C per week (average of 21°C); Early Spring Heat Wave (ESHW) – similar to NS, but in the fourth week of the experiment tadpoles were exposed to a one-week heat wave of 25°C, after which temperature was decreased to 21°C (treatment average temperature of 21.4°C; and Long Heat Wave (LoHW) – constant temperature of 25°C. Additionally, because *H. arborea* and *H. meridionalis* are spring breeders and more likely to face heat waves later in the season, we included a fifth temperature regime for these two species: Late Spring Heat Wave (LSHW) – similar to NS, but in the sixth week tadpoles were exposed to 25°C after which temperature was decreased to 23°C (treatment average temperature of 21.4°C; S2). The one-week-long heat wave of the ESHW and LSHW treatments matches the duration of current spring heat waves at the study area. The two-month-long heat wave (LoHW treatment) simulates an extremely long heat wave, such as the one that afflicted Europe in 2003, which are expected to become more frequent in the future (Jerez et al. 2013). The three average temperatures (17°C, ≈ 21°C and 25°C) were chosen to match the temperature range measured in the pond survey.

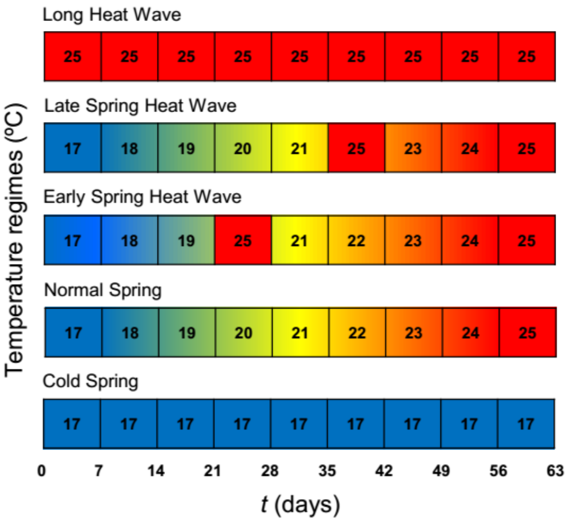


Figure 1. Schematic representation of temperature variation over time in each experimental treatment.

Isotope analysis

We used the whole froglet body to attain the minimum mass required per isotopic sample (0.8-1.2 mg of dry tissue). For *D. galganoi* and *H. arborea*, nine randomly chosen froglets per treatment were degutted, dried at 60°C for 24h and ground to a fine powder with mortar and pestle. For *H. meridionalis* we used between three and seven froglets for each treatment. We extracted storage lipids with chloroform-methanol (2:1; Dobush et al. 1985), and re-dried the samples at 60°C for 24h. Stable isotope ratios ($^{13}C/^{12}C$; $^{15}N/^{14}N$) and elemental analyses (C:N) were determined by continuous flow isotope mass spectrometry (CF-IRMS) (Preston and Owens 1983), on a Hydra 20-22 (Sercon, UK) stable isotope ratio mass spectrometer, coupled to a EuroEA (EuroVector, Italy) elemental analyzer for online sample preparation by Dumas-combustion. The standards used were IAEA-CH6 and IAEA-CH7 for carbon isotope ratio, and IAEA-N1 and USGS-35 for nitrogen isotope ratio; $\delta^{13}C$ results were referred to PeeDee Belemnite (PDB) and $\delta^{15}N$ to Air. Precision of the isotope ratio analysis was $\leq 0.2‰$ (6 to 9 replicates of laboratory standards in every batch). Although clutches and hatchlings were collected in the wild and we could not control for maternal effects in the isotopic signatures, tadpole mass at early Gosner stage 25 represented only 10 to 20% of the froglet mass, and potential initial differences should be negligible.

Statistical analyses

To estimate the relative contributions of the animal and the plant diets to the isotopic signature of tadpoles on the mixed diet, we used stable isotope mixing models, which offer reliable and time-integrated results. The Stable Isotope Analysis in R package (SIAR; Parnell et al. 2013) fits a Bayesian model to the proportions of the food sources contributing to the isotopic signature of the consumers, incorporating variability in the sources, trophic enrichment factors (TEF) and elemental composition (Parnell et al. 2010). We specified isotopic signatures and C:N ratios of the sources in each species model (Table 2), and accounted for the variability in the TEFs across species, sources and temperature conditions by using the isotopic signatures of the tadpoles fed on the pure diets (animal or plant) at each temperature. The food items differed considerably in their C:N ratio and more than 2‰ in their isotopic signatures at least in one of the isotopes. Half-life values for ^{13}C and ^{15}N turnover rates, determined in non-experimental tadpoles maintained at 20ºC and fed on commercial fish food, were found to be of ca. 1 month, in agreement with values obtained by Thomas and Crowther (2015) on 10mg-1g organisms at 10-30ºC.

We fitted a Cox proportional hazards regression model to test the treatment effects on tadpole survival (survival package of the R software). In the statistical analyses of larval period and mass at metamorphosis, we excluded the plant diet in *D. galganoi* and the animal diet in *H. meridionalis*, because mortality on these diets was close to 100% in several temperature treatments. We used general linear models (GLMs) to test the effects of temperature and diet (fixed effects) and their interaction on tadpole growth rate (mg.day^{-1}; mass divided by the experimental day at the end of the ESHW), on larval period (days from stage 25 to 42); and on froglet mass at metamorphosis (mg; mass at Gosner stage 46). Tadpole body size on day 1 was included as a covariate in all models, as it had a significant effect on tadpole growth rate in all species and on larval period in *H. arborea*. Post hoc pairwise comparisons were corrected for multiple comparisons (Bonferroni's test). The general linear models were performed using the software STATISTICA 12.6.255.0 (StatSoft 2012).

Results

Diet choice

The proportions of animal and plant diet assimilated by the tadpoles on the mixed diet differed among the species. *Discoglossus galganoi* was closer to the carnivorous end of the diet gradient, as the median proportion of plant material averaged 0.22 across the temperature treatments (Fig. 2). *Hyla arborea* assimilated a rather balanced proportion of the two food items as the median proportion of plant material averaged 0.57 across all treatments (Fig. 2). *Hyla meridionalis* was closer to the herbivorous end of the diet gradient, as the median proportion of plant material averaged 0.83 across the temperature treatments (Fig. 2).

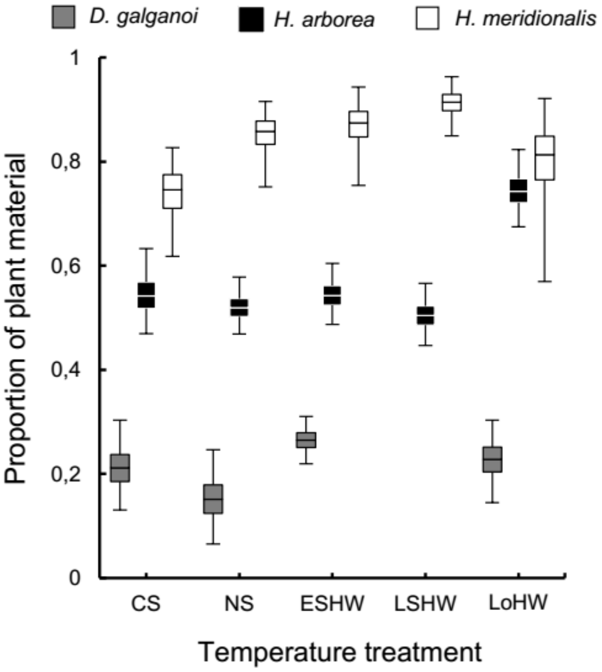


Figure 2. Proportion of plant material assimilated by the mixed diet tadpoles of each species in the temperature treatments: Cold Spring (CS), Normal Spring (NS), Early Spring Heat Wave (ESHW), Late Spring Heat Wave (LSHW) and Long Heat Wave (LoHW). Boxes show the median and the 25th – 75th percentiles; whiskers indicate the 2.5th – 97.5th percentiles.

Although still primarily carnivorous, in the early spring heat wave *D. galganoi* increased herbivory and assimilated almost two times more plant material (0.27; Bayesian credible interval (BCI) = 0.22-0.31) than in the normal spring (0.15; BCI = 0.07-0.25). However, this increase was not found in the long heat wave (0.23; BCI = 0.14-0.30), where herbivory was similar to the cold spring (0.21; BCI = 0.13-0.30). In *H. arborea* the proportion of plant material assimilated was similar across four temperature treatments, ranging from 0.50 (BCI = 0.45-0.57) to 0.54 (BCI = 0.47-0.63), but in the long heat wave tadpoles increased herbivory and the proportion of plant material rose to 0.74 (BCI = 0.67-0.82; Fig. 2). Mainly herbivorous, *H. meridionalis* also increased herbivory, although to a lesser extent. In the cold spring the proportion of plant material was 0.75

(BCI = 0.62-0.83), but in the other temperature treatments was consistently higher and varied between 0.81 (BCI = 0.57-0.92) in the long heat wave (the second lowest value) and 0.91 (BCI = 0.85-0.96) in the late spring heat wave (Fig. 2).

Survival

In *D. galganoi*, survival on the animal and mixed diets was over 90%, but dropped to 45% on the plant diet (Table 3, Fig. 3). The highest survival for *D. galganoi* in the temperature treatments was in the cold spring (95% survival), and lowest in the long heat wave (61%; Table 3, Fig. 3). In *H. meridionalis*, survival on the plant and mixed diets was over 86%, but dropped to 33% on the animal diet (Table 3, Fig. 3). Survival for *H. meridionalis* in the temperature treatments was 76%, with the exception of the long heat wave (55%), where complete mortality the animal diet treatment caused a marginally significant diet × temperature interaction (Table 3, Fig. 3). In *H. arborea* diet and temperature had no significant effect on survival (Table 3, Fig. 3).

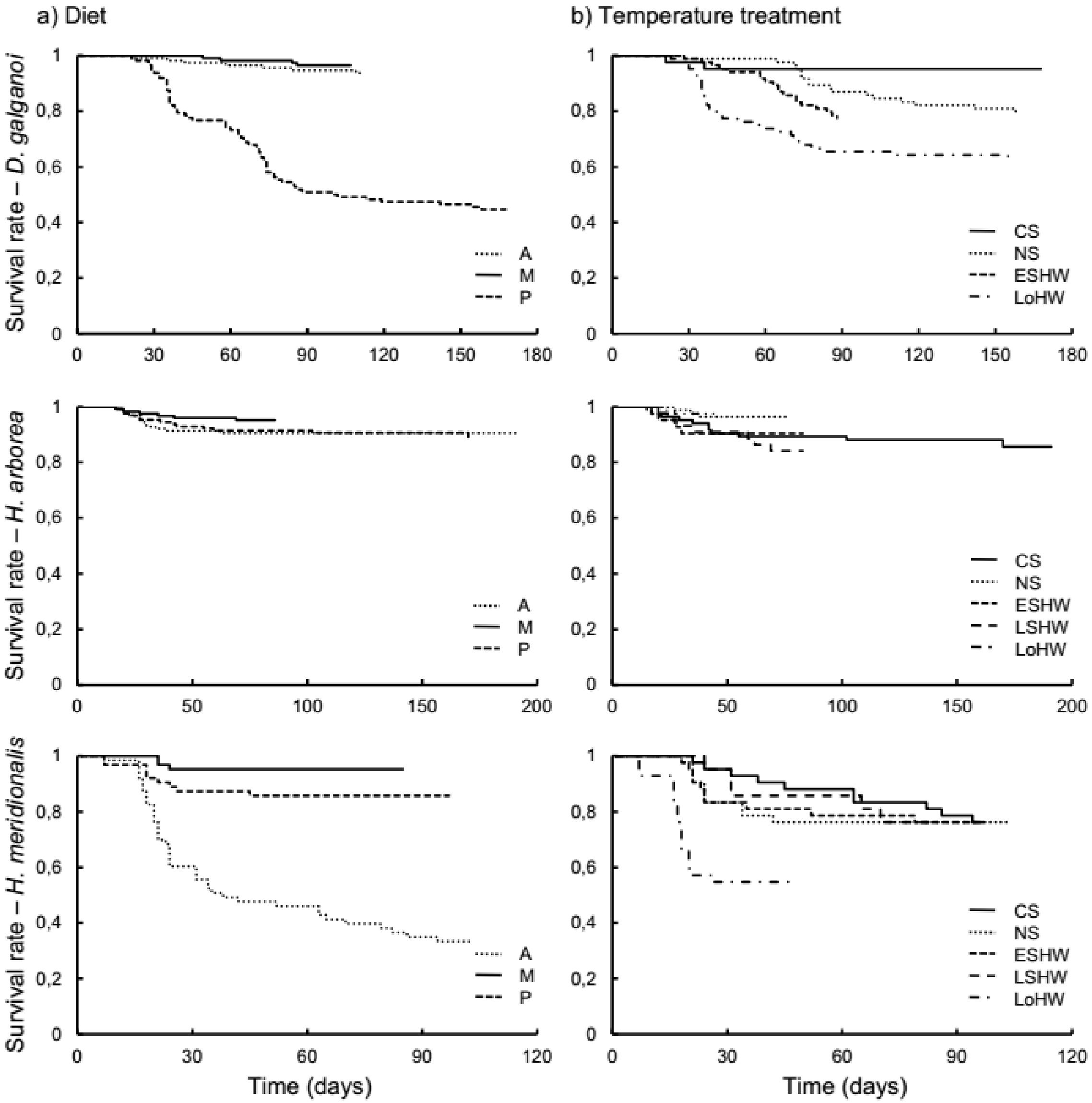


Figure 3. Cox proportional hazards regression for the survival rate of the study species on the different (a) diet treatments and (b) temperature treatments.

Table 3. Cox proportional hazards survival models and General Linear Models for growth rate, larval period and mass at metamorphosis.

	Survival			Growth rate			Larval Period			Mass at metamorphosis		
D. galganoi	*df*	χ^2	*P*	*df*	F	*P*	*df*	F	*P*	*df*	F	*P*
Diet	2	57.25	**<0.001**	2, 122	182.39	**<0.001**	1, 100	41.50	**<0.001**	1, 77	2.10	0.151
Temperature	3	78.09	**<0.001**	3, 122	53.48	**<0.001**	3, 100	48.62	**<0.001**	3, 77	65.92	**<0.001**
Diet × Temperature	6	4.39	0.625	6, 122	7.08	**<0.001**	3, 100	3.20	**<0.05**	3, 77	2.02	0.118
H. arborea												
Diet	2	1.21	0.548	2, 121	6.42	**<0.001**	2, 179	60.96	**<0.001**	2, 168	65.11	**<0.001**
Temperature	4	5.52	0.238	3, 121	15.99	**<0.001**	4, 179	225.60	**<0.001**	4, 168	24.31	**<0.001**
Diet × Temperature	8	5.20	0.736	6, 121	1.35	0.241	8, 179	21.55	**<0.001**	8, 168	7.22	**<0.001**
H. meridionalis												
Diet	2	74.95	**<0.001**	2, 51	50.51	**<0.001**	1, 50	5.13	**<0.05**	1, 50	15.82	**<0.001**
Temperature	4	43.28	**<0.001**	3, 51	10.88	**<0.001**	4, 50	70.19	**<0.001**	4, 50	10.39	**<0.001**
Diet × Temperature	8	15.37	0.052	6, 51	5.54	**<0.001**	4, 50	0.78	0.541	4, 50	1.23	0.310

P values <0.05 are marked in boldface

Life history – *D. galganoi*

Growth on the plant diet was lower than on the animal and mixed diets, ca. 63% and 71% respectively, while on the mixed diet growth was 23% greater than on the animal diet (Table 3, Fig. 4a). Growth was two times greater in the cold and normal spring than in the heat waves (Table 3, Fig. 4a). A temperature × diet interaction indicated that while in the cold and normal spring growth was similar on the animal and the mixed diets, in the long heat wave growth on the animal diet was lower than on the mixed diet (Table 3, Fig. 4a).

Larval period was 28% longer on the mixed diet than on the animal diet (Table 3, Fig. 4a). In the early spring and long heat waves larval period was almost 50% shorter than in the cold spring, and also 18% shorter than in the normal spring (Table 3, Fig. 4a). A diet × temperature interaction indicated that in the long heat wave and normal spring larval period on the mixed diet was longer than on the animal diet ($P < 0.01$), but in the cold spring and in the early spring heat wave there was no difference ($P = 1.0$; Table 3, Fig. 4a).

Mass at metamorphosis was not affected by diet, but in the cold spring it was almost two times higher than in the other temperature treatments (Table 3, Fig. 4a).

Life history – *H. arborea*

Growth on the plant and mixed diets was 24% greater than on the animal diet (Table 3, Fig. 4b). Growth in the long heat wave was 20% greater than in the normal and 41% greater than in the cold spring, for which growth was 28% lower than in the normal spring and 35% lower than in the early spring heat wave (Table 3, Fig 4b).

Larval period on the mixed diet was 13% shorter than on the animal and 25% shorter than on the plant diet (Table 3, Fig. 4b). Larval period in the long heat wave was shorter than in the other treatments, and in the early spring heat wave it was 14% shorter than in the normal spring and in the late spring heat wave (Table 3, Fig. 4b). A diet × temperature interaction indicated that in the cold spring larval period on the plant diet was much longer than in the other diets ($P < 0.001$), while in the long heat wave larval period on the animal diet was longer than on the other diets ($P < 0.05$; Table 3, Fig. 4b).

Mass at metamorphosis on the mixed diet was 9% higher than on the animal and 59% higher than on the plant diet (Table 3, Fig. 4b). Mass at metamorphosis in the cold spring was almost two times higher than in the long heat wave, in which mass at metamorphosis was 20% lower than in the normal spring, early and late spring heat waves (Table 3, Fig. 4b). A diet × temperature interaction showed that mass at metamorphosis on the plant diet was lower than on the mixed diet in all temperature treatments, except in the long heat wave ($P = 1.0$; Table 3, Fig. 4b); and lower than on the animal diet in all temperature treatments, except in the late spring ($P = 0.61$) and in the long heat wave ($P = 1.0$; Table 3, Fig. 4b). Furthermore, in the normal spring and in the late spring heat wave mass at metamorphosis on animal diet was lower than on the mixed diet ($P < 0.05$; Table 3, Fig. 4b), but not in the other temperature treatments.

Life history – *H. meridionalis*

Growth on the mixed diet was 33% higher than on the plant and 200% higher than on the animal diet, while growth on the plant diet was 66% higher than on the animal diet (Table 3, Fig. 4c). Growth in the heat waves was 46% higher than in the cold and normal spring (Table 3, Fig. 4c). A diet × temperature interaction showed that while in the cold spring diet had no effect on growth, in the heat waves growth increased on the mixed and plant diets, but not the animal diet (Table 3, Fig. 4c).

Larval period on the mixed diet was 7% shorter than on the plant diet (Table 3, Fig. 4c). Larval period in the long heat wave was shorter than in all the other treatments, while larval period in the cold spring was longer than in all the other treatments (Table 3, Fig. 4c).

Mass at metamorphosis on the mixed diet was 15% higher than on the plant diet (Table 3, Fig. 4c). Mass at metamorphosis in the cold spring was higher than in all the other treatments (Table 3, Fig. 4c).

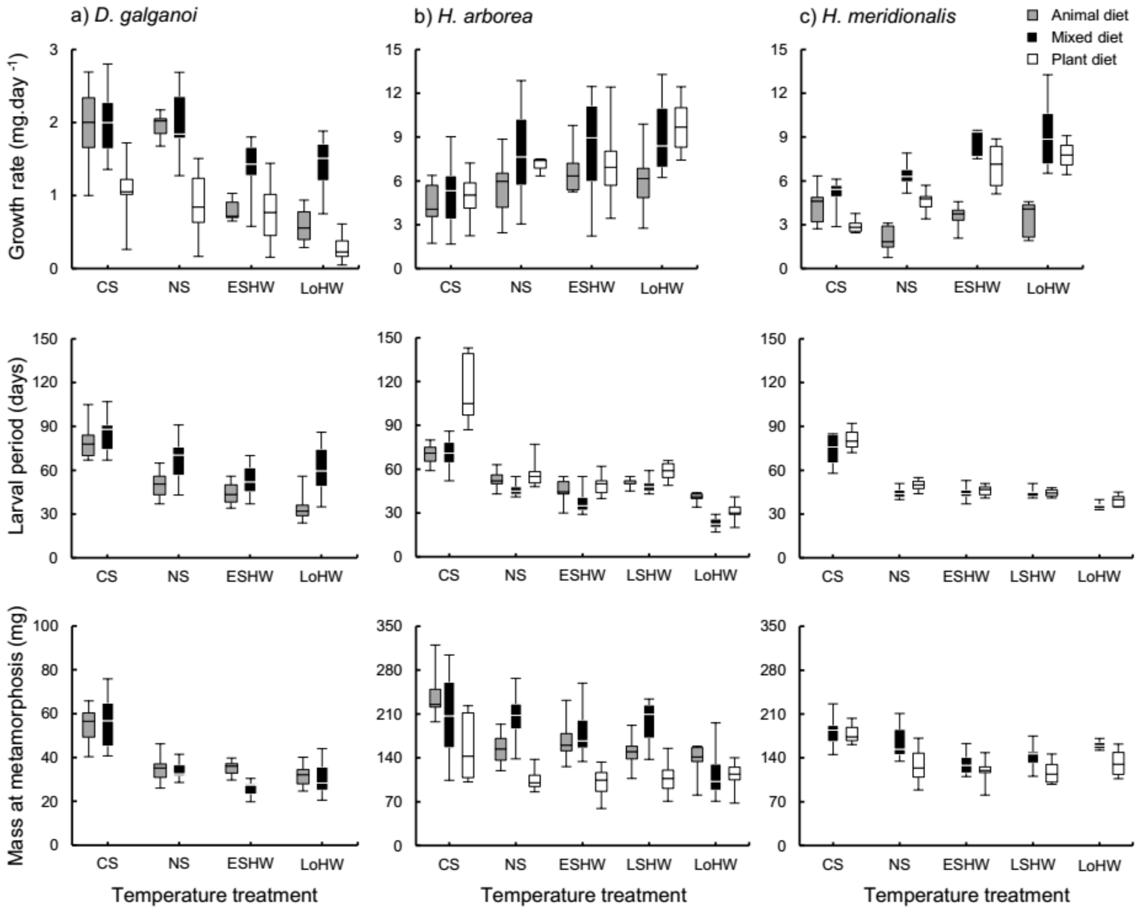


Figure 4. Growth rate (mg.day^{-1}), larval period (days) and mass at metamorphosis (mg) for a) *D. galganoi*, b) *H. arborea* and c) *H. meridionalis* tadpoles fed on animal, plant or mixed diet in the

temperature treatments: Cold Spring (CS), Normal Spring (NS), Early Spring Heat Wave (ESHW), Late Spring Heat Wave (LSHW) and Long Heat Wave (LoHW). Boxes show the median and the 25th – 75th percentiles; whiskers indicate the minimum and the maximum values.

