

Stable isotope analyses reveal predation on amphibians by a globally invasive fish (Gambusia holbrooki)

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ABSTRACT

1. Biodiversity loss caused by invasive species is particularly problematic in freshwater ecosystems, which are among the world's most threatened habitats. Invasive fish such as the eastern mosquitofish, *Gambusia holbrooki*, have been implicated in the decline of amphibians, which suffer high extinction rates globally. Although *G. holbrooki* is one of the most studied freshwater fish, its diet shows wide geographic variation and its impact on amphibian populations remains unclear.

2. Stable isotopes ¹³C and ¹⁵N were used in 10 urban ponds in Sydney, Australia to compare the diet of *G. holbrooki* in January, April and May 2013 using a stable isotope mixing model.

3. *Gambusia holbrooki* was carnivorous and fed on invertebrates (24–39%), tadpoles (25–32%) and conspecifics (20–45%). In contrast to previous studies, primary producers were a negligible part of *Gambusia holbrooki* diet (<10%). Its diet in late autumn comprised a high proportion of conspecifics (up to 45%) owing to the depletion of other food sources before winter (metamorphosis of larvae).

4. This study provides evidence of high rates of predation on native tadpoles and invertebrates by a highly invasive fish. This knowledge should be incorporated into amphibian releases through head-starting tadpoles or using soft releases where tadpoles are placed in predator-free enclosures until larvae are large enough to avoid predation. Considering the dire conservation status of amphibians globally and the growing interest for invertebrates, it is suggested that stable isotopes are valuable to identify threats from predation in order to target conservation practice toward suitable priorities.

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INTRODUCTION

Invasive species are a leading cause of biodiversity loss and pose serious threats to natural ecosystems (Clavero and García-Berthou, 2005). Adverse

impacts can be caused through competition (Finlay and Vredenburg, 2007), predation (Dickman, 1996), introduction of disease, or through structural habitat change (e.g. weeds). Invasive species are particularly problematic in fresh waters (García-Berthou, 1999;

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Vander Zanden *et al.*, 1999), which are home to a disproportionately high biodiversity and among the most threatened ecosystems globally. The high value provided by freshwater ecosystem services (Dudgeon *et al.*, 2006) and the reliance of humans on healthy systems (Foley *et al.*, 2005) make them a priority in research and conservation (Dudgeon *et al.*, 2006). In fresh waters, fish are among the most commonly introduced species (Copp *et al.*, 2005) because of their close relationship with human activities (fishing, pest control, aquaculture, etc.). An important challenge in conservation biology, therefore, is to estimate the impact of invasive fish in freshwater ecosystems and to mitigate the associated damage.

The eastern mosquitofish (*Gambusia holbrooki*, Girard, 1859) is native from North America and is possibly the most widespread invasive freshwater fish in the world. It has been introduced to more than 60 countries (García-Berthou *et al.*, 2005) as a mosquito control agent (Krumholz, 1948) but often rapidly attains high abundances and preys on more than just mosquito larvae (Miura *et al.*, 1979; Cabral *et al.*, 1998; García-Berthou, 1999). The diet of *G. holbrooki* includes a wide diversity of prey items (reviewed in Pyke, 2005) ranging from small components: algae, plants, detritus, zooplankton, to larger organisms: macroinvertebrates, conspecifics, amphibian eggs and tadpoles (Webb and Joss, 1997; Komak and Crossland, 2000; Babbitt *et al.*, 2003; Blanco *et al.*, 2004; Stanback, 2010; Gkenas *et al.*, 2012; Erguden, 2013). Its high abundances and voracious predation may contribute, together with climate change and emerging infectious diseases, to the global amphibian decline (Webb and Joss, 1997), which has seen more than 30% of amphibians threatened with extinction (Houlahan *et al.*, 2000; Hussain and Pandit, 2012).