Discussion

We found evidence for dietary specialization in larvae of three anuran species occurring along a seasonal temperature gradient, the degree of herbivory increasing with minimum temperature experienced in nature. Furthermore, we found that tadpoles shifted their diet in response to heat waves. However, these responses were not uniform as species responded differently to the various heat waves and none of the species responded to all types of heat wave. Nevertheless, whenever tadpoles performed diet shifts they always increased herbivory in response to higher temperature. This response was clearly adaptive in one species and potentially adaptive in another, but maladaptive in the most carnivorous species, indicating that the adaptive value of increasing herbivory at higher temperatures was species-specific. To our knowledge, this is one of the first studies to present hard evidence that ectotherms may maximize energy intake at higher temperatures by increasing herbivory, and the first study to investigate temperature-induced diet shifts in vertebrates (see Boersma et al. 2016 for a previous study on invertebrates).

Our results support the omnivorous character of anuran larvae (Petranka and Kennedy 1999, Caut et al. 2013, Arribas et al. 2015), since tadpoles of the three species assimilated nutrients from both animal and plant material. However, the species' trophic positions were clearly different, suggesting different degrees of specialization along the trophic gradient. As predicted, proportion of animal-based diet was higher in *D. galganoi*, the species that starts breeding earliest in the season (i.e. at the lowest temperature), while the proportion of the plant-based diet was higher in *H. meridionalis*, the species breeding at the highest temperature. The low survival rates of these species under the less-preferred diets (plant diet for *D. galganoi*, animal diet for *H. meridionalis*) further reinforced their relative specialist character. *Hyla arborea*, reproducing at an intermediate thermal environment, occupied a central position along the diet gradient, showing less diet specialization. Although the number of species in our study is limited, the present results suggest a general effect of temperature on the feeding preferences and the extent of herbivory in tadpoles. While previous studies have shown higher abundance and richness of herbivorous fishes in tropical latitudes (Floeter et al. 2005, Behrens and Lafferty 2007, Jeppesen et al. 2010, Gonzalez-Bergonzoni et al. 2012), this is the first study to experimentally demonstrate a relationship between the trophic niche of vertebrate omnivores and the temperatures to which they normally experience in natural environments.

Our study found support for lower benefits of the animal diet in the heat wave treatments in all species. When compared with the mixed diet, the animal diet markedly decreased tadpole growth in the early spring and long heat wave treatments in *D. galganoi*. In *H. arborea*, the animal diet hampered growth and extended the larval period in the long heat wave without an increase in mass at metamorphosis, the latter decreasing markedly on both the animal and mixed diets. In *H. meridionalis* the animal diet caused high mortality in the long heat wave. We also found support for increased benefits of the plant diet in the heat wave treatments in two species. In both hylids growth on the plant diet increased in the heat waves, especially in the long heat wave, in which the growth on the plant diet equaled growth on the mixed diet in *H. arborea*. Furthermore, in *H. arborea* the plant diet extended the larval period in the cold spring, but in the long heat wave larval period on this same diet was shorter than on the animal diet. These findings suggest that animal diet has a lower nutritional value than plant diet at higher temperatures, and support the adaptive value of increasing herbivory under these conditions, but also indicate that the temperature effect on animal diets may be stronger than on plant diets. While growth on the plant diet decreased with temperature in *D. galganoi*, the relative quality of the plant diet increased because in the heat waves growth on the plant diet was no longer lower than on the animal diet. However, in the long heat wave the mixed diet extended the larval period more than the animal diet, suggesting even a low proportion of plant diet was sufficient to hamper development in the late pre-metamorphic stages.

Although the study species shifted their diet towards increased herbivory, these responses were not uniform as the heat wave treatments inducing diet shifts varied among species, as did the magnitude of the diet shifts when they occurred. While *D. galganoi* and *H. meridionalis* appear to have performed modest diet shifts in the short heat wave treatments, the true extent of the diet shifts may have been greater, being lessened by a partial reversion in the isotopic signatures during the ca. 2 weeks the tadpoles took to reach metamorphosis. The essentially carnivorous *D. galganoi* (Richter-Boix et al. 2012, Arribas et al. 2015) performed a potentially maladaptive shift towards higher herbivory in the early spring heat wave, as suggested by the low mass at metamorphosis when compared to strictly carnivorous tadpoles. *Hyla meridionalis* increased herbivory in the late spring heat wave, suggesting a higher sensitivity in the late pre-metamorphic stages. The lack of notable benefits or costs in *H. meridionalis* may result from its already high herbivory in the experiments and from the small extent of the diet shift. The more specialized diets of *D. galganoi* (carnivory) and *H. meridionalis* (herbivory) may have prevented tadpoles from sustaining long-term diet shifts in the long heat wave. We found that both *D. galganoi* and *H. meridionalis* shortened larval period without detectable costs in mass at metamorphosis. In fact, each of these two species metamorphosed with a similar mass in all treatments (except the cold spring), suggesting a general response where, above a certain temperature threshold, tadpoles initiate metamorphosis at a minimum size required for its successful completion (Kuparinen et al. 2010). Since *D. galganoi* breeds in ephemeral and *H. meridionalis* in temporary ponds (Richter-Boix et al. 2006, Escoriza and Boix 2014), such sensitivity to heat is not surprising as they need to complete metamorphosis before pond desiccation.

In the long heat wave *H. arborea*, unlike *H. meridionalis*, sustained a rather large increase in herbivory. In this treatment, *H. arborea* shortened larval period without increasing growth rate, so this diet shift came at the cost of lower mass at metamorphosis. However, the benefits may outweigh the costs because a short larval period is adaptive in Mediterranean temporary ponds, given the mortality risk due to pond desiccation (Richter-Boix et al. 2011), and early metamorphosis may compensate for the negative effects of small body size in hylids (Smith 1987). *Hyla arborea* did not perform diet shifts in the early spring heat wave, but slightly decreased the larval period, which allowed tadpoles to complete metamorphosis earlier and without detectable costs in mass. *Hyla arborea* experienced the late spring heat wave earlier than *H. meridionalis* (Gosner stages 35-36 versus 37-38; data not shown). Therefore, despite the lack of response in this study, this species might still be sensitive to heat waves in later pre-metamorphic stages.

This study allows two important inferences regarding the mechanisms underlying diet shifts and their potential effects in freshwater communities. First, short term climatic events such as one-week heat waves increased the contribution of plants to the diet of tadpoles (*D. galganoi* and *H. meridionalis)*, either through an active change in the feeding preference, or through a temperature-mediated passive change in the digestion and/or assimilation efficiency of each diet. Our study suggests that beyond possible temperature mediated passive mechanisms, which may have increased the assimilation of the plant diet over the animal diet at higher temperatures, tadpoles actively regulated nutrient acquisition and changed feeding preferences according to the temperature. This is because a purely passive mechanism would have resulted in higher herbivory in the long heat wave than in the other heat waves, which did not occur in both *D. galganoi* and *H. meridionalis*. Second, the adaptive value of the temperature-induced diet shifts varied among species, suggesting the challenges imposed by heat waves may affect community structure, as diet shifts towards herbivory may favor some species and impair others, especially the species close to strict carnivory.

Although unavoidable, the use of different food items in the diet treatments may have affected our results. However, we maintain that this variation should have only a reduced effect when considering the overall difference between animal and plant diets and should have little impact on our conclusions. Unfortunately, logistic constraints did not allow cyclical dial variation in our experimental setup, which could have buffered the effects of high daytime temperatures and as such our results may overestimate responses in nature. Furthermore, the temperature range tested prevents generalizations to extreme temperatures, because under acute heat stress demand for nitrogen may increase (for e.g, synthesis of heat shock proteins; Podrabsky and Somero 2004). However, we focused on spring heat waves with mild temperatures (25ºC), because our study species inhabit Mediterranean temporary ponds which dry by the end of spring and the tadpoles are never exposed to the summer heat waves with temperatures above 35ºC. Although the study species have similar thermal performance ranges and upper critical thermal limits above

38°C (Duarte et al. 2011; Katzenberger 2015), the temperature range in the experiments may have induced different levels of stress among the species, especially in *D. galganoi*, in which overall growth decreased in the heat waves.

Climate change projections show heat waves are likely to become more frequent, intense, and longer lasting over the century. Although diet regulation may help omnivore ectotherms to cope with increased heat stress, the potential to perform diet shifts as well their adaptive value are likely to differ across species. More importantly, generalized diet shifts towards higher herbivory may trigger changes in freshwater ecosystems and disturb the structure and functioning of freshwater communities and food webs. Awareness of temperature effects on diet regulation may contribute to a better understanding and reassessment of the ecological effects of the ongoing climate change.

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References

Altig, R., M. R. Whiles, and C. L. Taylor. 2007. What do tadpoles really eat? Assessing the trophic status of an understudied and imperiled group of consumers in freshwater habitats. Freshwater Biology 52:386–395.

Arribas, R., C. Díaz-Paniagua, S.Caut, and I. Gomez-Mestre. 2015. Stable isotopes reveal trophic partitioning and trophic plasticity of a larval amphibian guild. Plos One 10:e0130897.

Behrens, M. D., and K. D. Lafferty. 2007. Temperature and diet effects on omnivorous fish performance: implications for the latitudinal diversity gradient in herbivorous fishes. Canadian Journal of Fisheries and Aquatic Sciences 64:867–873.

Boersma, M., K. A. Mathew, B. Niehoff, K. L. Schoo, R. M. Franco Santos, and C. L. Meunier. 2016. Temperature driven changes in the diet preference of omnivorous copepods: no more meat when it's hot? Ecology Letters 19:45–53.

Caut, S., E. Angulo, C. Díaz-Paniagua, and I. Gomez-Mestre. 2013. Plastic changes in tadpole trophic ecology revealed by stable isotope analysis. Oecologia 173:95–105.

Chapman, S., K. Mustin, A. R. Renwick, D. B. Segan, D. G. Hole, R. G. Pearson, and J. E. M. Watson. 2014. Publishing trends on climate change vulnerability in the conservation literature reveal a predominant focus on direct impacts and long time-scales. Diversity and Distributions 20:1221–1228.

Clusella-Trullas, S., T. M. Blackburn, and S. L. Chown. 2011. Climatic predictors of temperature performance curve parameters in ectotherms imply complex responses to climate change. The American naturalist 177:738–751.

Croll, S. L., and S. A. Watts. 2004. The effect of temperature on feed consumption and nutrient absorption in *Procambarus clarkii* and *Procambarus zonangulus*. Journal of the World Aquaculture Society 35:478–488.

Cross, W. F., J. M. Hood, J. P. Benstead, A. D. Huryn, and D. Nelson. 2015. Interactions between temperature and nutrients across levels of ecological organization. Global Change Biology 21:1025–1040.

Dasari, H. P., I. Pozo, F. Ferri-Yáñez, and M. B. Araújo. 2014. A regional climate study of heat waves over the Iberian Peninsula. Atmospheric and Climate Sciences 4:841–853.

Dobush, G. R., C. D. Ankney, and D. G. Krementz. 1985. The effect of apparatus, extraction time, and solvent type on lipid extractions of snow geese. Canadian Journal of Zoology 63:1917–1920.

Duarte, H., M. Tejedo, M. Katzenberger, F. Marangoni, D. Baldo, J. F. Beltrán, D. A. Martí, A. Richter-Boix, and A. Gonzalez-Voyer. 2011. Can amphibians take the heat? Vulnerability to climate warming in subtropical and temperate larval amphibian communities. Global Change Biology 18:412–421.

Dudgeon, D., A. H. Arthington, M. O. Gessner, Z. -I. Kawabata, D. J. Knowler, C. Lévêque, R. J. Naiman, A. -H. Prieur-Richard, D. Soto, M. L. J. Stiassny, and C. A. Sullivan. 2006. Freshwater biodiversity: importance, threats, status and conservation challenges. Biological reviews 81:163–182.

Earl, J. E., and H. H. Whiteman. 2015. Are commonly used fitness predictors accurate? A meta-analysis of amphibian size and age at metamorphosis. Copeia 103:297–309.

Eggers, S. L., B. K. Eriksson, and B. Matthiessen. 2012. A heat wave and dispersal cause dominance shift and decrease biomass in experimental metacommunities. Oikos 121:721–733.

Escoriza, D., and D. Boix. 2014. Reproductive habitat selection in alien and native populations of the genus *Discoglossus*. Acta Oecologica 59:97–103.

Fischer, K., M. Klockmann, and E. Reim. 2014. Strong negative effects of simulated heat waves in a tropical butterfly. Journal of Experimental Biology 217:2892–2898.

Floeter, S. R., M. D. Behrens, C. E. L. Ferreira, M. J. Paddack, and M. H. Horn. 2005. Geographical gradients of marine herbivorous fishes: Patterns and processes. Marine Biology 147:1435–1447.

Forster, J., A. G. Hirst, and G. Woodward. 2011. Growth and development rates have different thermal responses. The American Naturalist 178:668–678.

Frich, P., L. V. Alexander, P. Della-Marta, B. Gleason, M. Haylock, A. M. G. Tank Klein, and T. Peterson. 2002. Observed coherent changes in climatic extremes during the second half of the twentieth century. Climate Research 19:193–212.

Garrabou, J., R. Coma, N. Bensoussan, M. Bally, P. Chevaldonné, M. Cigliano, D. Diaz, J. G. Harmelin, M. C. Gambi, D. K. Kersting, J. B. Ledoux, C. Lejeusne, C. Linares, C. Marschal, T. Pérez, M. Ribes, J. C. Romano, E. Serrano, N. Teixido, O. Torrents, M. Zabala, F. Zuberer, and C. Cerrano. 2009. Mass mortality in Northwestern Mediterranean rocky benthic communities: effects of the 2003 heat wave. Global Change Biology 15:1090–1103.

Giorgi, F. 2006. Climate change hot-spots. Geophysical Research Letters 33:1–4.

Gonzalez-Bergonzoni, I., M. Meerhoff, T. A. Davidson, F. Teixeira-de Mello, A. Baattrup-Pedersen, and E. Jeppesen. 2012. Meta-analysis shows a consistent and strong latitudinal pattern in fish omnivory across ecosystems. Ecosystems 15:492–503.

Gosner, K. N. 1960. A simplified table for staging anuran embryos and larvae with notes on identification. Herpetologica 16:183–190.

Hoffmann, A. A., and C. M. Sgrò. 2011. Climate change and evolutionary adaptation. Nature 470:479–485.

Jeppesen, E., M. Meerhoff, K. Holmgren, I. González-Bergonzoni, F. Teixeira-de Mello, S. A. J. Declerck, L. De Meester, M. Søndergaard, T. L. Lauridsen, R. Bjerring, J. M. Conde-Porcuna, N. Mazzeo, C. Iglesias, M. Reizenstein, H. J. Malmquist, Z. Liu, D. Balayla, and X. Lazzaro. 2010. Impacts of climate warming on lake fish community structure and potential effects on ecosystem function. Hydrobiologia 646:73–90.

Jerez, S., J. P. Montavez, J. J. Gomez-Navarro, R. Lorente-Plazas, J. A. Garcia-Valero, and P. Jimenez-Guerrero. 2013. A multi-physics ensemble of regional climate change projections over the Iberian Peninsula. Climate Dynamics 41:1749–1768.

Karl, I., and K. Fischer. 2008. Why get big in the cold? Towards a solution to a life-history puzzle. Oecologia 155:215–225.

Katzenberger, M. 2015. Impact of Global Warming in Holarctic and Neotropical communities of amphibians. PhD thesis, University of Seville, Seville.

Kingsolver, J. G., S. E. Diamond, and L. B. Buckley. 2013. Heat stress and the fitness consequences of climate change for terrestrial ectotherms. Functional Ecology 27:1415–1423.

Kuparinen, A., A. T. Laugen, A. Laurila, and J. Merilä. 2010. Developmental threshold model challenged by temperature? Evolutionary Ecology Research 12:821–829.

Ma, G., V. H. W. Rudolf, and C. Sen Ma. 2015. Extreme temperature events alter demographic rates, relative fitness, and community structure. Global Change Biology 21:1794–1808.

Meehl, G. A., and C. Tebaldi. 2004. More intense, more frequent, and longer lasting heat waves in the 21st century. Science 305:994–997.

Myrick, C. A., and J. J. Jr. Cech. 2000. Temperature influences on California rainbow trout physiological performance. Fish Physiology Biochemestry 22:245–254.

Paaijmans, K. P., R. L. Heinig, R. A. Seliga, J. I. Blanford, S. Blanford, C. C. Murdock, and M. B. Thomas. 2013. Temperature variation makes ectotherms more sensitive to climate change. Global Change Biology 19:2373–2380.

Parnell, A. C., R. Inger, S. Bearhop, and A. L. Jackson. 2010. Source partitioning using stable isotopes: coping with too much variation. PloS One 5:e9672.

Parnell, A. C., D. L. Phillips, S. Bearhop, B. X. Semmens, E. J. Ward, J. W. Moore, A. L. Jackson, J. Grey, D. J. Kelly, and R. Inger. 2013. Bayesian stable isotope mixing models. Environmetrics 24:387–399.

Petranka, J. W., and C.A. Kennedy. 1999. Pond tadpoles with generalized morphology: Is it time to reconsider their functional roles in aquatic communities? Oecologia 120:621–631.

Pfenning, D.W. 1990. The adaptive significance of an environmentally-cued developmental switch in an anuran tadpole. Oecologia 85:101–107.

Podrabsky, J. E., and Romero. 2004. Changes in gene expression associated with acclimation to constant temperatures and fluctuating daily temperatures in an annual killifish *Austrofundulus limnaeus*. Journal of Experimental Biology 207:2237–2254.

Preston, T., and N. J. P. Owens. 1983. Interfacing an automatic elemental analyser with an isotope ratio mass sepectrometer: the potential for fully automated total nitrogen and nitrogen-15 analysis. Analyst 108:971–977.

Rebelo, R., and E. G. Crespo. 1999. Anfíbios. Pages 177-188 *in* M. Santos-Reis and A. I. Correia, editors. Caracterização da flora e da fauna do montado da herdade da Ribeira Abaixo (Grândola, Baixo Alentejo). Centro de Biologia Ambiental, Lisboa, Portugal.

Reusch, T. B. H., A. Ehlers, A. Hämmerli, and B. Worm. 2005. Ecosystem recovery after climatic extremes enhanced by genotypic diversity. Proceedings of the National Academy of Sciences of the United States of America 102:2826–2831.

Richter-Boix, A., G. A. Llorente, and A. Montori. 2006. A comparative analysis of the adaptive developmental plasticity hypothesis in six Mediterranean anuran species along a pond permanency gradient. Evolutionary Ecology Research 8:1139–1154.

Richter-Boix, A., M. Tejedo, and E. L. Rezende. 2011. Evolution and plasticity of anuran larval development in response to desiccation. A comparative analysis. Ecology and Evolution 1:15–25.

Richter-Boix, A., N. Garriga, A. Montori, M. Franch, O. San Sebastiám, D. Villero, and G. A. Llorente. 2012. Effects of the non-native amphibian species Discoglossus pictus on the recipient amphibian community : niche overlap, competition and community organization. Biological Invasions 15:799–815.

Schär, C., P. L. Vidale, D. Lüthi, C. Frei, C. Häberli, M. A. Liniger, and C. Appenzeller. 2004. The role of increasing temperature variability in European summer heatwaves. Nature 427:332–336.

Seifert, L. I., F. De Castro, A. Marquart, U. Gaedke, G. Weithoff, and M. Vos. 2014. Heated relations: Temperature-mediated shifts in consumption across trophic levels. Plos One 9:e95046.

Smith, D. C. 1987. Adult recruitment in chorus frogs: effects of size and date at metamorphosis. Ecology 68:344–350.

Tejedo, M., F. Marangoni, C. Pertoldi, A. Richter-Boix, A. Laurila, G. Orizaola, A. G. Nicieza, D. Álvarez, and I. Gomez-Mestre. 2010. Contrasting effects of environmental factors during larval stage on morphological plasticity in post-metamorphic frogs. Climate Research 43:31–39.

Thomas, S. M., and T. W. Crowther. 2014. Predicting rates of isotopic turnover across the animal kingdom: a synthesis of existing data. Journal of Animal Ecololy 84:861–870.

Thompson, R. M., J. Beardall, J. Beringer, M. Grace and P. Sardina. 2013. Means and extremes: building variability into community-level climate change experiments. Ecology Letters 16:799–806.

Vasseur, D. A., J. P. DeLong, B. Gilbert, H. S. Greig, C. D. G. Harley, K. S. McCann, V. Savage, T. D. Tunney, and M. I. O' Connor. 2014. Increased temperature variation poses a greater risk to species than climate warming. Proceedings of the Royal Society B 281:2013–2612.

Wernberg, T., D. A. Smale, F. Tuya, M. S.Thomsen, T. J. Langlois, T. de Bettignies, S. Bennett, and C. S. Rousseaux. 2012. An extreme climatic event alters marine ecosystem structure in a global biodiversity hotspot. Nature Climate Change 3:78–82.

Wilbur, H. M., and J. P. Collins. 1973. Ecological aspects of amphibian metamorphosis: nonnormal distributions of competitive ability reflect selection for facultative metamorphosis. Science 182:1305–1314.

CHAPTER 3

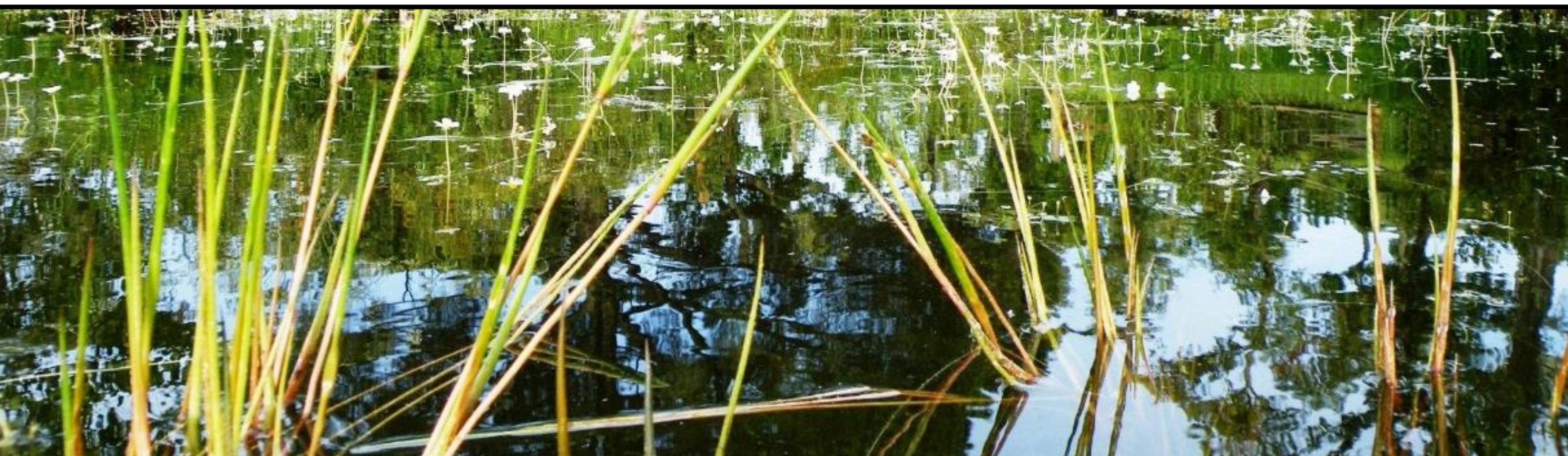
Disentangling stress: complex response of tadpole activity level to diet and temperature

V. Pinto & N. Gonçalves, B. M. Carreira, A. Laurila and R. Rebelo

Discoglossus galganoi

Hyla arborea

Hyla meridionalis



CHAPTER 3

Disentangling stress: complex response of tadpole activity level to diet and temperature

Abstract

Activity level is often used as a stress indicator in aquatic animals. However, besides the effects of the stressor agents studied, experiments subject animals to other potentially stress-causing factors, such as diet and temperature, whose effects on activity are often unaccounted for. We focused on tadpoles of three anuran species with known and contrasting thermal and feeding preferences, fed them on animal, plant or mixed diets and exposed them to different temperatures. We then measured tadpole growth and activity levels, using growth reduction as an indicator of stress. We predicted that tadpoles on single diets would compensate for the negative effects of stressful conditions by increasing activity, while tadpoles on a mixed diet should optimize nutrient intake by feeding selectively and display lower activity levels. All three species increased activity with suboptimal diets, but showed different responses under suboptimal temperatures, since activity increased in one species and decreased in the others. Increased activity allowed tadpoles on single diets to mitigate low to moderate stress, but was not sufficient to maintain growth rates under high stress. With one exception, diet regulation on mixed diets allowed tadpoles to decrease activity without growth costs. By demonstrating that activity varies according to the preferred diet, optimum temperature, and differential quality of the diet as temperature changes, our findings suggest that using activity as a stress indicator should be done carefully.

Keywords: Anurans; Behavior; Diet; *Discoglossus galganoi*; Growth; *Hyla arborea*; *Hyla meridionalis*; Tadpoles; Temperature

Introduction

Due to the natural change of seasons, the occurrence of extreme climatic events and human disturbance, environmental conditions in natural ecosystems are in constant change. When these changes disrupt homeostasis organisms experience stress (Maher *et al.*, 2013). In vertebrates the response to environmental stressors often includes behavioral changes, which for instance may affect their activity levels, sometimes with long-term fitness costs (Crespi & Denver, 2005; Maher *et al.*, 2013; Paull *et al.*, 2015). Easily measurable, the activity levels of vertebrates have been reported to vary under the effects of diverse stressors such as pesticides (Shuman-Goodier & Propper 2016), predation risk (Barry, 2014), food availability (Anholt & Werner, 1995; Krause, Steinfartz & Caspers, 2011) and temperature (Crespi & Denver, 2005; Maher *et al.*, 2013; Navas *et al.*, 2013).

In amphibians, tadpole responses to chemical agents in the water may be contrasting, since substances such as nitrate (Hatch & Blaustein, 2000) and carbaryl (Bridges, 1997) decrease activity levels, while others such as triclosan (Smith & Burgett, 2005) and caffeine (Fraker & Smith, 2004) increase activity. In the presence of predators, tadpoles generally reduce activity, as high activity levels increase predation risk (Werner & Anholt, 1993; Bridges, 2002; Davis, Purrenhage & Boone, 2012; Nunes *et al.*, 2013).

The amount and quality of the food available to tadpoles may influence their activity levels (Eklöv & Halvarsson, 2000). Increased activity has been interpreted as a positive measure (Richardson, 2001) because foraging time correlates positively with both feeding time and total food intake, and more active tadpoles should be better able to satisfy their nutritional demands and grow faster (Werner, 1992; Werner & Anholt, 1993; Semlitsch *et al.*, 1995; Nicieza & Metcalfe, 1997). However, increased activity has also been interpreted as negative because, unless tadpoles are hungry, greater foraging requires energy expenditure (Barry, 2014). Several studies reported tadpoles to forage more actively as the amount or quality of the food resources declined (Anholt & Werner, 1995; Laurila, Kujasalo & Ranta, 1998), suggesting increased activity helps hungry tadpoles to satisfy their nutritional demands (Eklöv & Halvarsson, 2000; Bridges, 2002; Barry, 2014). Conversely, by foraging less, well-fed tadpoles reduced energy expenditure and decreased predation risk.

Temperature also affects the activity levels of amphibian tadpoles, which at the cost of greater energy expenditure increase swimming speed at higher temperatures (Finkler & Claussen, 1999; Katzenberger *et al.*, 2014). Although warmer temperatures should increase activity levels, selective foraging may help tadpoles to cope with the effects of a warmer environment, allowing for changes in diet or increased feeding rates, and partly offsetting for the negative effects of activity increase (Kupferberg, 1997; Richter-Boix *et al.*, 2007). However, the scarce empirical data present both supportive and contradictory evidence for this hypothesis (e.g., Laurila, Lindgren & Laugen, 2008), which may stem from where the experimental temperatures were located along the species' thermal performance curves.

Recent studies on the metabolic ecology of ectotherms showed that the relative quality of diets may change with temperature (Boersma *et al.*, 2016; Carreira *et al.*, 2016). Assessing temperature and diet together, these studies showed higher temperatures to decrease performance on animal diets and increase performance on plant diets. While these findings may bring additional complexity, they may also provide new insights and contribute to a better understanding on how tadpole activity varies with stress. Prior knowledge, though often scarce, could constitute an additional tool to help in the clarification of this matter. Even though tadpoles are omnivorous, their trophic position is species-specific and varies widely along the herbivory-carnivory gradient (Altig, Whiles & Taylor, 2007; Schiesari, Werner & Kling, 2009). For instance, Carreira *et al.* (2016) showed tadpoles of three Iberian anurans to assimilate very different proportions of animal and plant diets.

We investigated diet and temperature stressor effects on three anurans by measuring changes in the activity levels of tadpoles fed on animal-based, plant-based or mixed diets under different temperature regimes. We used variation in growth as an indicator of stress and predicted that: 1) regardless of the stressor (diet or temperature), tadpole activity levels should follow the general shape of a performance curve with low activity on both tails explained by the absence of stress (left tail) or severe stress (right tail), while the high activity at the midpoint may be explained by moderate stress levels; 2) increasing activity should help tadpoles compensate for the lower quality of suboptimal diets and/or for the constraints imposed by stressful temperature regimes; 3) diet regulation by tadpoles on the mixed diet should promote lower activity levels than on the single diets, while maintaining or increasing growth.

Materials and Methods

This experiment was part of a broader study, where we determined the preferred diets of the larvae of three anuran species common in temporary ponds of the Southwest of the Iberian Peninsula (Carreira *et al.*, 2016). Mediterranean temporary ponds dry in late spring and early summer and tadpoles must complete metamorphosis or face pond drying.

High growth rates are expected to be an important trait in these habitat, and we used growth rate as a fitness proxy and investigated its relationship with activity levels.

Study species, collection and maintenance

We selected three anuran species breeding along a seasonal temperature gradient with known feeding preferences (Carreira *et al.*, 2016): the Iberian painted frog, *Discoglossus galganoi* (Capula, Nascetti, Lanza, Bullini & Crespo 1985), which breeds from autumn through early spring and is mostly carnivorous; the European tree frog, *Hyla arborea* (Linnaeus, 1758), which breeds during spring and has an omnivorous balanced diet; and the Mediterranean tree frog, *Hyla meridionalis* (Boettger 1874), which also breeds during spring and is mostly herbivorous (Table 1).

Egg clutches, or newly hatched tadpoles in *H. meridionalis,* were collected in ponds of the southwest of Portugal (Table 1, Carreira *et al.* 2106). The eggs were kept in 1L plastic trays with aged tap water and taken to the facilities of the Faculty of Sciences, University of Lisbon (Portugal), where they were kept at room temperature (ca. 20ºC) under a 12L:12D photoperiod. Water was changed twice a week and tadpoles were fed commercial fish food (Tetra PondSticks®, Germany) until reaching Gosner stage 25 (Gosner, 1960). At this stage, tadpoles were photographed for determination of initial body size with ImageJ software and transferred into the experimental setup under a 12L:12D photoperiod. On experimental day 1, randomly selected tadpoles were assigned to the individual experimental units consisting of cylindrical plastic containers (9 cm diameter x 10 cm height) filled with 0.3L of aged tap water. The experimental units were assigned to a treatment and inserted into the corresponding water bath (plastic tank: 70 x

45 cm). Tadpoles were fed *ad libitum* three times per week when water was changed. Water bath temperatures were checked daily and adjusted when necessary.

Table 1. Species origin and experimental procedures details. Collection site; date of collection; number of clutches collected; duration of the experimental period; and dates of the behavioral observations for each study species.

Species	Collection site (coordinates)	Date of collection	# Clutches collected	Experimental period	Behavioral observations (day)
Discoglossus galganoi	Grândola (38º06'N, 8º34'W)	6 January 2014	3	4 February to 17 July 2014	29, 38 and 45
Hyla arborea	Verdizela (38°34'N, 9°08'W)	20 March to 20 May 2014	60	25 June to 16 December 2014	21, 28 and 35
Hyla meridionalis	Grândola (38º06'N, 8º34'W)	26 April 2012	-	20 June to 9 October 2012	14, 21 and 28

Experimental Design

We performed a factorial experiment, with three diets and three temperature treatments, resulting in nine treatment combinations, each replicated 28 (*D. galganoi* and *H. arborea*) or 14 times (*H. meridionalis*). Tadpoles were fed either a plant diet (P), composed of defrosted stalks of *Ranunculus peltatus* (Hylids) or *Juncus heterophyllus* (*D. galganoi*); an animal diet (A) composed of defrosted commercially obtained chironomid larvae (*H. arborea* and *D. galganoi*) or ephemeropteran larvae (*H. meridionalis*) collected in a stream (Lisbon, Portugal); or a mixed diet (M), with both food items offered *ad libitum*. Macrophytes offered on the plant diet differed among the species because *D. galganoi* preferred *J. heterophyllus* in the preliminary diet tests, while the Hylids preferred *R. peltatus*. Invertebrates offered on the animal diet varied among the species because it was difficult to capture sufficient amounts of ephemeropteran larvae. Tadpoles were exposed to three temperature treatments: cold spring (CS) – constant temperature of 17ºC; normal spring (NS) – temperature was gradually increased from 17ºC to 25ºC (1ºC per week); and long heat wave (LoHW) – constant temperature of 25ºC.

Activity was recorded in three dates approximately one week apart (Table 1). On each date tadpoles were checked five times (*D. galganoi* and *H. arborea*) or two times (*H. meridionalis*), in intervals of 30 to 60 minutes. Tadpoles were considered active when actively swimming, feeding or undulating the tail. After concluding activity recordings on the last date, half of the tadpoles in each treatment were randomly selected and weighed to nearest mg for determination of growth rate (mg.day^{-1}; mass measured at a fixed time divided by the experimental day; see Table 1). The remaining tadpoles were kept in the experiment for another study (Carreira *et al.*, 2016).

Statistical analyses

We used generalized linear mixed models to test treatment effects and interactions on tadpole activity. Since date had no significant effects, we used a simplified model with only diet and temperature as fixed effects. We specified a binomial distribution with a logit link function and estimated tadpole activity as the number of times a tadpole was active out of the total number of times its activity was checked; pairwise comparisons were corrected for multiple comparisons (Bonferroni's test). We used general linear models to test treatment effects and interactions on tadpole growth rate and included tadpole body size on day 1 as a covariate in all models; pairwise comparisons were corrected for multiple comparisons (Bonferroni's test).

For an easier comprehension of the results, we used a graphic approach and plotted tadpole growth and activity levels together. Each species biplot shows the difference between treatment average and the overall mean for: growth (x-axis) and activity (y-axis). Starting at the bottom left and proceeding clockwise, the quadrants define four regions of the activity performance curve as a function of growth, used as a measure of stress: i) low growth and low activity (debilitated state, G^-A^-); ii) low growth and high activity (inefficient stress compensation by increased activity, G^-A^+); iii) high growth and high activity (efficient stress compensation by increased activity, G^+A^+); iv) high growth and low activity (satiation in the absence of stress, G^+A^-).

Results

Discoglossus galganoi

Tadpoles on the plant and mixed diets were 44% more active than tadpoles on the animal diet (Table 2, Fig. 1a). The activity levels in the normal spring and in the long heat wave were almost two times higher than in the cold spring (Table 2, Fig. 1a). The diet × temperature interaction was not significant (Table 2).

Growth on the animal and the mixed diets was about three times greater than on the plant diet (Table 2, Fig. 1a). Growth was greatest in the cold spring, but decreased by 5% in the normal spring and to less than half in the long heat wave growth (Table 2, Fig. 1a). The diet × temperature interaction was significant, since in the long heat wave growth on the animal diet was three times lower than on the mixed diet, unlike in the other temperature treatments (Table 2, Fig. 1a).

Hyla arborea

Tadpoles on the animal diet were twice as active as tadpoles on the mixed and plant diets (Table 2, Fig. 1b). Tadpole activity levels were highest in the long heat wave, but decreased to half in the normal spring and even more so in the cold spring (Table 2, Fig. 1b). The diet × temperature interaction was significant, since in the cold and normal spring treatments tadpoles on the animal diet were more active, but in the long heat wave diet had no effect on activity levels (Table 2, Fig. 1b).

Growth on the plant and the mixed diets was 30% greater than on the animal diet (Table 2, Fig. 1b). Growth was greatest in the long heat wave, but decreased 17% in the normal spring and 40% in the cold spring (Table 2, Fig. 1b). The diet × temperature interaction was not significant (Table 2).

Hyla meridionalis

Tadpole activity was highest on the plant and the mixed diets, more than two times higher than on the animal diet (Table 2, Fig. 1c). Temperature had no significant effect on tadpole activity and there was no significant diet × temperature interaction (Table 2, Fig. 1c).

Growth was greatest on the mixed diet, but decreased 28% on the plant diet and to less than half on the animal diet (Table 2, Fig. 1c). Growth in the long heat wave was 35% greater than in the cold and normal spring (Table 2, Fig. 1c). The diet × temperature interaction was significant, since in the normal spring and in the long heat wave growth on the animal diet was lower, but in the cold spring diet had no effect on growth (Table 2, Fig. 1c).

Table 2. Generalized linear mixed models for the proportion of active tadpoles and general linear models for growth rate of the study species.

	D. galganoi			*H. arborea*			*H. meridionalis*		
Activity	*df*	F	*P*	*df*	F	*P*	*df*	F	*P*
Diet	2, 232	6.17	**<0.01**	2, 231	12.72	**<0.001**	2, 101	5.04	**<0.01**
Temperature	2, 232	17.70	**<0.001**	2, 231	28.08	**<0.001**	2, 101	0.93	0.400
Diet × Temperature	4, 232	1.49	0.205	4, 231	3.52	**<0.01**	4, 101	0.35	0.842
Growth rate									
Diet	2, 96	172.77	**<0.001**	2, 90	7.34	**<0.01**	2, 39	32.57	**<0.001**
Temperature	2, 96	47.76	**<0.001**	2, 90	24.05	**<0.001**	2, 39	12.22	**<0.001**
Diet × Temperature	4, 96	4.56	**<0.01**	4, 90	1.78	0.139	4, 39	6.66	**<0.001**

P values <0.05 are marked in boldface

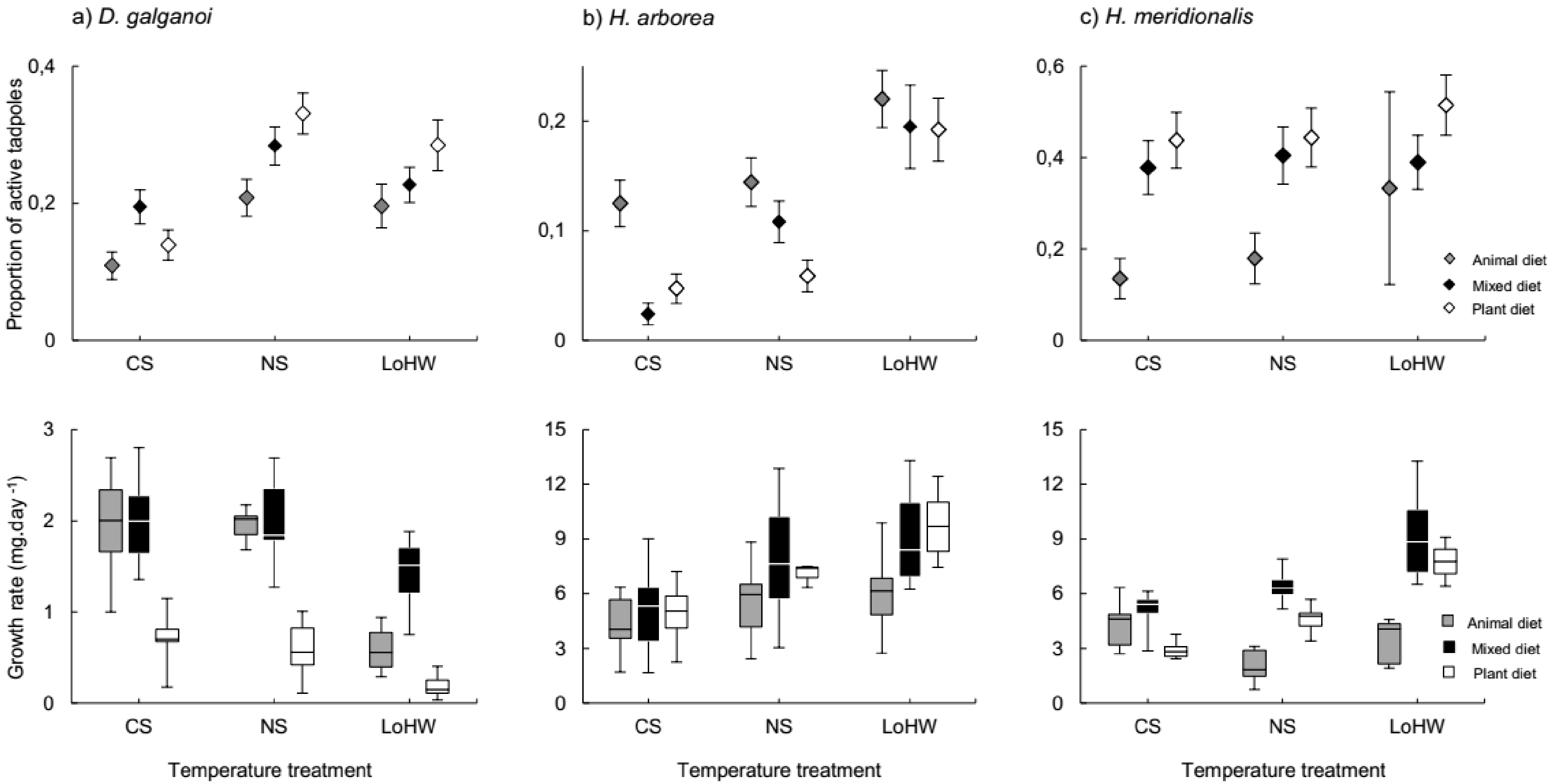


Figure 1. Proportion of active tadpoles of a) *D. galganoi*, b) *H. arborea* and c) *H. meridionalis* fed on animal, plant or mixed diet in the temperature treatments: Cold Spring (CS), Normal Spring (NS) and Long Heat Wave (LoHW); diamonds show the mean and whiskers indicate the standard error. Corresponding growth rates (mg.day^{-1}) for each of the study species; boxes show the median and the 25th – 75th percentiles and whiskers indicate the minimum and the maximum values.

Growth-activity biplots

All species had treatments in the quadrant corresponding to the debilitating tail of the activity curve (G^-A^-, Fig. 2), but treatments occupying the quadrant corresponding to the satiation tail of the activity curve were only found in *D. galganoi* (G^+A^-, Fig. 2a). In *H. arborea* all animal diet treatments were clustered in the “inefficient stress compensation” quadrant ($G^-A^{+)}$, while in *H. meridionalis* they were clustered in the debilitating quadrant and all mixed diet treatments in the “efficient stress compensation” (G^+A^+, Fig. 2b and 2c). Finally, in *D. galganoi* diet treatments did not cluster in any of the quadrants. Regarding the temperature treatments, no clusters were found in any of the species.

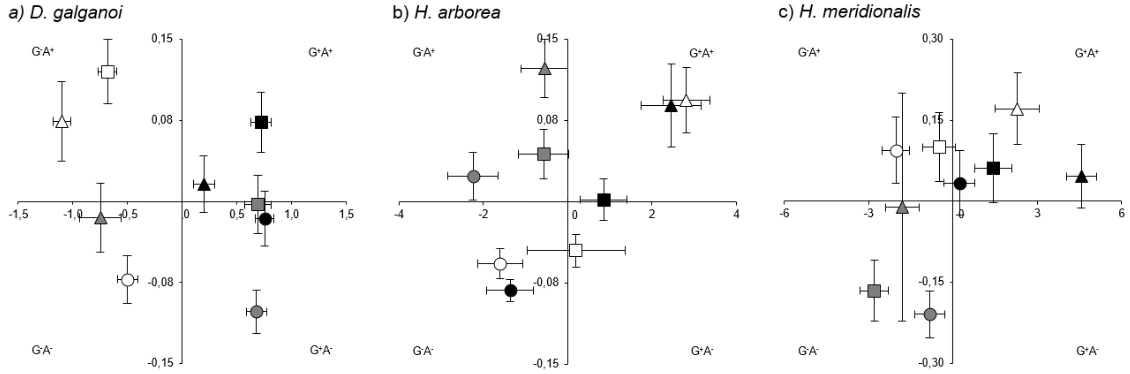


Figure 2. Difference between treatment mean and the overall mean for the proportion of active tadpoles (y-axis) in function of the respective growth rates (x-axis) in a) *D. galganoi*, b) *H. arborea* and c) *H. meridionalis*. Starting at the bottom left and proceeding clockwise, the quadrants define four general regions of the performance curve: G^-A^- – a debilitated state with low growth and low activity; G^-A^+ – an inefficient stress compensation with low growth and high activity; G^+A^+ – an efficient stress compensation with high growth and high activity; G^+A^- – satiation with high growth and low activity. Diet treatments are color coded: animal diet in gray, mixed diet in black and plant diet in white; temperature treatments are shape coded: Cold Spring in circles, Normal Spring in squares and Long Heat Wave in triangles. Bars represent standard deviation.

Discussion

We found that diet and temperature affected the activity of all species. However, the interpretation of the variation in tadpole activity levels required accounting for the independent and combined effects of the two stressors. Furthermore, by using growth reductions as an indicator of stress, our study shows that the curve defined by the variation in tadpole activity levels follows the general shape of a performance curve, in which low activity levels may either indicate high or low stress levels. Additionally, high activity levels may indicate an attempt to cope with moderate stress levels, but effectiveness of this response depends on the severity of the stress experienced by tadpoles.

Diet

Given that *D. galganoi* is mostly carnivorous (Carreira *et al.*, 2016), we expected the plant diet to be stressful and to increase activity levels. The greater growth on the animal diet suggests it was less stressful and, in agreement with our predictions, tadpoles on this diet displayed low activity levels. In accordance, growth on the plant diet was lower and tadpoles displayed higher activity levels, supporting that stress increases activity. Despite the high growth, tadpoles on the mixed diet also displayed high activity levels (against our prediction), suggesting that the added plant material decreased diet quality, and therefore that this species is specialized. While tadpoles on the mixed diet successfully compensated for the lower food quality by increasing activity, tadpoles on the plant diet were unable to do so (Fig. 2a).

The lower growth on the animal diet suggests the omnivorous tadpoles of *H. arborea* experienced more stressful conditions when on this diet. As expected, these tadpoles displayed higher activity levels than tadpoles on the plant and mixed diets, also supporting that stress increases activity. However, this response did not compensate for the lower food quality of the animal diet (Fig. 2b).

Being mostly herbivorous, the tadpoles of *H. meridionalis* experienced higher stress on the animal diet as predicted, which decreased growth and strongly diminished tadpole activity. This suggests that tadpoles on the animal diet experienced severe stress, debilitating them beyond their ability to compensate for the low food quality through increased foraging. The high growth and activity levels on the plant and mixed diets indicate that tadpoles were under a more moderate stress. Despite the lack of significant differences between tadpole activity under these diets, the mixed diet treatments were close to the satiation quadrant (G^+A^-, Fig. 2c), indicating the mixed diet was less stressful and closer to an optimal diet.

Temperature

The greater growth and the reduced activity levels in the cold spring suggest *D. galganoi* experienced low stress levels, as expected. Higher thermal stress, indicated by lower growth, may have increased activity and allowed tadpoles in the normal spring to partially compensate for the thermal stress, but in the long heat wave this response was insufficient (Fig. 2a).

The low growth in the cold spring suggests *H. arborea* tadpoles experienced thermal stress that restricted their activity. Yet, while thermal stress decreased at higher temperatures and allowed for greater growth rates in the normal spring and in the long heat wave, tadpole activity increased. Although higher temperatures increase activity (Gillooly *et al.*, 2001), and consequently energetic demands, tadpoles benefited from the greater foraging and increased growth (Fig. 2b), suggesting a transition from severe stress in the cold spring to more moderate stress levels in the other treatments.

In *H. meridionalis* the overall low growth in the cold and normal spring treatments suggests tadpoles experienced severe thermal stress, which they were unable to cope with despite the high activity levels (Fig. 1c). Like in *H. arborea*, high growth in the long heat wave also suggests that high activity was beneficial and that tadpoles experienced only moderate stress.

Diet × Temperature

As the relative quality of diets changes with temperature (Boersma *et al.*, 2016; Carreira *et al.*, 2016), a careful investigation of diet × temperature interactions may explain conflicting results. Tadpoles of *D. galganoi* on the animal diet displayed similar activity levels in the normal spring and in the long heat wave, but experienced a marked growth decrease in the latter. This suggests a higher stress in the long heat wave than in the normal spring and that maintaining high activity levels was not sufficient to compensate for the decreased quality of the animal diet at higher temperatures (Figs. 1a and 2a).

In *H. arborea* the stress imposed by low temperature restricted tadpole activity, but less so on the animal diet, suggesting compensatory activity (Fig. 1b). Alternatively, as the relative quality of animal diets increases at lower temperatures, temperature may have imposed a lower stress and allowed tadpoles to display higher activity levels without affecting growth (Fig. 2b). The transition from severe to moderate stress as treatment average temperature increased allowed tadpoles to display higher activity levels, which increased growth on the plant and mixed diets, but not on the animal diet since the lower quality of the animal diet at higher temperatures increased stress (Fig. 2b).

In *H. meridionalis*, the high activity levels on the plant and mixed diets suggest compensatory activity, which was effective in the normal spring and especially in the long heat wave, suggesting a transition to more moderate thermal stress probably enhanced by the increased quality of plant diets at higher temperatures. The low activity levels on the animal diet at no growth cost suggest tadpoles in the cold spring were satiated and, although not significantly, *H. meridionalis* tadpoles seem to have increased activity levels under the long heat wave, unsuccessfully attempting to compensate for the lower quality of animal diet (Figs. 1c and 2c).

Conclusions

The absence of Hylid treatments in the satiation quadrant may suggest that not all species reduce activity when satiated, but it is also possible that tadpoles were under stress in all treatments. Although our results advise species-specific interpretations, the variation of activity levels in function of stress followed the shape of performance curve in *D. galganoi* and may hold true also for other species.

The study shows that the efficiency of increased activity in mitigating stressor effects may depend of the severity of the stress, since tadpoles maintained high growth rates under low to moderate stress, but failed to do so under moderate to high stress. Furthermore, the positive effects of diet regulation were clear in both *D. galganoi* and *H. arborea*, for which the mixed diet promoted growth rates as high as on the best single diets and lower activity levels than on the most stressful single diets. Interestingly, the growth variation across the temperature treatments indicates differences in the species' optimum temperature ranges, with a sharp contrast between *D. galganoi*, performing best in the cold spring, and the two *Hyla* species, performing best in the long heat wave. Furthermore, while *H. arborea* performance was higher in the normal spring than in the cold spring, in *H. meridionalis* performance was equally low in both treatments, suggesting a higher optimum temperature range for *H. meridionalis*.

The interpretation of the variation in activity levels as a function of stress must account for the single and combined effects of the stressors – diet and temperature are no exception, as the quality of diets changes with temperature. Given the increasing ethical restrictions on stress-inducing experiments, many studies (including ours) use moderate stress treatments. Although unavoidable, this may further complicate the results interpretation, because of the gradual and hardly detectable transition from a stress level at which increased activity is an effective response, to a stress level at which it is no longer effective.

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References

Altig, R., Whiles, M.R. & Taylor, C.L. (2007). What do tadpoles really eat? Assessing the trophic status of an understudied and imperiled group of consumers in freshwater habitats. *Freshw. Biol.* **52**, 386–395.

Anholt, B.R. & Werner, E.E. (1995). Interaction between food availability and predation mortality mediated by adaptive behavior. *Ecology* **76**, 2230–2234.

Barry, M.J. (2014). The energetic cost of foraging explains growth anomalies in tadpoles exposed to predators. *Physiol. Biochem. Zool.* **87**, 829–36.

Boersma, M., Mathew, K.A., Niehoff, B., Schoo, K.L., Franco Santos, R.M. & Meunier, C.L. (2016). Temperature driven changes in the diet preference of omnivorous copepods: no more meat when it's hot? *Ecol. Lett.* **19**, 45–53.

Bridges, C.M. (1997). Tadpole swimming performance and activity affected by acute exposure to sub-lethal levels of carbaryl. *Environ. Toxicol. Chem.* **16**,1935–1939.

Bridges, C.M. (2002). Tadpoles balance foraging and predator avoidance: effects of predation, pond drying, and hunger. *J. Herpetol.* **36**, 627–634.

Carreira, B.M, Segurado, P., Orizaola, G., Gonçalves, N., Pinto, V., Laurila, A. & Rebelo, R. (2016). Warm vegetarians? Heat waves and diet shifts in tadpoles. *Ecology* **97**, 2964–2974.

Crespi, E.J. & Denver, R.J. (2005). Roles of stress hormones in food intake regulation in anuran amphibians throughout the life cycle. *Comp. Biochem. Physiol. - A Mol. Integr. Physiol.* **141**, 381–390.

Davis, M.J., Purrenhage, J.L. & Boone, M.D. (2012). Elucidating predator-prey interactions using aquatic microcosms: complex effects of a crayfish predator, vegetation, and atrazine on tadpole survival and behavior. *J. Herpetol.* **46**, 527–534.

Finkler, M.S. & Claussen, D.L. (1999). Influence of temperature, body size, and inter-individual variation on forced and voluntary swimming and crawling speeds in *Nerodia sipedon* and *Regina septemvittata*. *J. Herpetol.* **33**, 62–71.

Fraker, S.L. & Smith, G.R. (2004). Direct and interactive effects of ecologically relevant concentrations of organic wastewater contaminants on *Rana pipiens* tadpoles. *Environ. Toxicol.* **19**, 250–256.

Gillooly, J.F., Brown, J.H., West, G.B., Savage, V.M. & Charnov, E.L. (2001). Effects of size and temperature on metabolic rate. *Science* **293**, 2248–2251.

Gosner, K.L. (1960). A simplified table for staging anuran embryos larvae with notes on identification. *Herpetologica* **16**, 183–190.

Hatch, A & Blaustein, A. (2000). Combined effects of UV-B, nitrate, and low pH reduce the survival and activity level of larval cascades frogs (*Rana cascadae*). *Arch. Environ. Contam. Toxicol.* **39**, 494–499.

Katzenberger, M., Hammond, J., Duarte, H., Tejedo, M., Calabuig, C. & Relyea, R.A. (2014). Swimming with predators and pesticides: How environmental stressors affect the thermal physiology of tadpoles. *PLoS One* **9**, e98265.

Krause, E.T., Steinfartz, S. & Caspers, B.A. (2011). Poor nutritional conditions during the early larval stage reduce risk-taking activities of fire salamander larvae (*Salamandra salamandra*). *Ethol.* **117**, 416–421.

Kupferberg, S.J. (1997). The Role of Larval Diet in Anuran Metamorphosis 1. *Am. Zool.* **159**, 146–159.

Laurila, A., Kujasalo, J. & Ranta, E. (1997). Different antipredator behaviour in two anuran tadpoles: effects of predator diet. *Behav. Ecol. Sociobiol.* **40**, 329–336.

Laurila, A., Kujasalo, J. & Ranta, E. (1998). Predator-induced changes in life history in two anuran tadpoles: effects of predator diet. *Oikos* **83**, 307–317.

Laurila, A., Lindgren, B. & Laugen, A.T. (2008). Antipredator defenses along a latitudinal gradient in Rana temporaria. *Ecology* **89**, 1399–1413.

Shuman-Goodier, M. E. & Propper, C. R. (2016). A meta-analysis synthesizing the effects of pesticides on swim speed and activity of aquatic vertebrates. Sci. Total Environ. 565:758–766.

Maher, J.J.M., Werner, E.E., Denver, R.J. & B, P.R.S. (2013). Stress hormones mediate predator-induced phenotypic plasticity in amphibian tadpoles. *Proc. R. Soc. B Biol. Sci.* **280**, 1–9.

Navas, C.A., Carvajalino-Fernández, J.M., Saboyá-Acosta, L.P., Rueda-Solano, L.A. & Carvajalino-Fernández, M.A. (2013). The body temperature of active amphibians along a tropical elevation gradient: Patterns of mean and variance and inference from environmental data. *Funct. Ecol.* **27**, 1145–1154.

Nicieza, A.G. & Metcalfe, N.B. (1997). Growth compensation in juvenile Atlantic salmon: responses to depressed temperature and food availability. *Ecology* **78**, 2385–2400.

Nunes, A.L., Richter-Boix, A., Laurila, A. & Rebelo, R. (2013). Do anuran larvae respond behaviourally to chemical cues from an invasive crayfish predator? A community-wide study. *Oecologia* **171**, 115–127.

Paull, S.H., Raffel, T.R., Lafonte, B.E. & Johnson, P.T.J. (2015). How temperature shifts affect parasite production: testing the roles of thermal stress and acclimation. *Funct. Ecol.* **29**, 941–950.

Richardson, J.M.L. (2001). A comparative study of activity levels in larval anurans and response to the presence of different predators. *Behav. Ecol.* **12**, 51–58.

Richter-Boix, A., Llorente, G.A., Montori, A. & Garcia, J. (2007). Tadpole diet selection varies with the ecological context in predictable ways. *Basic Appl. Ecol.* **8**, 464–474.

Schiesari, L., Werner, E.E. & Kling, G.W. (2009). Carnivory and resource-based niche differentiation in anuran larvae: implications for food web and experimental ecology. *Freshw. Biol.* **54**, 572–586.

Semlitsch, R.D., Foglia, M., Mueller, A., Steiner, I., Fioramonti, E. & Fent, K. (1995). Short term exposure to triphenyltin affects the swimming and feeding behavior of tadpoles. *Environ. Toxicol. Chem.* **14**, 1419–1423.

Smith, G. & Burgett, A. (2005). Effects of three organic wastewater contaminants on American toad, *Bufo americanus*, tadpoles. *Ecotoxicol.* **14**, 477–482.

Werner, E.E. (1992). Competitive interactions between wood frog and northern leopard frog larvae – the influence of size and activity. *Copeia* **1992**, 26–35.

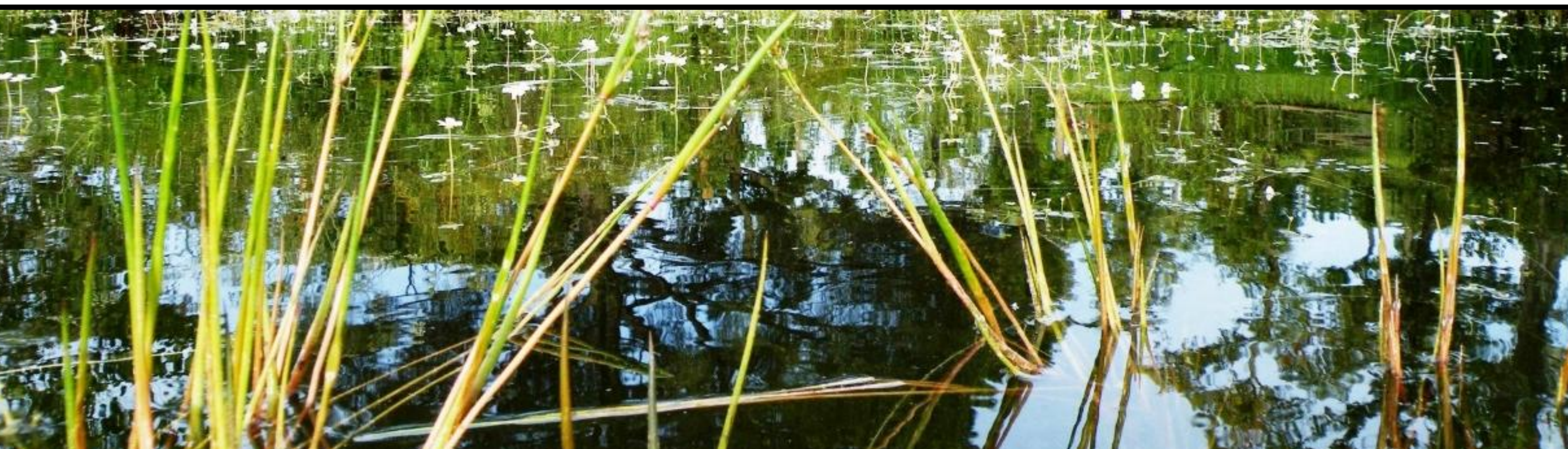
Werner, E. & Anholt, B. (1993). Ecological consequences of the trade-off between growth and mortality rates mediated by foraging activity. *Am. Nat.* **142**, 242–272.

CHAPTER 4

Heat waves in a temperate climate: diet shifts and increased performance of a subtropical invasive crayfish?

B. M. Carreira, P. Segurado, A. Laurila and R. Rebelo

Procambarus clarkii



CHAPTER 4

Heat waves in a temperate climate: diet shifts and increased performance of a subtropical invasive crayfish?

Abstract

The globally increasing temperatures, commonly assumed to benefit cold-limited invasive alien species, may also change their feeding preferences especially in the case of omnivorous ectotherms. We investigated heat wave effects on diet choice, growth and energy reserves in the invasive red swamp crayfish, *Procambarus clarkii*. In laboratory experiments, we fed juvenile and adult crayfish on animal, plant or mixed diets and exposed them to a short or a long heat wave. We measured crayfish survival, growth, body reserves and Fulton's condition index; diet choices of the crayfish maintained on the mixed diet were estimated using stable isotopes (^{13}C and ^{15}N). We found evidence for a decreased efficiency of carnivorous diets at higher temperatures and herbivorous diets at lower temperatures. Heat wave treatments induced diet shifts towards increased herbivory in juvenile crayfish, but affected adult feeding preferences only to a small extent. Increasing herbivory allowed juveniles subject to the long heat wave to sustain high growth rates, suggesting adaptive value. Contrary to our expectations, crayfish performance decreased in the long heat wave, suggesting heat waves may have negative effects and are unlikely to boost *P. clarkii* populations in Mediterranean climates. However, by causing diet shifts and changing the trophic niche of this keystone species, they may aggravate crayfish impacts on aquatic vegetation and economic damage to rice fields.

Keywords: alien species, climate change, diet regulation, herbivory, *Procambarus clarkii*

Introduction

Global change drivers may interact synergistically with each other and cause severe damage to communities and ecosystems (Vitousek et al. 1997; Pereira et al. 2010). By weakening geographic barriers, climate change may provide invasive alien species (IAS) with new habitats and enhance their expansion, while the impacts of IAS at the invaded areas may increase ecological susceptibility to climate change (Dukes and Mooney 1999; Pyšek and Richardson 2010; Leishman and Gallagher 2015). These global change drivers are expected to severely afflict freshwater ecosystems (Sala et al. 2000; Theoharides and Dukes 2007), due to the facilitated spread of IAS and the high proportion of ectothermic species in these environments, the latter being especially sensitive to climate change (Dudgeon et al. 2006).

Given the strong influence of temperature on the metabolic processes and the stoichiometric balance of ectotherms, the ongoing climatic changes have fostered a strong interest for studies addressing the nutrient acquisition by these organisms at higher temperatures (for a review see Cross et al. 2015). Higher temperatures increase the demand for carbon over nitrogen, due to the stronger thermal effect on catabolic than on anabolic processes, which promotes a greater increase in respiration than in growth (Karl and Fischer 2008; Forster et al. 2011). Furthermore, the overall digestion efficiency of large and complex nutrients such as proteins decreases at higher temperatures, because the higher assimilation rate does not fully compensate for the greater increase in gut passage and evacuation rates (Myrick and Cech 2000; Seifert et al. 2014). In crayfish – important omnivorous ectotherms in freshwater ecosystems – the protein to carbohydrate assimilation ratio shifts to a greater assimilation of carbohydrates at higher temperatures (Croll and Watts 2004), and there is also evidence that higher temperatures enhance the digestion of plant material (Floeter et al. 2005). These facts suggest omnivorous ectotherms such as crayfishes should optimize energy intake by avoiding protein-rich diets and increasing herbivory at higher temperatures. In fact, recent studies showed that both copepods and anuran larvae perform diet shifts towards increased herbivory at higher temperatures (Boersma et al. 2016; Carreira et al. 2016).

While invasions are more likely to succeed when climatic conditions in the new environments are similar to those in the invasive species native distribution range, this is often not the case (Walther et al. 2009; Davidson et al. 2011). Like many of the most abundant IAS in Europe, the invasive red swamp crayfish *Procambarus clarkii* (Girard, 1852) is native to warm regions with tropical and subtropical climates (Robinet and Roques 2010). Ranking among Europe’s worst IAS (Gallardo 2014), this omnivorous crayfish is native to central-southern USA and northeastern Mexico, where its optimum growth temperature ranges from 20 to 27ºC (Croll and Watts 2004). In Europe, the colder temperatures have prompted changes in the life-history of *P. clarkii*, causing a switch from multivoltine to uni- or bivoltine life cycles and the onset of the breeding period to vary latitudinally according to water temperature (Chucholl 2011; Peruzza et al. 2015).

The dual nature of *P. clarkii*, as both an omnivorous ectotherm and an IAS, may manifest through multiple effects on aquatic communities as this species experiences the projected climatic changes. First, shifts towards increased herbivory under higher temperatures may aggravate *P. clarkii*’s negative impacts on freshwater macrophyte communities and rice crops, where invertebrates currently constitute a significant proportion of its diet (Correia 2002; Alcorlo et al. 2004; Souty-Grosset et al. 2016). Second, the climatic changes projected for Europe, and particularly the Iberian Peninsula may alleviate or temporarily lift the constraints imposed by cold temperatures on *P. clarkii*’s life-cycle (Chucholl 2011) and distribution (Capinha and Anastácio 2011), intensifying its ecological impacts.

In this study, we investigated the effects of simulated heat waves on juvenile and adult *P. clarkii*. Crayfish were fed animal-based, plant-based or mixed diets and exposed to temperature treatments simulating either the current common short heat waves or long heat waves expected to become more frequent in the near future. We reconstructed dietary choices of crayfish fed mixed diets by using stable isotopes and recorded crayfish fitness in terms of survival and life-history traits (growth rate, body reserves and Fulton's index). We predicted that in the heat wave treatments this subtropical crayfish should: (1) increase herbivory on the mixed diet; (2) have improved performance on the plant diet; (3) have decreased performance on the animal diet; (4) display a higher fitness than in colder treatments.

Materials and Methods

Study area

In the Iberian Peninsula, *P. clarkii* experiences average minimum temperatures 2 to 5ºC lower than in its native range, 7 to 8ºC lower in spring and summer. However, winter minima are projected to rise 3ºC in this region (Jerez et al. 2012), which may enhance habitat conditions and, similarly to other IAS, boost *P. clarkii* populations by extending growing and reproductive seasons (Walther et al. 2009; Bradley et al. 2010; Walther 2010). Heat waves in this region are projected to become longer, more frequent and more intense (Meehl and Tebaldi 2004; Dasari et al. 2014). Using a 10-year data set of air temperatures (2002-2012), we found that typical heat waves occurring in the southwest of the Iberian Peninsula lasted one to two weeks (B. M. Carreira, *unpubl. data*). Following Frich et al. (2002), we considered a heat wave occurred when the daily maximum temperature exceeded the average maximum temperature by 5ºC for more than five consecutive days (reference period: 1961–1990).

Collection, maintenance and experimental procedures

Crayfish were captured with dip-net sweeps in rice field ditches near Samora Correia (38º52'N, 8º51'W), before the main reproductive episode in the fall (Correia 1995). Adults were collected on the 30th of May 2013 (total body length: 60-90mm) and immature juveniles from the autumn cohort on the 25th of July 2013 (total body length: 40 to 45mm), when average water temperatures range from 18 to 23ºC and maximum water temperature averages 25ºC (Correia 1995). Crayfish were acclimatized for two months at room temperature (ca. 20ºC) under 12L:12D photoperiod, juveniles in groups of 15 individuals in 5L aquaria and adults individually in 1.5L aquaria. All animals were fed commercial fish food every other day.

At the start of the experiments individuals were blotted dry in paper towel, weighed and measured (carapace length – CL; post orbital carapace length – POCL). Crayfish were transferred to individual 1.5L aquaria placed in water baths, following a fully factorial experimental design with diet (three levels) and temperature (four levels) as factors (see sections *Diet* and *Temperature*). We balanced the replicates' sex ratio and randomly assigned each treatment combination ten juveniles (5 Females:5 Males) and seven adult crayfish (4 Females:3 Males). Crayfish were fed overnight every other day and the position of each aquarium in the water bath was randomized; the following morning food was removed along with water renewal, and water bath temperatures were checked and adjusted when necessary. Experiments lasted for two months to exceed the half-life of ^{13}C and ^{15}N turnover rates (ca. 6-8 weeks for 10-100g organisms at 10-30ºC; Thomas and Crowther 2015).

Deaths were recorded throughout the experiments. The crayfish were weighed and measured at the end of the experiment. Growth rate was calculated as weight variation (mg) divided by the experiment duration (days); Fulton's index, the species expected weight at a given length, was determined with CL following Banha (2015). Individuals were then euthanized by rapid freezing at -18ºC, to avoid contamination of the isotopic signature with euthanizing agents. Most of the juveniles matured during the experiments, as indicated by the greater percentage of body reserves in females (see Results), and by the change to form I (presence of copulatory hooks and cornified gonopodia) in 50-60% of the males, regardless of diet or temperature. Hepatopancreas and gonads were difficult to separate in defrosted male crayfish, and so were weighed together to estimate body reserves (gonads plus hepatopancreas mass as a percentage of the body mass), allocated to both maintenance (hepatopancreas) and reproduction (gonads). Additionally, abdominal muscle samples were prepared for stable isotope analysis: dried at 60 °C for 24 h, ground to fine powder with mortar, cleansed of storage lipids with chloroform-methanol (2:1; Dobush et al. 1985) and re-dried at 60°C for 24h.

Diet

We tested three experimental diets, all offered *ad libitum*: Animal diet (A) – composed of defrosted Chironomidae larvae (favored by *P. clarkii* and common in temporary ponds and rice fields (Correia 2002; Trigal et al. 2007)); Plant diet (P) – composed of defrosted stalks of *Juncus heterophyllus*, an emergent macrophyte abundant in Mediterranean temporary ponds and favored by *P. clarkii* (Carreira et al. 2014); and Mixed diet (M) – composed of both food items in diets A and P offered in similar proportions of fresh mass.

Temperature

The experiments included four temperature treatments (Fig. 1): Cold (C) – constant temperature of 17°C; Normal Spring (NS) – temperature was gradually increased from 17 to 25°C, at the rate of 1°C per week (average 21°C); Short Heat Wave (SHW) – similar to NS, but on day 28 crayfish were exposed to a two-week heat wave of 25°C, after which temperature was decreased to 23°C (going back to the same regime as in NS); and Long Heat Wave (LHW) – constant temperature of 25°C. The two-week long heat wave (SHW) aimed to simulate the maximum duration of current spring heat waves in the southwest of Portugal. The two-month long heat wave (LHW) aimed to simulate extremely long heat waves similar to the one that afflicted Europe in 2003 and which are expected to become more frequent due to climate change (Jerez et al. 2013; Dasari et al. 2014).

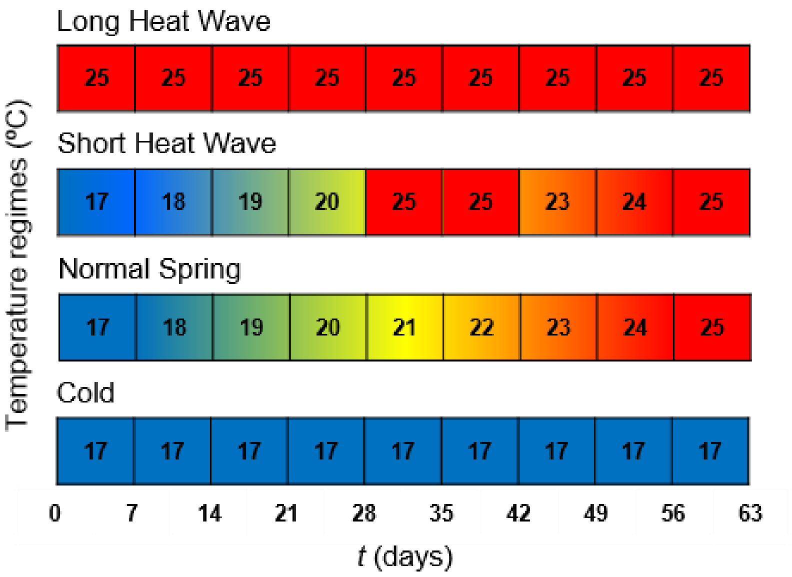


Figure 1. Schematic representation of temperature variation over time in each experimental treatment.

Isotope analysis

Stable isotope ratios ($^{13}C/^{12}C$, $^{15}N/^{14}N$) and elemental analyses (C:N) were determined by continuous flow isotope mass spectrometry (CF-IRMS) (Preston and Owens 1983), on a Hydra 20-22 (Sercon, UK) stable isotope ratio mass spectrometer, coupled to a EuroEA (EuroVector, Italy) elemental analyzer for online sample preparation by Dumas-combustion. The standards for carbon isotope ratio were IAEA-CH6 and IAEA-CH7, and the standards for nitrogen isotope ratio were IAEA-N1 and USGS-35. $\delta^{13}C$ results were referred to PeeDee Belemnite (PDB) and $\delta^{15}N$ to Air. Estimated precision of the isotope ratio analysis was $\leq$ 0.2‰ (6 to 9 replicates of laboratory standard material in every batch analysis).

Statistical analyses

We used stable isotope mixing models to obtain time-integrated estimates of the proportions of animal and plant material contributing to the isotopic signature of crayfish fed on the mixed diet. The Stable Isotope Analysis package in R (SIAR; Parnell et al. 2013) fits a Bayesian model to the proportions of the sources contributing to the consumers' isotopic signature and incorporates variability in the sources, trophic enrichment factors and elemental composition (Parnell et al. 2010). For each life stage, we built separate models for female and male crayfish and specified the isotopic signatures and C:N ratios of the sources, which differed substantially in the $\delta^{13}C$ and C:N ratios (Table 1). The isotopic signatures of crayfish fed on single diets (animal or plant diet) at each temperature treatment were used to control for differences in the sources' trophic enrichment factors and in temperature conditions.

Table 1. Isotopic signatures and C:N ratios of the food items provided to juvenile and adult crayfish (average ± standard deviation).

	Animal diet	Plant diet
δ ^{13}C	-24.61 ± 0.13	-28.63 ± 0.19
δ ^{15}N	5.09 ± 0.92	4.41 ± 0.44
C:N	3.79 ± 0.05	23.69 ± 11.52

We tested treatment effects on the survival of adult crayfish with the Cox proportional hazards regression model in the survival package of R software. We used general linear models (GLMs), performed on STATISTICA 12.6.255.0 (StatSoft), to test for temperature, diet and sex effects (fixed factors) and their interactions on growth rate, body reserves (including POCL as a covariate) and on Fulton's index. The non-significant three-way interaction diet × temperature × sex was removed to simplify the models. Post hoc pairwise comparisons were corrected for multiple comparisons (Bonferroni's test).

Results

Juveniles

Juveniles on the mixed diet were mostly carnivorous (Fig. 2a), with females generally more herbivorous than males (overall proportion of plant material 0.38 versus 0.30; Fig. 2a). Regardless of sex, crayfish were most carnivorous in the normal spring treatment (females: 0.20, Bayesian Credible Interval (BCI) = 0.01-0.55; males: 0.18, BCI = 0.01-0.67; Fig. 2a). Herbivory increased in the short heat wave (females: 0.35, BCI = 0.02-0.77; males: 0.27, BCI = 0.01-0.85) and even more in females in the long heat wave treatment (0.47, BCI = 0.02-0.77; Fig. 2a). However, herbivory in the long heat wave was similar to herbivory in the cold treatment (females: 0.49, BCI = 0.05-0.94; males: 0.45, BCI = 0.05-0.94).

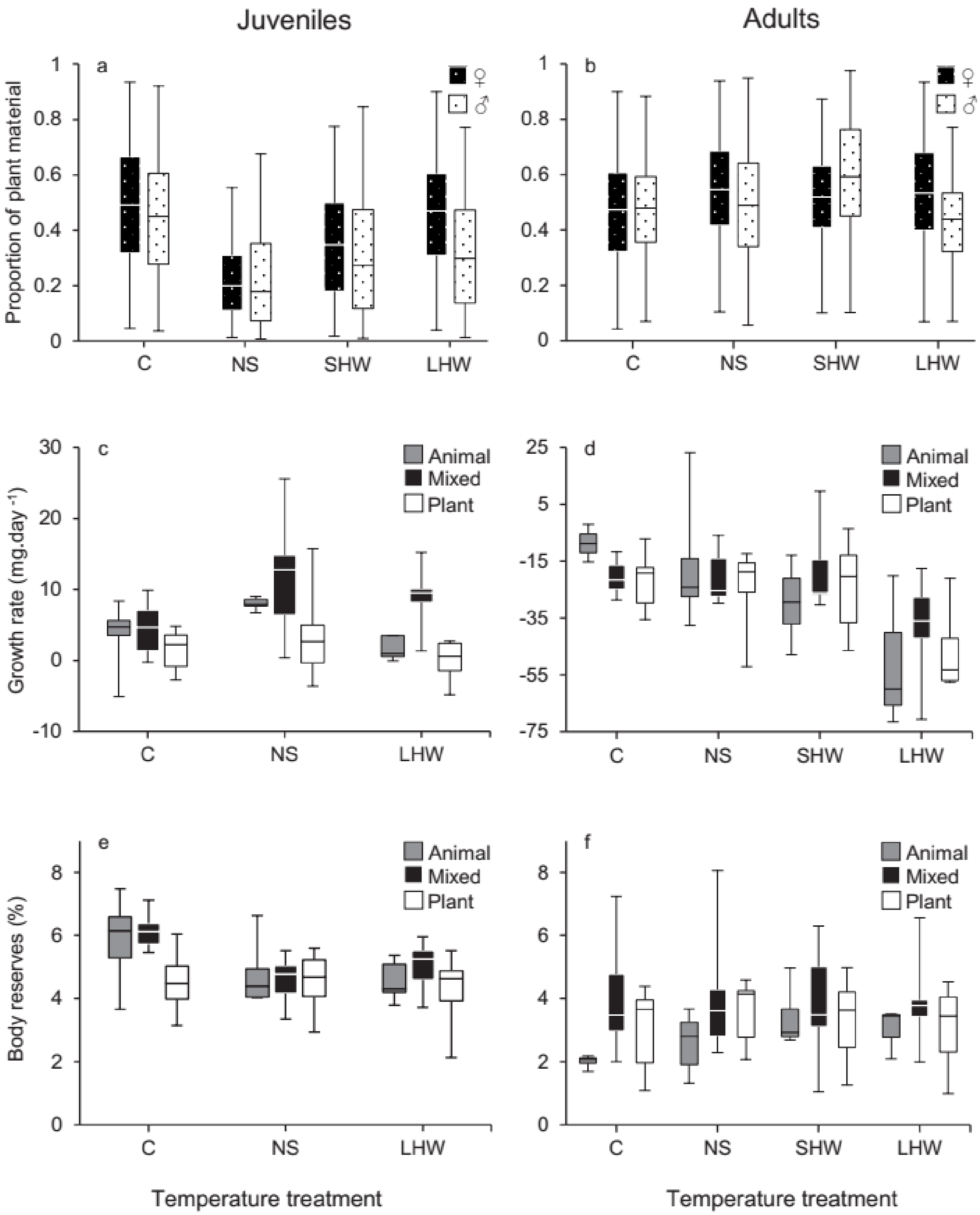


Figure 2 The effect of the temperature treatments: Cold (C), Normal Spring (NS), Short Heat Wave (SHW), and Long Heat Wave (LHW) on the assimilation of plant material by *Procambarus clarkii* and on its growth rate and body reserves. Proportion of plant material assimilated by male and female (a) juveniles and (b) adults fed on the mixed diet; the boxes show the median and the 25th – 75th percentiles and the whiskers indicate the 2.5th – 97.5th percentiles. (c) Juvenile and (d) adult growth rate (mg.day^{-1}) of crayfish fed on the animal, plant and mixed diets, and their body reserves (%), (e) and (f) respectively; the boxes show the median and the 25th – 75th percentiles and the whiskers indicate the minimum and the maximum values.

No crayfish died because of the experimental treatments. However, a spoiled batch of chironomid larvae caused an episode of high mortality on the animal (67%) and mixed diets (17%). The death of all individuals in the “animal diet × short heat wave” forced us to discard this treatment from the analyses.

Growth on the mixed diet was two times greater than on the animal diet and five times greater than on the plant diet (Table 2; Fig. 2c). Growth in normal spring was two times greater than in cold spring and long heat wave treatments (Table 2; Fig. 2c). However, a significant diet × temperature interaction showed a higher growth on the mixed diet in the normal spring and in the long heat wave treatments, but no diet effect in the cold treatment (Table 2; Fig. 2c). Sex had no significant effect on growth (Table 2; Fig. 2c).

Body reserves on the animal and mixed diets were 14 % greater than on the plant diet (Table 2; Fig. 2e). However, a significant diet × temperature interaction showed that unlike in the other temperature treatments, in the cold treatment body reserves on the plant diet were lower (Table 2; Fig. 2e). In general, body reserves in the cold treatment were 16% greater than in the normal spring and long heat wave treatments (Table 2; Fig. 2e). Body reserves were 8% greater in females than in males (Table 2; Fig. 2e).

Fulton’s index on the animal diet (0.22 ± 0.02) was higher than on the plant and mixed diets (0.20 ± 0.02; Table 2). Fulton’s index in the cold and in the normal spring (0.21 ± 0.02) was higher than in the long heat wave (0.20 ± 0.02; Table 2). Sex had no effect on Fulton’s index, but a significant diet × sex interaction showed that Fulton’s index on the mixed diet was lower in females (females: 0.20 ± 0.01; males: 0.22 ± 0.02; Table 2).

Table 2. General Linear Models for growth rate, Fulton's index and body reserves of juvenile crayfish.

	Growth rate			Body reserves			Fulton's index		
Juveniles	*df*	F	*P*	*df*	F	*P*	*df*	F	*P*
Diet	2, 65	22.02	**<0.001**	2, 65	7.67	**<0.01**	2, 66	5.60	**<0.01**
Temperature	2, 65	10.09	**<0.001**	2, 65	9.82	**<0.001**	2, 66	5.55	**<0.01**
Sex	1, 65	0.99	0.323	1, 65	3.99	0.050	1, 66	0.08	0.772
Diet × Temperature	4, 65	3.17	**<0.05**	2, 65	0.30	0.743	4, 66	0.53	0.713
Diet × Sex	2, 65	1.94	0.152	2, 65	0.06	0.945	2, 66	4.19	**<0.05**
Temperature × Sex	2, 65	0.12	0.886	2, 65	0.30	0.743	2, 66	1.10	0.339

P values <0.05 are marked in boldface

Adults

Overall, proportions of animal and plant material on the mixed diet were similar in females (0.48:0.52) and males (0.50:0.50; Fig. 2b). However, in females, herbivory increased from the cold treatment (0.47; BCI = 0.04-0.90) to the other temperature treatments (median average = 0.53; Fig. 2b), whereas in males, the median proportion of plant material in the cold and the normal spring treatments averaged 0.49, but in the short heat wave increased to 0.59 (BCI = 0.10-0.98) and in the long heat wave decreased to 0.44 (BCI = 0.07-0.77; Fig. 2b).

Survival was high for both plant (93%) and mixed diets (76%), but dropped to 50% on the animal diet (Table 3). Survival was higher in the cold treatment (95%) and ranged in the other temperature treatments from 62% in normal spring to 81% in long heat wave (Table 3). Survival was similar among female (74%) and male (71%) crayfish (Table 3).

There was a general loss of body mass during the experiment, which was more pronounced in the long heat wave treatment, where crayfish lost ca. two times more mass than in the other temperature treatments (Table 3; Fig. 2d). Diet and sex had no significant effect on mass loss (Table 3; Fig. 2d).

Body reserves on the mixed diet were 31% greater than on the animal diet, and marginally greater than on the plant diet (Table 3; Fig. 2f). Temperature had no significant effect on body reserves (Table 3; Fig. 2f), but sex had a strong effect and body reserves were 40% greater in females than in males (Table 3; Fig. 2f).

Fulton’s index averaged 0.24 ± 0.03 and was not significantly affected by diet, temperature or sex (Table 3).

Table 3. Cox proportional hazards survival model and General Linear Models for growth rate, body reserves and Fulton's index of adult crayfish.

	Survival			Growth rate			Body reserves			Fulton's index		
Adults	*df*	χ^2	*P*	*df*	F	*P*	*df*	F	*P*	*df*	F	*P*
Diet	2	20.76	**<0.001**	2, 57	0.09	0.910	2, 57	6.44	**<0.01**	2, 58	0.14	0.871
Temperature	3	11.74	**<0.01**	3, 57	7.95	**<0.001**	3, 57	0.83	0.484	3, 58	0.35	0.787
Sex	1	0.18	0.672	1, 57	0.10	0.753	1, 57	33.73	**<0.001**	1, 58	2.11	0.152
Diet × Temperature	6	8.00	0.238	6, 57	0.92	0.491	6, 57	0.79	0.585	6, 58	0.41	0.868
Diet × Sex	2	0.40	0.819	2, 57	1.49	0.234	2, 57	1.44	0.247	2, 58	2.37	0.102
Temperature × Sex	3	2.24	0.525	3, 57	1.20	0.318	3, 57	0.15	0.932	3, 58	0.47	0.703

P values <0.05 are marked in boldface

Discussion

We found that especially juvenile *P. clarkii* increased herbivory in the heat waves, for which high growth rates in the long heat wave treatment indicated an adaptive value of the diet shifts. However, we found evidence for decreased performance of both *P. clarkii* life stages in the long heat wave. Although generally heat waves may have negative effects on *P. clarkii* populations, diet shifts towards higher herbivory may aggravate its economic impacts in rice fields, especially in the more carnivorous young life stages. To our knowledge, this is the first study to investigate temperature-induced diet shifts in crayfish and to show the potential for heat waves to increase the impact of IAS on aquatic vegetation.

Diet quality

The contrasting temperature effects on single diets support the notion that relative quality of diets changes with temperature. The decreased performance in the long heat wave, estimated either by growth or Fulton's index, suggests a lower quality of the animal diet at higher temperatures. The negative effects of higher temperatures on animal diet appear to be stronger than the positive effects on plant diet, as also found in a recent study on tadpoles (Carreira et al. 2016). Evidence for higher performance or higher relative performance of the plant diet in the heat wave treatments was not clear, as the effects of plant diet changed very little across temperature treatments. Nonetheless, the lower body reserves of juveniles on the plant diet in the cold treatment suggest a decreased performance of the plant diet at lower temperatures, as also found in other ectotherms (Carreira et al. 2016).

Diet shifts

Changes in the relative quality of diets in heat waves – either through passive mechanisms or active feeding choices – translated into diet shifts towards increased herbivory, like in other ectotherms (Boersma et al. 2016; Carreira et al. 2016). When compared with the normal spring, juveniles increased herbivory in both heat wave treatments, but more so in the long heat wave, where the beneficial effects of mixed diet on growth were very clear. Although the lower Fulton´s index and body reserves on the plant diet suggests pure herbivory would be maladaptive, increasing herbivory in the long heat wave allowed crayfish to maintain high growth rates. The surprisingly high herbivory in the cold treatment, also found in larvae of the Iberian painted frog (Carreira et al. 2016), may originate in a potential advantage of consuming plant diets, which are richer in polyunsaturated lipids and may help ectotherms to maintain membrane fluidity and high performance at low temperatures (e.g., Martin-Creuzberg et al. 2012; Alhazzaa et al. 2013). Although against our predictions, this advantage may outweigh the low performance of plant diets at low temperatures, just as an increase in nitrogen intake for synthesis of heat shock proteins may outweigh the low performance of animal diets at very high temperatures.

The higher herbivory in adult crayfish supports the ontogenetic shift in the feeding preferences of the more carnivorous juveniles documented for this species (Ilhéu and Bernardo 1993). Male herbivory increased in the short heat wave and decreased in the long heat wave, with no apparent costs or benefits in any of the cases. While the lower adult survival on the animal diet may suggest increasing carnivory could be maladaptive, this may be a normal response, as another study showed male *P. clarkii* to increase carnivory in the summer (Perez-Bote 2004).

Heat waves – general effects

Contrary to our expectations, we found no evidence that heat waves improve the performance of this subtropical species, instead the long heat wave decreased performance of both life stages. Although optimal growth occurs at 20-27ºC (Croll and Watts 2004), juvenile growth in the long heat wave was lower than in the normal spring treatment. Furthermore, Fulton’s index in the long heat wave (0.19 ± 0.02) was lower than in the other treatments (0.21± 0.02) and in wild Portuguese populations (0.23 ± 0.03; Banha 2015), suggesting the increase in size was not accompanied by a proportional increase in weight. Performance of adult crayfish also decreased in the long heat wave with weight loss being greater than in the other treatments. General weight loss could indicate physiological stress, since it typically occurs when crayfish are kept out of their optimum temperatures for extended periods (Croll and Watts 2004), but we believe it was also a result of the lower quality of the experimental diets, as compared to the commercial fish food. This is supported by the decrease of Fulton’s index from 0.29 ± 0.03 at the start of the experiment to 0.24 ± 0.03 at the end, which is still within normal values for wild *P. clarkii* (0.23 ± 0.03; Banha 2015). Furthermore, since starvation reduces hepatopancreas mass (Huner et al. 1985), weight loss may have overridden temperature effects on crayfish body reserves.

We suggest the life-history changes observed in European populations of *P. clarkii* are accompanied with lowered tolerance to warmer temperatures (Chucholl 2011; Gallardo 2014). Acclimatization allows physiological function over wide temperature ranges (Chown et al. 2007; Kelley 2014), and is often key to successful invasions, but Powell and Watts (2006) showed *P. clarkii* acclimatized to 10ºC to have a lower survival at 30ºC. Also, genetic adaptation occurring over a short temporal scale, as documented in other studies (Whitney and Gabler 2008; Buswell et al. 2011; Nunes et al. 2014), may have shifted the optimum temperature range or the overall shape of the performance curve of our study population. Recent studies show this is a common mechanism driving the expansion of IAS, suggesting limited niche conservatism (McCann et al. 2014; Sillero et al. 2014; Krehenwinkel et al. 2015). Originating from a warmer region, *P. clarkii* could revert recent cold adaptations and tolerate high temperatures imposed by longer, more frequent and intense heat waves. But, as projected by Capinha et al. (2012), its distribution may still contract in Mediterranean climate regions, because in shallow water bodies heat waves may expose *P. clarkii* to drought. However, in central and northern Europe, where climate changes are projected to increase winter minima, higher temperatures may have positive effects on *P. clarkii* distribution.

This study shows that the occurrence, extent and adaptive value of diet shifts in response to heat waves may not only vary across species (Carreira et al. 2016), but also between different life-stages of the same species. Our results show clear differences in diet regulation between the two *P. clarkii* life stages, stressing the importance of assessing them separately. Juveniles were more responsive to temperature changes and even though we did not measure temperature effects on consumption rates, diet shifts alone may increase crayfish impact on aquatic vegetation. Increased herbivory at higher temperatures may greatly extend the economic damage caused by *P. clarkii*, since the growth season of rice plants coincides with the highest abundance of this juvenile class (Correia 1995). Although the effects were relatively small, our study also provides some indication that differences between the sexes may add complexity to the responses to heat waves. Imbalanced temperature effects like these may also occur in other IAS and impacts in the invaded areas may change as the species life stages or sexes respond differently to temperature increases.

While agreeing with the findings of other studies, the relatively small diet effects on crayfish performance may have originated in our aim for realistic conditions. Unlike Croll and Watts (2004), we used natural food items, instead of laboratory-assembled fodder. Additionally, the temperature range in our experiments was narrower and included temperatures at which the shift in the protein to carbohydrate assimilation ratio is relatively small (Croll and Watts, 2004), which may have caused low variation in total protein and carbohydrate assimilated. However, diet effects may get stronger as temperature approaches the upper limit of optimal growth (27ºC), a scenario that is likely to become more frequent under the current climate changes. Furthermore, logistic constraints did not allow cyclical dial variation in our experimental setup. Although experimental temperatures realistically covered water temperatures at the collection sites and temperatures for optimum growth, cyclical dial variation could have partially offset the effects of high daytime temperatures.

The diet shifts and the changes in the performance of *P. clarkii* add to the results obtained in other taxonomic groups, such as copepods (Boersma et al. 2016) and anurans (Carreira et al. 2016). Our findings strengthen the hypothesis that temperature effects on nutrient acquisition may form a general and consistent pattern in ectotherms. Diet shifts towards increased herbivory proved adaptive and, thus, diet regulation may help *P. clarkii* to cope with increased heat stress from longer heat waves. While heat waves are unlikely to boost *P. clarkii* populations in Mediterranean climates, diet shifts may change the trophic niche of this keystone species. The potential changes in the trophic interactions of *P. clarkii* in freshwater ecosystems may aggravate both its ecological impact on aquatic vegetation and economic damage to rice fields.

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References

Alcorlo P, Geiger W, Otero M (2004) Feeding preferences and food selection of the red swamp crayfish, *Procambarus clarkii*, in habitats differing in food item diversity. Crustaceana 77:435–453.

Alhazzaa R, Bridle AR, Nichols PD, Carter CG (2013) Coping with sub-optimal water temperature: Modifications in fatty acid profile of barramundi as influenced by dietary lipid. Comp Biochem Physiol A 165: 243–253. doi:10.1016/j.cbpa.2013.03.019

Banha F (2015) Human dispersal of freshwater invasive fauna. PhD dissertation, Universidade de Évora, Évora, Portugal.

Boersma M, Mathew KA, Niehoff B, Schoo KL, Franco-Santos RM, Meunier CL (2016) Temperature driven changes in the diet preference of omnivorous copepods: no more meat when it’s hot? Ecol Lett 19:45–53. doi:10.1111/ele.12541

Bradley BA, Wilcove DS, Oppenheimer M (2010) Climate change increases risk of plant invasion in the Eastern United States. Biol Invasions 12:1855–1872. doi:10.1007/s10530-009-9597-y

Buswell JM, Moles AT, Hartley S (2011) Is rapid evolution common in introduced plant species? J Ecol 99:214–224. doi:10.1111/j.1365-2745.2010.01759.x

Capinha C, Anastácio P (2011) Assessing the environmental requirements of invaders using ensembles of distribution models. Divers Distrib 17:13–24. doi:10.1111/j.1472-4642.2010.00727.x

Capinha C, Anastácio P, Tenedório JA (2012) Predicting the impact of climate change on the invasive decapods of the Iberian inland waters: An assessment of reliability. Biol Invasions 14:1737–1751. doi:10.1007/s10530-012-0187-z

Carreira BM, Dias MP, Rebelo R (2014) How consumption and fragmentation of macrophytes by the invasive crayfish *Procambarus clarkii* shape the macrophyte communities of temporary ponds. Hydrobiologia 721:89–98. doi:10.1007/s10750-013-1651-1

Carreira BM, Segurado P, Orizaola G, Gonçalves N, Pinto V, Laurila A, Rebelo R (2016) Warm vegetarians? Heat waves and diet shifts in tadpoles. Ecology 97:2964–2974. doi:10.1002/ecy.1541

Chown SL, Slabber S, McGeoch MA, Janion C, Leinaas HP (2007) Phenotypic plasticity mediates climate change responses among invasive and indigenous arthropods. Proc R Soc B 274:2531–2537. doi:10.1098/rspb.2007.0772

Chucholl C (2011) Population ecology of an alien "warm water" crayfish (*Procambarus clarkii*) in a new cold habitat. Knowl Manag Aquat Ecosyst 401:29. doi:10.1051/kmae/2011053

Correia AM (1995) Biologia Populacional e interacções tróficas de *Procambarus clarkii* (Crustacea, Decapoda, Cambaridae) no sistema hidrográfico do rio Tejo (Ribatejo, Portugal). PhD dissertation, Universidade de Lisboa, Lisboa, Portugal.

Correia AM (2002) Niche breadth and trophic diversity: feeding behaviour of the red swamp crayfish (*Procambarus clarkii*) towards environmental availability of aquatic macroinvertebrates in a rice field (Portugal). Acta Oecol 23:421–429. doi:10.1016/S1146-609X(02)01166-9

Croll SL, Watts SA (2004) The effect of temperature on feed consumption and nutrient absorption in *Procambarus clarkii* and *Procambarus zonangulus*. J World Aquac Soc 35:478–488.

Cross WF, Hood JM, Benstead JP, Huryn AD, Nelson D (2015) Interactions between temperature and nutrients across levels of ecological organization. Glob Chang Biol 21:1025–1040. doi:10.1111/gcb.12809

Dasari HP, Pozo I, Ferri-Yáñez F, Araújo MB (2014) A regional climate study of heat waves over the Iberian Peninsula. Atmos Clim Sci 4:841–853. doi:10.4236/acs.2014.45074

Davidson AM, Jennions M, Nicotra AB (2011) Do invasive species show higher phenotypic plasticity than native species and, if so, is it adaptive? A meta-analysis. Ecol Lett 14:419–431. doi:10.1111/j.1461-0248.2011.01596.x

Dobush GR, Ankney CD, Krementz DG (1985) The effect of apparatus, extraction time, and solvent type on lipid extractions of snow geese. Can J Zool 63:1917–1920. doi:10.1139/z85-285

Dudgeon D, Arthington AH, Gessner MO, Kawabata Z-I, Knowler DJ, Lévêque C, Naiman RJ, Prieur-Richard A-H, Soto D, Stiassny MLJ, Sullivan CA (2006) Freshwater biodiversity: importance, threats, status and conservation challenges. Biol Rev Camb Philos Soc 81:163–182. doi:10.1017/S1464793105006950

Dukes JS, Mooney HA (1999) Does global change increase the success of biological invaders? Trends Ecol Evol 14:135–139. doi:10.1016/S0169-5347(98)01554-7

Floeter SR, Behrens MD, Ferreira CEL, Paddack MJ, Horn MH (2005) Geographical gradients of marine herbivorous fishes: patterns and processes. Mar Biol 147:1435–1447. doi:10.1007/s00227-005-0027-0

Forster J, Hirst AG, Atkinson D (2012) Warming-induced reductions in body size are greater in aquatic than terrestrial species. Proc Natl Acad Sci USA 109:19310–19314.

Frich P, Alexander LV, Della-Marta P, Gleason B, Haylock M, Klein Tank AMG, Peterson T (2002) Observed coherent changes in climatic extremes during the second half of the twentieth century. Clim Res 19:193–212. doi:10.3354/cr019193

Gallardo B (2014) Europe’s top 10 invasive species: relative importance of climatic, habitat and socio-economic factors. Ethol Ecol Evol 26:130–151. doi:10.1080/03949370.2014.896417

Huner JV, Lindqvist OV, Kononen H (1985) Responses of intermolt noble crayfish *Astacus astacus* L. (Decapoda, Astacidae), to short-term and long-term holding condition at low temperature. Aquacult 47:213–221.

Ilhéu M, Bernardo JM (1993) Experimental evaluation of food preference of red swamp crayfish, *Procambarus clarkii*: vegetal versus animal. Freshwat Crayfish 9:359–364.

Jerez S, Montavez JP, Gomez-Navarro JJ, Lorente-Plazas R, Garcia-Valero JA, Jimenez-Guerrero P (2012) The role of the land-surface model for climate change projections over the Iberian Peninsula. J Geophys Res 117:D01109. doi:10.1029/2011JD016576

Jerez S, Montavez JP, Gomez-Navarro JJ, Lorente-Plazas R, Garcia-Valero JA, Jimenez-Guerrero P (2013) A multi-physics ensemble of regional climate change projections over the Iberian Peninsula. Clim Dyn 41:1749–1768. doi:10.1007/s00382-012-1551-5

Karl I, Fischer K (2008) Why get big in the cold? Towards a solution to a life-history puzzle. Oecologia 155:215–225. doi:10.1007/s00442-007-0902-0

Kelley AL (2014) The role thermal physiology plays in species invasion. Conserv Physiol 2:cou045. doi:10.1093/conphys/cou045

Krehenwinkel H, Rödder D, Tautz D (2015) Eco-genomic analysis of the poleward range expansion of the wasp spider *Argiope bruennichi* shows rapid adaptation and genomic admixture. Glob Chang Biol 21:4320–4332. doi:10.1111/gcb.13042

Leishman MR, Gallagher RV (2015) Will there be a shift to alien-dominated vegetation assemblages under climate change? Divers Distrib 21:848–852. doi:10.1111/ddi.12338

Martin-Creuzberg D, Wacker A, Ziese C, Kainz MJ (2012) Dietary lipid quality affects temeprature-mediated reaction norms of a freshwater key herbivore. Oecologia 168:901-912

McCann S, Greenlees MJ, Newell D, Shine R (2014) Rapid acclimation to cold allows the cane toad to invade montane areas within its Australian range. Funct Ecol 28:1166–1174. doi:10.1111/1365-2435.12255

Meehl GA, Tebaldi C (2004) More intense, more frequent, and longer lasting heat waves in the 21st century. Science 305:994–997. doi:10.1126/science.1098704

Miller GA, Clissold FJ, Mayntz D, Simpson SJ (2009) Speed over efficiency: locusts select body temperatures that favour growth rate over efficient nutrient utilization. Proc Biol Sci 276:3581–3589. doi:10.1098/rspb.2009.1030

Myrick CA, Cech JJJr. (2000) Temperature influences on California rainbow trout physiological performance. Fish Physiol Biochem 22:245–254.

Nunes AL, Orizaola G, Laurila A, Rebelo R (2014) Rapid evolution of constitutive and inducible defenses against an invasive predator. Ecology 95:1520–1530. doi:10.1890/13-1380.1

Parnell AC, Inger R, Bearhop S, Jackson AL (2010) Source partitioning using stable isotopes: coping with too much variation. PLoS One 5:e9672. doi:10.1371/journal.pone.0009672

Parnell AC, Phillips DL, Bearhop S, Semmens BX, Ward EJ, Moore JW, Jackson AL, Grey J, Kelly DJ, Inger R (2013) Bayesian stable isotope mixing models. Environmetrics 24:387–399. doi:10.1002/env.2221

Pereira HM, Leadley PW, Proença V, Alkemade R, Scharlemann JPW, Fernandez-Manjarrés JF, Araújo MB, Balvanera P, Biggs R, Cheung WWL, Chini L, Cooper HD, Gilman EL, Guénette S, Hurtt GC, Huntington, HP, Mace GM, Oberdorff T, Revenga C, Rodrigues P, Scholes RJ, Sumaila UR, Walpole M (2010) Scenarios for global biodiversity in the 21st century. Science 330:1496–1501. doi:10.1126/science.1196624

Pérez-bote AJL (2004) Feeding ecology of the exotic red swamp crayfish, *Procambarus clarkii* (Girard, 1852) in the Guadiana river (SW Iberian Peninsula). Crustaceana 77:1375–1387.

Peruzza L, Piazza F, Manfrin C, Bonzi LC, Battistella S, Giulianini PG (2015) Reproductive plasticity of a *Procambarus clarkii* population living 10ºC below its thermal optimum. Aquat Invasions 10:199–208. doi:10.3391/ai.2015.10.2.08

Powell ML, Watts SA (2006) Effect of temperature acclimation on metabolism and hemocyanin binding affinities in two crayfish, *Procambarus clarkii* and *Procambarus zonangulus*. Comp Biochem Physiol A Mol Integr Physiol 144:211–217. doi:10.1016/j.cbpa.2006.02.032

Preston T, Owens NJP (1983) Interfacing an automatic elemental analyser with an isotope ratio mass sepectrometer: the potential for fully automated total nitrogen and nitrogen-15 analysis. Analyst 108:971–977. doi:10.1039/an9830800971

Pyšek P, Richardson DM (2010) Invasive species, environmental change and management, and health. Annu Rev Environ Resour 35:25–55. doi:10.1146/annurev-environ-033009-095548

Robinet C, Roques A (2010) Direct impacts of recent climate warming on insect populations. Integr Zool 5:132–142. doi:10.1111/j.1749-4877.2010.00196.x

Sala OE, Chapin FS, Armesto JJ, Berlow E, Bloomfield J, Dirzo R, Huber-Sanwald E, Huenneke LF, Jackson RB, Kinzig A, Leemans R, Lodge DM, Mooney HA, Oesterheld M, Poff NL, Sykes MT, Walker BH, Walker M, Wall DH (2000) Global biodiversity scenarios for the year 2100. Science 287:1770–1774.

Seifert LI, De Castro F, Marquart A, Gaedke U, Weithoff G, Vos M (2014) Heated relations: temperature-mediated shifts in consumption across trophic levels. PLoS One 9:e95046. doi:10.1371/journal.pone.0095046

Sillero N, Reis M, Vieira CP, Vieira J, Morales-Hojas R (2014) Niche evolution and thermal adaptation in the temperate species Drosophila americana. J Evol Biol 27:1549–1561. doi:10.1111/jeb.12400

Souty-Grosset C, Anastácio PM, Aquiloni L, Banha F, Choquer J, Chucholl C, Tricarico E (2016) The red swamp crayfish *Procambarus clarkii* in Europe: impacts on aquatic ecosystems and human well-being. Limnologica 58:78–93. doi:10.1016/j.limno.2016.03.003

Spicer JI, Gaston, KJ (1999) Physiological diversity and its ecological implications. Blackwell Science Ltd, Oxford

Theoharides KA, Dukes JS (2007) Plant invasion across space and time: factors affectig nonindigenous species success during four stages of invasion. New Phytol 176:256–273. doi:10.1111/j.1469-8137.2007.02207.x

Thomas SM, Crowther TW (2014) Predicting rates of isotopic turnover across the animal kingdom: a synthesis of existing data. J Anim Ecol 84:861–870. doi:10.1111/1365-2656.12326

Trigal C, García-Criado F, Aláez CF (2007) Macroinvertebrate communities of mediterranean ponds (North Iberian Plateau): importance of natural and human-induced variability. Freshw Biol 52:2042–2055. doi:10.1111/j.1365-2427.2007.01805.x

Vitousek PM, Mooney HA, Lubchenco J, Melillo JM (1997) Human domination of Earth ecosystems. Science 277:494–499. doi:10.1126/science.277.5325.494

Walther G-R, Roques A, Hulme PE, Sykes MT, Pyšek P, Kühn I, Zobel M, Bacher S, Botta-Dukát Z, Bugmann H (2009) Alien species in a warmer world: risks and opportunities. Trends Ecol Evol 24:686–693. doi:10.1016/j.tree.2009.06.008

Walther G-R (2010) Community and ecosystem responses to recent climate change. Philos Trans R Soc Lond B Biol Sci 365:2019–2024. doi:10.1098/rstb.2010.0021

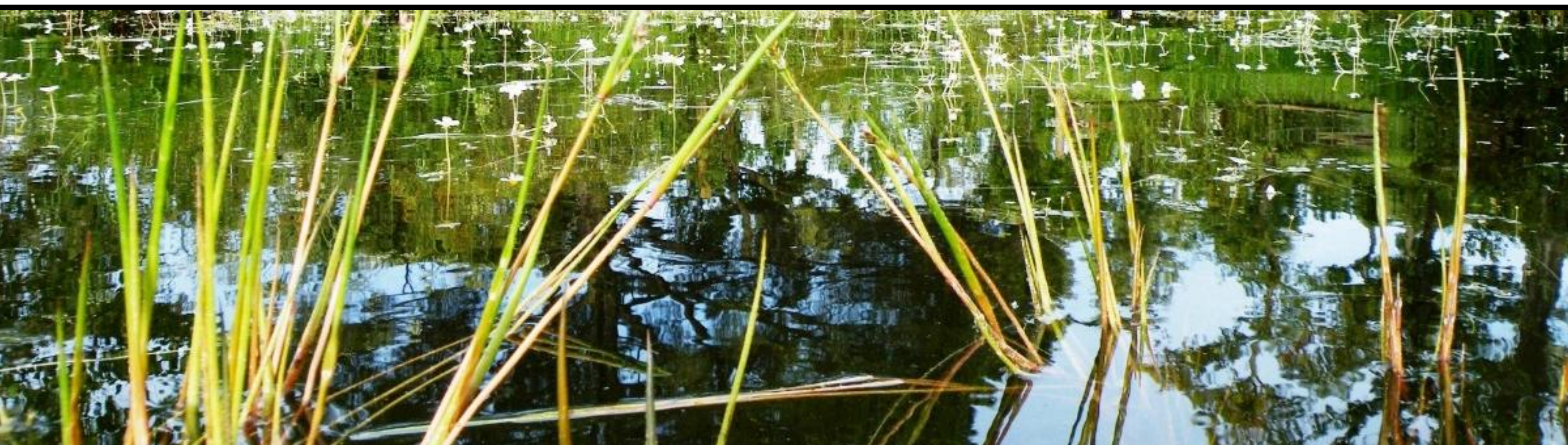
Whitney KD, Gabler CA (2008) Rapid evolution in introduced species, “invasive traits” and recipient communities: challenges for predicting invasive potential. Divers Distrib 14:569–580. doi:10.1111/j.1472-4642.2008.00473.x

CHAPTER 5

Heat waves increase herbivory in a freshwater snail

B. M. Carreira, P. Segurado, A. Laurila and R. Rebelo

Radix balthica



CHAPTER 5

Heat waves increase herbivory in a freshwater snail

Abstract

Recent theoretical and empirical studies suggest that higher temperatures increase herbivory among omnivorous ectotherms, which is likely to have important ramifications to food webs, given the current climate change. However, increased herbivory at higher temperatures remains controversial, and it is unclear whether temperature affects ectotherm feeding preferences or simply the balance upon nutrient assimilation rates. We investigated diet shifts in *Radix balthica* exposed to short and long heat waves and found that the greater assimilation of the plant material allowed for greater growth rates without an effect on egg production. Furthermore, we found a disproportional shift in the assimilation of plant nutrients in the short heat wave, suggesting that the higher temperatures in the heat wave changed snails' feeding preference and increased herbivory.

Keywords:

Radix balthica, feeding preferences, omnivory, herbivory, temperature

Introduction

Several studies on marine and freshwater fish communities have found that the prevalence of herbivory increases towards lower latitudes [1-4]. Among several potential explanations, a plausible hypothesis suggests that temperature influences the feeding preferences of ectotherms, which considering the ongoing climatic changes may echo through the structure of food webs worldwide. Consequently, the influence of temperature on nutrient acquisition by ectothermic organisms has recently become a hot topic on climate change biology [5].

Experimental evidence suggests that temperature may influence nutrient intake in ectotherms through imbalanced effects on different aspects of metabolism scaling differently with temperature. Recent studies show that the greater assimilation of plant material at higher temperatures is adaptive – contrary to the consumption of animal diet which decreased ectotherm performance [6-7], suggesting that at higher temperatures omnivorous ectotherms may optimize energy intake by avoiding protein-rich diets. Given the stronger temperature effect on catabolic than on anabolic processes [8-9], higher temperatures should increase demand for carbon over nitrogen, as they promote a greater increase in respiration than in growth. Furthermore, since metabolic rates increase faster with temperature than feeding rates [10-11], the energetic deficit at higher temperatures can be compensated through the active consumption of fast energy sources – carbohydrates [12]. Interestingly, by promoting a greater increase in gut passage rates than in gut assimilation rates [13-14], higher temperatures passively favor the assimilation of carbohydrates, since these smaller and structurally less complex nutrients are easier to digest. Therefore, even in the absence of temperature-induced changes in ectotherm feeding preferences, higher temperatures may shift the protein to carbohydrate assimilation ratio to a greater assimilation of carbohydrates [15].

The importance of unveiling the underlying mechanisms through which temperature affects nutrient acquisition in ectotherms resides on whether warmer climates may affect food webs directly by inducing diet shifts in omnivorous ectotherms leading to trophic downgrading, or indirectly by simply affecting individual nutrient assimilation. Despite the convincing arguments, actual demonstrations of changes in the feeding preferences under increased temperatures are controversial. While some studies found evidence for temperature-induced changes in the feeding preferences of omnivorous ectotherms [6,7,12], others found no evidence for such shifts [16-17]. This controversy may stem from the use of different organisms or the relatively short duration of some experiments, but also from the different approaches used to assess feeding preferences. Quantifying the food ingested through consumption tests is a challenging task in small species, especially if they are aquatic. By contrast, stable isotopes analysis offers time-integrated estimates of the food assimilated by the consumers [18], even in small organisms, and better reflects the consumers' true dependence on the food items.

Here we investigated temperature-induced diet shifts in the common pond snail, *Radix balthica*. Snails were fed animal, plant or mixed diets and exposed to short or long heat waves. We recorded performance in terms of growth rate and egg production and used stable isotope mixing models to estimate the proportions of animal and plant material assimilated by the mixed-diet snails. We predicted that the proportion of plant material assimilated on the mixed diet should increase in the heat wave treatments and that such shifts would be adaptive. Furthermore, we predicted that the proportion of plant material assimilated should be proportional to heat wave duration under the assumption that disproportionate responses indicate increased herbivory, rather than simple passive temperature-induced differences in the assimilation of plant material.

Materials and Methods

The common pond snail, *Radix balthica*, is a widespread pulmonate gastropod in freshwater habitats throughout Europe and Asia [19]. This model species is highly suitable to test heat wave effects in the Iberian Peninsula, for which projections indicate an increase in the severity of heat waves [20]. *R. balthica* is a hermaphrodite capable of selfing and may store sperm up to six months. Snails (N=200) were collected on the 18th of April 2014 in Sintra, southwestern Portugal (38º47’30.6’’N, 9º25’9.0’’W), kept at 20ºC (12L:12D) in two 15L aquaria for a month and fed commercial fish food twice a week along with water change.

At the start of the experiment snails were blotted dry in paper towel and weighed to nearest 0.1mg. Shell length was measured as the distance between the apex and the edge of the outer shell lip (average ± SD = 10.5 ± 1.4 mm). Each treatment was assigned 18 randomly selected snails transferred to individual 80mL plastic cups placed in water baths. The snails were fed every other day along with water renewal, the position of each cup in the water baths was randomized, egg batches collected and the total number of eggs counted.

The experiment lasted for seven weeks to exceed the half-life of ^{13}C and ^{15}N turnover rates [21]. At the end of the experiment the snails were weighed, and growth rate was calculated as LN of weight increase (mg) per day. Individuals were euthanized by rapid freezing at -18ºC. Muscle tissue was dried at 60 °C for 24 h, ground to fine powder, cleansed of storage lipids with chloroform-methanol (2:1) and re-dried. Stable isotope ratios ($^{13}C/^{12}C$) and elemental analyses (C:N) were determined by continuous flow isotope mass spectrometry (CF-IRMS).

We tested three experimental diets offered *ad libitum*: Animal diet (A) – composed of defrosted Chironomidae larvae; Plant diet (P) – composed of defrosted spinach leaves; and Mixed diet (M) – composed of both food items in diets A and P offered in similar proportions of fresh weight. Following the heat wave definition of Frich et al. (2002) [22], we exposed snails to three temperature treatments: Cold (C) – 7 weeks of constant 20^{o}C; Short Heat Wave (SHW) – after 5 weeks of temperatures identical to C, two weeks before the end of the experiment snails experienced a one-week 25^{o}C heat wave; and Long Heat Wave (LHW) – 7 weeks of constant 25^{o}C. The SHW treatment simulated current spring heat waves in the southwest of Portugal [7], while the LHW simulated extremely long heat waves projected to become more frequent [20].

The estimates of animal and plant material assimilated by snails on the mixed diet were obtained on Stable Isotope Analysis package in R [23]. Using Bayesian statistics, the stable isotope mixing models calculate a probability distribution function for the proportions of diets A and P assimilated, based on 10 000 iterations. We tested treatment effects on growth rate with a general linear model and using Bonferroni's test for post hoc pairwise comparisons in STATISTICA 12. We tested treatment effects on egg production with a generalized linear model, specifying a negative binomial distribution with a log link function and using Bonferroni's test for post hoc pairwise comparisons in SPSS (IBM). Initial shell length was included as a covariate in both models due to its significant effect on growth and egg production (Table 1).

Table 1. General linear model for growth rate and generalized linear model for egg production.

	Growth			Egg production		
	df	F	*P*	*df*	χ^2	*P*
Diet	2, 67	5.14	**<0.01**	2	11.37	**<0.01**
Temperature	2, 67	3.88	**<0.05**	2	3.24	0.197
Diet × Temperature	4, 67	1.45	0.227	4	10.82	**<0.05**
Initial shell length	1, 67	13.42	**<0.001**	1	4.06	**<0.05**

P values <0.05 are marked in boldface

Results

All the individuals fed on the mixed diet assimilated both animal and plant material, but the median proportion of plant material across the temperature treatments (0.69) indicates they were closer to the herbivorous end of the omnivory gradient (Fig. 1a). The median proportion of plant material assimilated increased with treatment average temperature from 0.59 (Bayesian credible interval (BCI) = 0.34-0.81) in C to 0.70 (BCI = 0.52-0.85) in SHW and to 0.78 (BCI = 0.63-0.89) in LHW (Fig. 1a).

Growth rate on diets P and M was over two times greater than on diet A (Table 1; Fig. 1b). Growth rate in LHW was 31% greater than in SHW, and more than two times greater than in C ($P < 0.001$), but there was no difference between growth in C and in SHW ($P = 0.557$; Table 1; Fig. 1b). We found no significant diet × temperature interaction.

Egg production on diet P was 2.5 times greater than on diet A, but not significantly greater than on diet M (Table1; Fig. 1c). Although temperature had no significant effect on egg production, we found diet × temperature interactions involving

SHW. Under this treatment, snails fed on diet P produced only half of the eggs produced in C and LHW, and snails fed on diet M produced five times more eggs than in LHW and 50% more eggs than in C (Table 1; Fig. 1c). However, we found no significant pairwise differences after correcting for multiple comparisons.

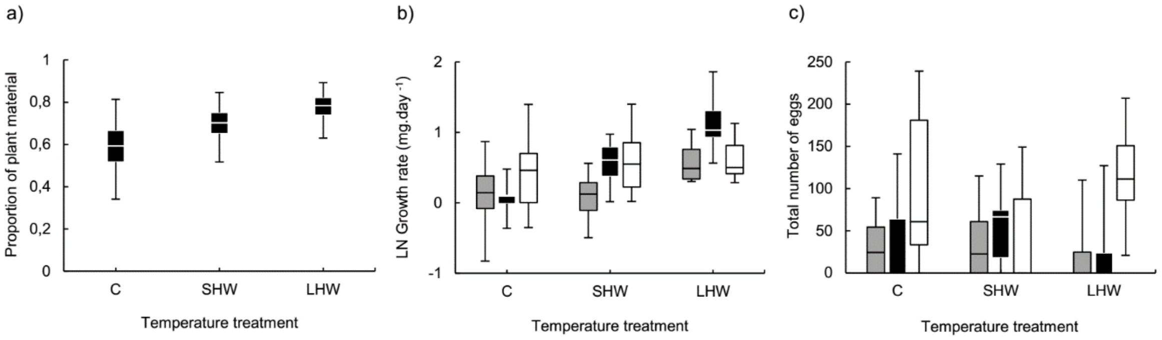


Figure 1. Proportion of plant material assimilated by mixed diet snails calculated as a probability distribution function (a); the boxes show the median and the 25th – 75th percentiles, and the whiskers indicate the 2.5th – 97.5th percentiles (10 000 iterations). LN of growth rate in mg.day^{-1} (b), and total number of eggs produced by *Radix balthica* (c); the boxes show the median and the 25th – 75th percentiles, and the whiskers indicate minimum and maximum values.

Discussion

We found a temperature-induced shift toward greater assimilation of plant nutrients in both heat waves. In LHW this allowed snails fed on diet M to achieve greater growth rates with no differences in the number of eggs produced. We also detected a disproportional shift in the assimilation of plant material in SHW, which experienced temperature conditions similar to C, except for the one-week heat wave two weeks before the end of the experiment. Furthermore, this SHW effect in assimilation of plant nutrients was twice as strong as its effect in growth, suggesting snails may have increased herbivory.

Both the greater growth and egg production on diet P support that, as indicated by the stable isotopes mixing models, the natural feeding preferences of *R. balthica* should be closer to the herbivory end of the omnivory gradient. The increased growth experienced in LHW was not accompanied by changes in egg production, indicating differences in growth and reproduction strategies of the snails across the temperature treatments. Although eggs contain large amounts of lipids, and their production could interfere with the snail isotopic signature, this is unlikely to have occurred since egg production was similar across temperature treatments, unlike in Northern European snails that fail to reproduce at 24ºC [24]. Additionally, despite the differences in the rate of tissue turnover caused by the greater growth in LHW, the duration of the experiment was carefully planned to exceed the half-live of the turnover rates and reflect food item isotopic signatures, even at 20ºC [21].

A recent short-term study in the freshwater snail *Lymnaea stagnalis* showed that higher temperatures increased consumption rates without changing feeding preferences [17]. However, consumption tests in a moth caterpillar showed increased preference for carbohydrates at higher temperatures [12]. The greater assimilation of diet P by *R. balthica* in the heat wave treatments, also found in anuran tadpoles [7], may have been caused by a temperature-induced shift in the protein to carbohydrate assimilation ratio [15] or by altered feeding preferences. The non-linear relationship between the proportion of plant material assimilated and the duration of the heat wave treatments may suggest a shift in the snails' diet preferences. This is further supported by a disproportionate effect of SHW on the assimilation of plant material, two times greater than on growth, which increased very little in comparison to C. Temperature effects upon nutrient assimilation and altered feeding preferences are not necessarily mutually exclusive, and probably co-occur, but our results cannot be explained under a purely a passive mechanism acting on the assimilation rates.

Our findings raise important questions about the underlying mechanisms and the linearity of the temperature effects, but the identification of these effects is a significant advancement in our understanding of nutrient acquisition by ectotherms. Even in the absence of increased herbivory, ectotherms' dependency on plant diets may increase under the current climate changes through the positive discrimination of plant diets upon assimilation. To fully unveil the temperature effects on nutrient acquisition by ectotherms, future studies should preferentially address both food consumption and its assimilation.

Ethics. Animal handling and experimentation procedures were approved by the Body for Regulation of Animal Welfare of the Faculty of Sciences of the University of Lisbon.

Author's contributions. BMC, AL and RR designed the experiment. BMC and PS carried out the experiment and the statistical analysis. BMC, AL and RR wrote the manuscript.

Competing interests. We have no competing interests.

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References

1. Floeter SR, Behrens MD, Ferreira CEL, Paddack MJ, Horn MH. 2005 Geographical gradients of marine herbivorous fishes: patterns and processes. *Mar. Biol.* **147**, 1435–1447. (doi:10.1007/s00227-005-0027-0)

2. Behrens MD, Lafferty KD. 2007 Temperature and diet effects on omnivorous fish performance: implications for the latitudinal diversity gradient in herbivorous fishes. *Can. J. Fish. Aquat. Sci.* **64**, 867–873. (doi:10.1139/f07-063)

3. Jeppesen E, Meerhoff M, Holmgren K, González-Bergonzoni I, Teixeira-de Mello F, Declerck SAJ, De Meester L, Søndergaard M, Lauridsen TL, Bjerring R, et al. 2010 Impacts of climate warming on lake fish community structure and potential effects on ecosystem function. *Hydrobiologia* **646**, 73–90.

4. Gonzalez-Bergonzoni I, Meerhoff M, Davidson TA, Teixeira-de Mello F, Baattrup-Pedersen A, Jeppesen E. 2012 Meta-analysis shows a consistent and strong latitudinal pattern in fish omnivory across ecosystems. *Ecosystems* **15**, 492–503.

5. Cross WF, Hood JM, Benstead JP, Huryn AD, Nelson D. 2015 Interactions between temperature and nutrients across levels of ecological organization. *Glob. Chang. Biol.* **21**, 1025–1040. (doi:10.1111/gcb.12809)

6. Boersma M, Mathew KA, Niehoff B, Schoo KL, Franco-Santos RM, Meunier CL. 2016 Temperature driven changes in the diet preference of omnivorous copepods: no more meat when it’s hot? *Ecol. Lett.* **19**, 45–53. (doi:10.1111/ele.12541)

7. Carreira BM, Segurado P, Orizaola G, Gonçalves N, Pinto V, Laurila A, Rebelo R. 2016 Warm vegetarians? Heat waves and diet shifts in tadpoles. *Ecology* **97**, 2964–2974. (doi: 10.1002/ecy.1541)

8. Karl I, Fischer K. 2008 Why get big in the cold? Towards a solution to a life-history puzzle. *Oecologia* **155**, 215–225. (doi:10.1007/s00442-007-0902-0)

9. Forster J, Hirst AG, Woodward G. 2011 Growth and development rates have different thermal responses. *Am. Nat.* **17**, 668–678.

10. Kingsolver JG, Woods HA. 1997 Thermal sensitivity of growth and feeding in *Manduca sexta* caterpillars. *Physiol. Zool.* **70**, 631–638.

11. Lemoine NP, Burkepile DE. 2012 Temperature-induced mistaches between consumption and metabolism reduce consumer fitness. *Ecology* **93**, 2483–2489.

12. Lee KP, Jang T, Ravzanaadii N, Rho MS. 2015 Macronutrient balance modulates the temperature-size rule in an ectotherm. *Am. Nat.* **186**, 212–222. (doi:10.1086/682072)

13. Myrick CA, Cech JJJr. 2000 Temperature influences on California rainbow trout physiological performance. *Fish. Physiol. Biochem.* **22**, 245–254.

14. Seifert LI, De Castro F, Marquart A, Gaedke U, Weithoff G, Vos M. 2014 Heated relations: temperature-mediated shifts in consumption across trophic levels. *PLoS One* **9**, e95046. (doi:10.1371/journal.pone.0095046)

15. Croll SL, Watts SA. 2004 The effect of temperature on feed consumption and nutrient absorption in *Procambarus clarkii* and *Procambarus zonangulus*. *J. World Aquac. Soc.* **3**, 478–488.

16. Miller GA, Clissold FJ, Mayntz D, Simpson SJ. 2009 Speed over efficiency: locusts select body temperatures that favor growth rate over efficient nutrient utilization. *Proc. R. Soc. B* **276**, 3581–3589. (doi:10.1098/rspb.2009.1030)

17. Zhang P, Blonk BA, van den Berg RF, Bakker ES. 2016 The effect of temperatue on herbivory by the omnivorous ectotherm snail *Lymnaea stagnalis*. *Hydrobiologia* in press. (doi:10.1007/s10750-016-2891-7)

18. Szepanski MM, Ben-David M, Van Ballenberghe V. 1999 Assessment of anadromous salmon resources in the diet of the Alexander Archipelago wolf using stable isotope analysis. *Oecologia* **120**, 327–335.

19. Pfenninger M, Salinger M, Haun T, Feldmeyer B. 2011 Factors and processes shaping the population structure and distribution of genetic variation across the species range of the freshwater snail *Radix balthica* (Pulmonata, Basommatophora). *BMC Evol. Biol.* **11**, 135. (doi:10.1186/1471-2148-11-135)

20. Dasari HP, Pozo I, Ferri-yáñez F, Araújo MB. 2014 A regional climate study of heat waves over the Iberian Peninsula. *Atmos. Clim. Sci.* **4**, 841–853. (doi:10.4236/acs.2014.45074)

21. Thomas SM, Crowther TW. 2014 Predicting rates of isotopic turnover across the animal kingdom: a synthesis of existing data. *J. Anim. Ecol.* **84**, 861–870. (doi:10.1111/1365-2656.12326)

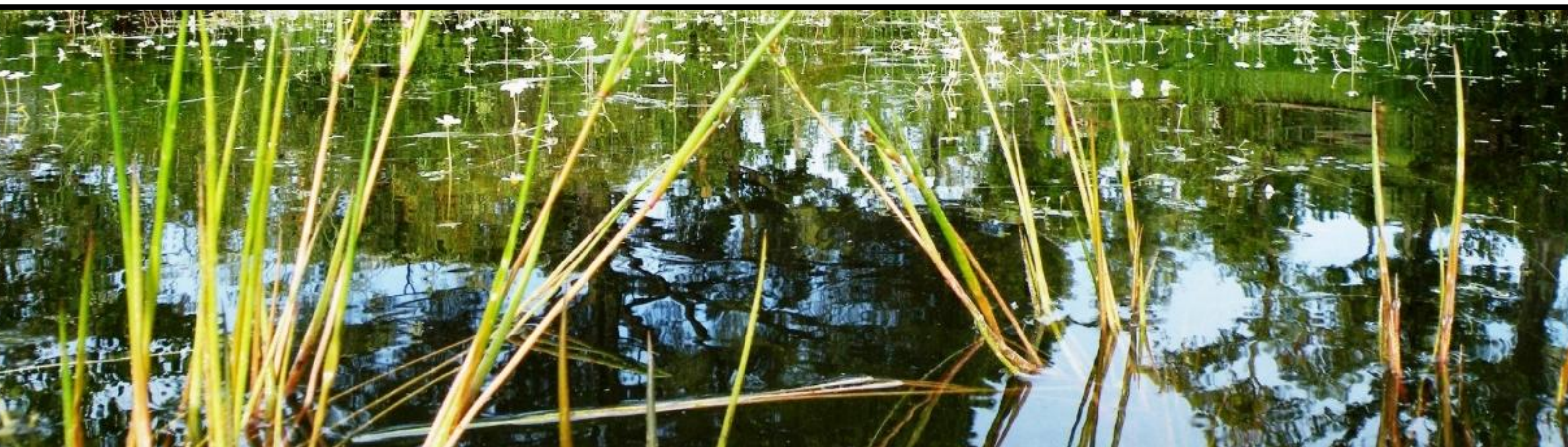
22. Frich P, Alexander LV, Della-Marta P, Gleason B, Haylock M, Klein Tank AMG, Peterson T. 2002 Observed coherent changes in climatic extremes during the second half of the twentieth century. *Clim. Res.* **19**, 193–212. (doi:10.3354/cr019193)

23. Parnell AC, Phillips DL, Bearhop S, Semmens BX, Ward EJ, Moore JW, Jackson AL, Grey J, Kelly DJ, Inger R. 2013 Bayesian stable isotope mixing models. *Environmetrics* **24**, 387–399. (doi:10.1002/env.2221)

24. Johansson MP, Ermold F, Kristjánsson BK, Laurila A. 2016 Divergence of gastropod life history in contrasting thermal environments in a geothermal lake. *J. Evol. Biol., in press*. (doi:10.1111/jeb.12928)

CHAPTER 6

General Discussion



CHAPTER 6

General Discussion

In this thesis, I investigated heat waves effects on the most important taxonomic groups of omnivorous ectotherms inhabiting Mediterranean temporary ponds, assessing the effects of these extreme climatic events on the feeding preferences, survival and life history of these organisms, and how they may respond to longer heat waves projected for the future. More specifically, I assessed how the diet quality changed with temperature and if higher temperatures could trigger shifts in the assimilation of animal and plant diets, addressing the adaptive value of the assimilation shifts by analyzing effects on the survival and life history of the study species. Finally, I investigated the nature of the mechanisms behind the assimilation shifts and whether it rooted in altered feeding preferences or in imbalanced temperature effects on ectotherm metabolism.

The work presented in this thesis highlights the role of temperature on nutrient acquisition by omnivorous ectotherms and contributes to a better understanding of the ecological effects of the ongoing climate change. This chapter summarizes and integrates the main findings of the papers presented in the previous chapters with a focus on the potential consequences of greater herbivory at higher temperatures for the structure of freshwater food webs.

Performance on animal diets

This work showed decreased performance on the animal diets in the heat waves, indicating a lower nutritional value of these diets at higher temperatures. Although not evident in all the traits studied, there was a substantial amount of evidence supporting this pattern, which was mostly evident in the long heat wave. Growth rate was a particularly important trait revealing this trend – in the heat waves the animal diet markedly decreased the growth rate of *D. galganoi*, *H. arborea* and adult *P. clarkii*. Decreased performance on the animal diet in the heat waves was to a lesser extent also evident in other traits. For instance, in *H. arborea* the larval period lasted longer and mass at metamorphosis markedly decreased, *H. meridionalis* experienced a high mortality rate, and the Fulton index in *P. clarkii* was lower, also indicating that the quality of the animal diet decreased at higher temperatures.

Even though animal diets have a high nutrient content, it seems that as temperature increases ectotherms experience a greater difficulty in obtaining the energy necessary to meet the energetic demands imposed by an accelerated metabolism. This difficulty may originate in the fact that temperature effects are stronger in feeding and gut passage rates than in assimilation rates (Myrick & Cech 2000, Seifert et al. 2014). Proteins are structurally complex nutrients, and protein-rich animal diets may not provide energy fast enough to keep up with the increase in feeding and gut passage rates at higher temperatures. While this constraint may have had negligible effects in the short heat waves, the same was not true in the long heat wave.

Performance on plant diets

This work also showed increased performance on the plant diets in the heat waves, indicating a greater nutritional value of these diets at higher temperatures. Although this pattern was less obvious, the anurans provided some evidence supporting increased performance or higher relative quality of plant diets. In the heat waves, the plant diet increased the growth rates of both *H. arborea* and *H. meridionalis*, and despite decreased growth in *D. galganoi*, the relative quality of the plant diet increased. Furthermore, the greater performance of plant diets at higher temperatures was also evident in the larval period of *H. arborea*, which decreased sharply in the long heat wave. However, in *P. clarkii* the performance on the plant diet changed very little across the temperature treatments, with no noticeable increase in the heat waves.

The low nutritional value of plant diets seems to be at odds with the increased quality of these diets at higher temperatures, precisely when ectotherms face greater energetic demands motivated by an accelerated metabolism. The low nutritional value of these diets together with slow feeding rates may impair energy acquisition at lower temperatures. However, plant diets are rich in carbohydrates, which make up for a fast source of energy that, together with faster feeding rates at higher temperatures, provide a steady source of easily available energy, which compensates for the greater metabolic demands (Kingsolver & Woods 1997, Lemoine & Burkepile 2012).

Changes in the food quality

The temperature effects on the performance of the study species found in Chapters 2 and 4 showed that temperature may influence the nutritive quality of animal and plant diets, with contrasting trends for the two diets: a decreased performance of animal diets and an increased performance of plant diets at higher temperatures. However, the strength of these temperature effects appeared to differ considerably among animal and plant diets. While performance on the animal diets decreased markedly as temperatures rose, the performance on plant diets increased only slightly, sometimes changing very little across temperature treatments. The freshwater snail *R. balthica* was apparently an exception, as there was no supporting evidence for either of the trends in the species life history as shown in Chapter 5. But since variation in the temperature treatments was low in this experiment (20-25°C), it is possible that a wider temperature range would revealed similar trends, especially at lower temperatures where the effects are stronger (Malzahn et al. 2016).

The changes in food quality described here confirmed the hypothesis advanced by other studies suggesting that the consumption of plant diets, rich in carbohydrates, would allow ectotherms to better cope with the greater energetic demands at higher temperatures (Kingsolver & Woods 1997, Lemoine & Burkepile 2012). Nevertheless, my results suggest this seems to result from a greater constraint to carnivory at higher temperatures, rather than from a particularly strong increase in the efficiency of herbivory. While partially unexpected, the weaker effects of the temperature on the performance of the plant diets may originate in their generally low nutritional value, which may limit their benefits as temperature increases.

Assimilation shifts

The temperature-induced changes in the quality of the animal and plant diets caused assimilation shifts at higher temperatures. The assimilation of the plant diet in the heat waves increased at least to some extent in all of the study species. Nevertheless, this response was not uniform between vertebrates and invertebrates, since while *P. clarkii* and *R. balthica* (Chapters 4 and 5, respectively) incorporated more plant nutrients in both the short and the long heat waves, the different anuran species (Chapter 2) responded differently to each type of heat wave. For instance, the assimilation of the plant diet in *H. arborea* increased only in the long heat wave, whereas in *D. galganoi* and *H. meridionalis* it increased mostly in the short heat waves. Unsurprisingly, the extension of the shifts identified in the different species may have been conditioned by their position within the omnivory gradient. The smallest shifts were observed in the species that showed the greatest specialization in their feeding preferences – *D. galganoi* (the most carnivorous) and *H. meridionalis* (the most herbivorous). On the contrary, the greatest shifts that were observed in *H. arborea*, *P. clarkii* and *R. balthica* may have been allowed by the greater plasticity conferred by more balanced feeding preferences.

The shifts found in Chapters 2, 4 and 5, at the very least confirm that the temperature-induced changes in different metabolic processes may have noticeable effects on nutrient assimilation by ectotherms. The greater assimilation of plant material at higher temperatures found in these chapters conflicts with results showing that beetles select plants with higher N-content at higher temperatures (Lemoine et al. 2013). However, it agrees both with the studies suggesting a positive discrimination of plant diets (Myrick & Cech 2000, Croll & Watts 2004, Clements et al. 2009, Seifert et al. 2014) and a greater demand for plant diets (Karl & Fischer 2008, Forster et al. 2011, Lemoine & Burkepile 2012). While the physiological or behavioral nature of these shifts can be questioned, originating either in unbalanced temperature effects on the metabolism or in altered feeding preferences, their implications for the omnivores are clear. These findings indicate a greater contribution of plant diets upon nutrient assimilation and, perhaps, a greater dependency on plant diets as temperature increases. Additionally, these findings suggest climate change can potentially interfere with nutrient assimilation in ectotherms, even during discrete climatic events such as short heat waves.

Adaptive value

The generalized occurrence of shifts toward greater assimilation of plant material across the study species raised an important question regarding their adaptive value. Life history traits revealed variation in the adaptive value of increased plant assimilation, which appeared to be linked to the species' feeding preferences and the character of the heat wave. The lower mass at metamorphosis and extended larval period in the heat waves suggest that the increased assimilation of plant diet in *D. galganoi* was maladaptive. As a mostly carnivorous species, *D. galganoi* may lack the plasticity to efficiently satisfy its energetic demands with an increased assimilation of plant material. On the opposite end of the omnivory gradient, no notable benefits or costs were found in *H. meridionalis*, possibly because, given the essentially herbivorous character of this species, the greater assimilation of plant material may have had very little impact. By contrast, life history traits suggested the greater assimilation of plant material in the heat waves to be adaptive in *H. arborea*, as the benefits of shorter larval period most likely outweigh the costs of the lower mass at metamorphosis. The assimilation shifts were also adaptive in *P. clarkii*, allowing juveniles to maintain high growth rates in the heat wave; and in *R. balthica*, promoting a greater growth rate without negative effects on egg production.

Overall, these findings suggest that the effects of a greater assimilation of plant material at higher temperatures may depend on the species position within the omnivory gradient. The species closer to the carnivorous end of the omnivory gradient may be unable to incorporate a greater proportion of plant material in their diet and meet their nutritional demands, while the species closer to the herbivorous end of the gradient may simply not be affected by the assimilation of additional plant material. The species in intermediate positions within the omnivory gradient, benefiting from a greater trophic plasticity, may be positively affected by the assimilation of additional plant material, taking advantage of the temperature-induced changes in food quality. Finally, although these examples may be indicative of how increased assimilation of plant diets may affect species life histories, the effects on individual fitness are yet to be assessed.

Altered feeding preferences

The underlying mechanisms responsible for the shifts in the assimilation of the animal and plant diets observed in Chapters 2, 4 and 5 are yet to be fully unveiled. In

Chapter 5, the contribution of this thesis to the controversy surrounding the physiological or behavioral nature of these shifts issue was limited by the methodological approach. Measuring consumption was challenging because of the small size and the aquatic character of the species, which imposed logistic difficulties and rendered consumption measurements impractical. As such, the perspective presented here is based only on stable isotopes analysis, i.e. what individuals assimilated rather than what they consumed. This distinction is important because temperature effects on assimilation indirectly affect food webs through effects on individual fitness, while temperature effects on consumption rates will directly affect food webs through changes in the species' trophic interactions. Although consumption patterns can be inferred from assimilation estimates and to a certain degree from mismatches between these estimates and the temperatures in the experimental treatments (see Chapter 5), accurate consumption measurements would indubitably confirm the existence of temperature-induced changes in the feeding preferences of ectotherms.

The evidence from this thesis suggests that the assimilation shifts identified here are unlikely to arise solely from a temperature-induced discrimination of plant material upon nutrient assimilation. In the absence of altered feeding preferences, a passive temperature effect upon nutrient assimilation should generate a greater contribution of plant nutrients in the long heat wave than in the short heat waves. However, this was not the case in in *D. galganoi* and *H. meridionalis* (Chapter 2), for which the assimilation of plant material was greater in the short heat waves than in the long heat wave. This suggests that tadpoles of *D. galganoi* and *H. meridionalis* may have actively changed feeding preferences according to the temperature and increased herbivory. Additionally, the disproportional temperature effect of the short heat wave on the assimilation of the plant diet by *R. balthica* also suggests altered feeding preferences (Chapter 5).

Nevertheless, a temperature-induced passive change in the assimilation of plant material could explain both the maladaptive assimilation of plant material by *D. galganoi,* and the relationship between treatment average temperature and the proportion of plant material assimilated in the heat waves by *P. clarkii*.

The scarce literature on the temperature effects on nutrient acquisition by ectotherms is divided. There is strong evidence for temperature-induced changes in feeding preferences in amphipods (Sotka & Giddens 2009), beetles (Lemoine et al. 2013), and moth caterpillars (Lee et al. 2015), while others found no evidence for such shifts in locusts (Miller et al. 2009) and gastropods (Zhang et al. 2016). At the very least, the findings presented here show for the first time an unequivocal positive discrimination of plant material upon nutrient assimilation at higher temperatures in vertebrates, also recently shown to occur in copepods (Boersma et al. 2016, Malzahn et al. 2016). Beyond that, the variation in the assimilation of plant material in the short and long heat waves suggests some of the species may have regulated nutrient intake and increased herbivory, supporting ectotherms change feeding preferences in response to higher temperatures.

Thermal background and feeding preferences

Apart from inducing short term changes in the feeding preferences of ectotherms, temperature may also drive long term selective pressure leading to dietary specialization. The anuran species studied in Chapter 2 have similar thermal performance limits (Katzenberger 2015), but were selected according to the minimum temperatures their larvae experience in nature to define a seasonal temperature gradient established by the onset of breeding. This gradient in the thermal background of the study species, also supported by the variation in the tadpole activity of the three species across the temperature treatments reported in Chapter 3, was found to be associated to the species trophic position along the omnivory gradient. Assimilation of plant material increased from the earliest breeding species experiencing lower water temperatures (*D. galganoi*), to the following species experiencing intermediate water temperatures (*H. arborea*), and to the latest breeding species, which experiences the highest water temperatures (*H. meridionalis*). The thermal backgrounds of *P. clarkii* and *R. balthica* and the proportion of plant material assimilated by these invertebrate species provide additional support for the important role of temperature in determining the feeding preferences of ectotherms. Although the optimum temperature for growth in *P. clarkii* is 21-27^{o}C in the native distribution range (Croll & Watts 2004), the results in Chapter 4 suggest adaptation to the cold. Furthermore, the optimum temperature for growth in *R. balthica* (ca. 20^{o}C), described in Nordic populations, was found to be the same in both cold and warm-adapted populations (Johansson et al. 2016). In the absence of more evidence we assume that the thermal optima of these snails is similar to that of *H. arborea* populations in the southwest of Portugal. As such, both these invertebrates seem to occupy an intermediate position along the omnivory gradient, as the anuran from an intermediate temperature background.

Despite the small number of species, my results contribute to the growing evidence for a general influence of temperature on the feeding preferences of ectotherms, with the extent of herbivory increasing with temperature. Similar trends have been identified in marine communities of herbivorous fishes (Floeter et al. 2005, Behrens & Lafferty 2007, Jeppesen et al. 2010) and in freshwater communities of omnivorous fishes (Pennings et al. 2009, Schemske et al. 2009, Gonzalez-Bergonzoni et al. 2012). However, these patterns were obtained with large scale latitudinal surveys assessing the relative abundance and richness of herbivorous and omnivorous fishes. Interestingly, my results suggest that similar patterns can potentially be found at regional scales driven by the seasonality in the species that compose these communities.

Heat waves: present and future

The two types of heat wave in this thesis were investigated with a double purpose: i) to assess the effects of the current short heat waves afflicting omnivorous ectotherms in the Mediterranean temporary ponds of the southwest of the Iberian Peninsula, and to which these species should already be adapted; and ii) investigate how these organisms may respond to longer heat waves that may affect them for a very significant part of their life. Generally, both the short and long heat waves had noticeable effects on survival and life history traits, but even though the effects were not catastrophic, the long heat waves had a greater impact in all species. Interestingly, the assimilation of plant nutrients in the Mediterranean species (*D. galganoi* and *H. meridionalis*) appeared more responsive to the short heat waves. This could either indicate acclimatization by these species in the long heat wave treatment or that their generalized response to temperatures beyond a certain threshold (see Chapter 2) may have conditioned the response to the long heat waves.

Interpreting the heat wave impacts in the context of the projected changes for the heat wave regime in the Iberian Peninsula, it becomes clear that in the future these climatic events may take a greater role in the dynamic of Mediterranean temporary ponds. The accentuated severity of the June heat waves may prove dramatic, as this period coincides with the pond drying, which may lead to sudden drought and cause episodes of mass mortality. Furthermore, as a result of the higher frequency and longer duration of the heat waves, organisms are far more likely to spend a substantial part of their life in the ponds under heat wave conditions, approaching the conditions of the “long heat wave” treatment used in this thesis. Short lived and with larval stages completely dependent on the ponds, ectotherm species from Mediterranean temporary ponds face a serious challenge in the future heat wave regime, which may impair annual recruitment and ultimately affect population dynamics.

Implications for freshwater food webs

The influence of the species thermal background on their feeding preferences, the temperature-induced changes in diet quality, the greater assimilation of plant diet and – possibly – the increased herbivory at higher temperatures, all suggest that the ongoing climatic changes may affect the functioning and the structure of freshwater food webs worldwide.

The evidence gathered in Chapter 3 showed that the temperature-induced changes in the quality of the experimental diets were accompanied by changes in tadpole activity according to the levels of stress experienced. This suggests that as environmental conditions change because of long-term trend effects or extreme climatic events, ectotherms may adjust their behavior. Individual behavioral changes may have population- or even ecosystem-level consequences (Lowe et al. 2006, Abrams 2008, Hawlena & Schmitz 2010). For instance, greater foraging activity was shown to increase exposure to predation in *Pseudacris triseriata* (Bridges 2002), *Rana clamitans* (Davis et al. 2012), and several species of Iberian anurans (Nunes et al. 2013). Strongly affecting nutrient recycling, zooplankton diversity and macrophyte biomass (Arribas et al. 2015), tadpoles are key components of freshwater communities and changes in their population dynamics may affect ecosystem functioning.

Impacts in the structure of freshwater food webs may arise essentially from trophic downgrading. Buffering the effects of strong perturbations, omnivory confers stability to the structure of food webs (Ingram et al. 2009). Therefore, increased herbivory by omnivorous ectotherms may decrease the resilience of food webs, which may become more prone to cascading effects and extinctions (Thompson et al. 2007). Furthermore, changes in the strength of trophic interactions are to be expected as higher temperatures push ectotherms toward lower trophic levels. Increasing the strength of herbivory links and decreasing the strength of predatory interactions, in extreme cases, trophic downgrading may, respectively, open new trophic pathways or close previously existing ones. Such changes may even alter the landscape, e. g. driving freshwater ecosystems to alternative stable states with turbid waters, as herbivory pressure on macrophytes increases (Rodríguez et al. 2003). While changes in food webs may be more easily detected and monitored in permanent waters, the stability of these ecosystems should reduce vulnerability to strong perturbations. In addition, ecosystems such as Mediterranean temporary ponds undergo cyclical successions that may conceal changes in their food webs. Although pond drying may halt the propagation of the perturbations in time, the lower stability of these ecosystems renders them vulnerable to strong perturbations that may break their successional cycle.

Final considerations

The repeatability of the results in species from three phyla strongly suggests that temperature plays an important role in modulating nutrient acquisition in ectotherms – a latent and generalized temperature effect on ectotherms that was unknown until very recently. While this thesis suggests that temperature may influence the demand for nutrients such as carbon and nitrogen, the same may be true for other nutrients such as phosphorus, which is key in the synthesis of the lipids controlling membrane fluidity. Furthermore, although the chapters presented here addressed temperature and nutrient interactions at the individual level, for more realistic predictions of ectotherm responses to climate change it would be important to do so across ecological organization levels. Even though the patterns described here may unveil only a part of the temperature influence on nutrient acquisition by ectotherms, the awareness of these effects will contribute to a better understanding and reassessment of the ecological effects of the ongoing climate change.

References

Abrams, P. A. 2008. Measuring the population-level consequences of predator-induced prey movement. Evolutionary Ecology Research 10:333–350.

Arribas, R., C. Díaz-Paniagua, S. Caut, and I. Gomez-Mestre. 2015. Stable isotopes reveal trophic partitioning and trophic plasticity of a larval amphibian guild. Plos One 10:e0130897.

Behrens, M. D., and K. D. Lafferty. 2007. Temperature and diet effects on omnivorous fish performance: implications for the latitudinal diversity gradient in herbivorous fishes. Canadian Journal of Fisheries and Aquatic Sciences 64:867–873.

Boersma, M., K. A. Mathew, B. Niehoff, K. L. Schoo, R. M. Franco Santos, and C. L. Meunier. 2016. Temperature driven changes in the diet preference of omnivorous copepods: no more meat when it’s hot? Ecology Letters 19:45–53.

Bridges, C. M. 2002. Tadpoles Balance Foraging and Predator Avoidance: Effects of Predation, Pond Drying, and Hunger. Journal of Herpetology 36:627–634.

Clements, K. D., D. Raubenheimer, and J. H. Choat. 2009. Nutritional ecology of marine herbivorous fishes: Ten years on. Functional Ecology 23:79–92.

Croll, S. L., and S. A. Watts. 2004. The effect of temperature on feed consumption and nutrient absorption in *Procambarus clarkii* and *Procambarus zonangulus*. Journal of the World Aquaculture Society 35:478–488.

Davis, M. J., J. L. Purrenhage, and M. D. Boone. 2012. Elucidating predator-prey interactions using aquatic microcosms: Complex effects of a crayfish predator, vegetation, and atrazine on tadpole survival and behavior. Journal of Herpetology 46:527–534.

Floeter, S. R., M. D. Behrens, C. E. L. Ferreira, M. J. Paddack, and M. H. Horn. 2005. Geographical gradients of marine herbivorous fishes: Pptterns and processes. Marine Biology 147:1435–1447.

Forster, J., A. G. Hirst, and G. Woodward. 2011. Growth and Development Rates Have Different Thermal Responses. American Naturalist 178:668–678.

González-Bergonzoni, I., M. Meerhoff, T. A. Davidson, F. Teixeira-de Mello, A. Baattrup-Pedersen, and E. Jeppesen. 2012. Meta-analysis shows a consistent and strong latitudinal pattern in fish omnivory across ecosystems. Ecosystems 15:492–503.

Hawlena, D., M. S. Strickland, M. a Bradford, and O. J. Schmitz. 2012. Fear of predation slows plant-litter decomposition. Science (New York, N.Y.) 336:1434–8.

Ingram, T., L. Harmon, and J. Shurin. 2009. Niche evolution, trophic structure, and species turnover in model food webs. The American Naturalist 174:56–67.

Jeppesen, E., M. Meerhoff, K. Holmgren, I. González-Bergonzoni, F. T. Mello, S. A. J. Declerck, L. Meester, M. Søndergaard, T. L. Lauridsen, R. Bjerring, J. M. Conde-Porcuna, N. Mazzeo, C. Iglesias, M. Reizenstein, H. J. Malmquist, Z. Liu, D. Balayla, and X. Lazzaro. 2010. Impacts of climate warming on lake fish community structure and potential effects on ecosystem function. Hydrobiologia 646:73–90.

Johansson, M. P., F. Ermold, B. K. Kristjánsson, and A. Laurila. 2016. Divergence of gastropod life history in contrasting thermal environments in a geothermal lake. Journal of Evolutionary Biology, in press.

Karl, I., and K. Fischer. 2008. Why get big in the cold? Towards a solution to a life-history puzzle. Oecologia 155:215–225.

Katzenberger, M. 2015. Impact of Global Warming in Holarctic and Neotropical communities of amphibians. PhD thesis, University of Seville, Seville.

Kingsolver, J. G., and H. A. Woods. 1997. Thermal sensitivity of growth and feeding in *Manduca sexta* caterpillars. Physiological Zoology 70:631–638.

Lee, K. P., T. Jang, N. Ravzanaadii, and M. S. Rho. 2015. Macronutrient balance modulates the temperature-size rule in an ectotherm. The American Naturalist 186:212–222.

Lemoine, N. P., and D. E. Burkepile. 2012. Temperature-induced mismatches between consumption and metabolism reduce consumer fitness. Ecology 93:2483–2489.

Lemoine, N. P., W. A. Drews, D. E. Burkepile, and J. D. Parker. 2013. Increased temperature alters feeding behavior of a generalist herbivore. Oikos 122:1669–1678.

Lowe, W. H., G. E. Likens, and B. J. Cosentino. 2006. Self-organisation in streams: The relationship between movement behaviour and body condition in a headwater salamander. Freshwater Biology 51:2052–2062.

Malzahn, A. M., D. Doerfler, and M. Boersma. 2016. Junk food gets healthier when it' s warm. Limnology and Oceanography, *in press.*

Miller, G. A., F. J. Clissold, D. Mayntz, and S. J. Simpson. 2009. Speed over efficiency: locusts select body temperatures that favour growth rate over efficient nutrient utilization. Proceedings of the Royal Society B: Biological Sciences 276:3581–3589.

Myrick, C. A., and J. J. Cech. 2000. Temperature influences on California rainbow trout physiological performance. Fish Physiology and Biochemistry 22:245–254.

Nunes, A. L., A. Richter-Boix, A. Laurila, and R. Rebelo. 2013. Do anuran larvae respond behaviourally to chemical cues from an invasive crayfish predator? A community-wide study. Oecologia 171:115–127.

Pennings, S. C., C. K. Ho, C. S. Salgado, K. Wieski, N. Davé, A. E. Kunza, and E. L.

Wason. 2009. Latitudinal variation in herbivore pressure in Atlantic Coast salt marshes. Ecology 90:183–195.

Rodríguez, C. F., E. Bécares, and M. Fernández-Aláez. 2003. Shift from clear to turbid phase in Lake Chozas (NW Spain) due to the introduction of American red swamp crayfish (*Procambarus clarkii*). Hydrobiologia:421–426.

Schemske, D. W., G. G. Mittelbach, H. V Cornell, J. M. Sobel, and K. Roy. 2009. Is there a latitudinal gradient in the importance of biotic interactions? Annual Review of Ecology Evolution and Systematics 40:245–269.

Seifert, L. I., F. De Castro, A. Marquart, U. Gaedke, G. Weithoff, and M. Vos. 2014. Heated relations: temperature-mediated shifts in consumption across trophic levels. PLoS ONE 9:e95046.

Sotka, E. E., and H. Giddens. 2009. Seawater temperature alters feeding discrimination by cold-temperate but not subtropical individuals of an ectothermic herbivore. Biological Bulletin 216:75–84.

Thompson, R. M., M. Hemberg, B. M. Starzomski and J. B. Shurin. 2007. Trophic levels and trophic tangles: the prevalence of omnivory in real food webs. Ecology 88:612–617.

Zhang, P., B. A. Blonk, R. F. van den Berg, and E. S. Bakker. 2016. The effect of temperature on herbivory by the omnivorous ectotherm snail *Lymnaea stagnalis*. Hydrobiologia, *in press*.