Although *G. holbrooki* is probably one of the most studied freshwater fish, its diet (based on gut contents or faecal samples) shows wide variation among locations and studies (Pyke, 2005). Amphibian consumption by *G. holbrooki* is surprisingly absent in previous work despite laboratory experiments and field surveys demonstrating predation on amphibian eggs and tadpoles by *G. holbrooki* (Komak and Crossland, 2000; Babbitt *et al.*, 2003; Stanback, 2010; Mahony *et al.*, 2013). This might occur because tadpoles and eggs of amphibians

disintegrate rapidly in the guts of fish when ingested making them difficult to identify (Reynolds, 1995) cited in (Pyke, 2005). Furthermore, dietary studies are limited by gut content analysis because ingested materials are not always assimilated, different dietary contents are digested at different rates and they represent only a snapshot of the diet at the moment of collection (Rudnick and Resh, 2005). Stable isotopes of carbon ($\delta^{13}\text{C}$, $^{13}\text{C}:^{12}\text{C}$) and nitrogen ($\delta^{15}\text{N}$, $^{15}\text{N}:^{14}\text{N}$) are an effective alternative for understanding energy flows and trophic positions of organisms in food webs (Post, 2002; Fry, 2006) and are able to reveal seasonal variations in the diet of organisms (Gillespie, 2013). Isotopic ratios reflect long-term predation and dietary patterns rather than snapshot studies or controlled condition experiments, which may not be the true representation of field conditions. Tracking of invasive fish and their effects in aquatic food chains using stable isotope analysis has been studied since 1999 (Vander Zanden *et al.*, 1999). Further applications will improve the understanding of the effect of invasive fish on biodiversity (Bodey *et al.*, 2011; Cucherousset *et al.*, 2012).

The objective of the present study was to use stable isotope analysis of ^{13}C and ^{15}N to reveal dietary patterns of *G. holbrooki* in invaded urban ponds comprising native flora and fauna. Particular attention was given to the impact of *G. holbrooki* on an amphibian community including the threatened green and golden bell frog (*Litoria aurea*). *Gambusia holbrooki* does not usually exhibit prey preferences (Pyke and White, 2000; Pyke, 2005). Its diet is also known to change in late autumn toward consuming primary producers because of the depletion of other food sources (Morton *et al.*, 1988; García-Berthou, 1999; Pyke, 2005). Hence, the aims were to (1) determine the isotopic signatures of *G. holbrooki* and its potential food sources, and (2) assess whether the diet of *G. holbrooki* shows a shift toward primary producers in autumn. These findings were used to estimate (3) the proportion of native amphibian larvae consumed by this invasive fish and the conservation strategies that may improve amphibian demographic dynamics in wetlands.

MATERIAL AND METHODS

Study site

Sydney Olympic Park (SOP), Australia, is a 320 ha park on remediated and fabricated landscapes located 14 km west of the Sydney central business district. In its present state, Sydney Olympic Park consists of 111 ponds varying in size. A large restoration strategy was developed in 1995 (Darcovich and O'Meara, 2008), which included the protection and enhancement of habitat for one of the last remaining populations of the endangered green and golden bell frog (*Litoria aurea*). Half of SOP is now dedicated to parklands with habitat management for this species. Sampling occurred in Narawang Wetland, a 1.6 km corridor of 22 artificial, permanent, freshwater ponds (mean pond area = $716 \pm 96 \text{ m}^2$) and three large water storage ponds. Each pond is attached to an irrigated water reticulation system, allowing managers to manipulate water levels and dry periods. The Sydney Olympic Park Authority has drained a subset of ponds annually since 2003 to remove invasive fish (*Gambusia holbrooki*) for the benefit of *L. aurea*. Ponds are drained in August/September and kept dry for up to a month at which time water is pumped back into the ponds

(O'Meara and Darcovich, 2008). However, *G. holbrooki* rapidly recolonize ponds after heavy rain causing flooding, because ponds are very close together in this human modified environment. Ten ponds were sampled for invertebrates, fish, tadpoles, macrophytes and algae (Figure 1).

Sampling aquatic animals

In January, April and May 2013, 10 mesh fish traps (23×23×43 cm) with inverted funnels at either end were randomly positioned half-submerged in each pond. A 13 cm yellow glow-stick (Glowstix, Glendenning, Australia) was positioned inside each trap as an attractant. Traps were deployed between 15:00 and 21:00 h and retrieved the following day between 08:00 and 12:00 h. *Gambusia holbrooki*, invertebrates and tadpoles were collected from traps. Dip nets (triangular 30×30×30 cm, 1 mm mesh size) were also used to sample additional fauna, when abundance in traps was low. Tadpoles were identified to species (Anstis, 2007) and tail clips were taken using metallurgical scissors. Care was taken to collect the minimum of tissue in the shortest possible time to minimize harm to tadpoles. Tail clips were stored in individual Eppendorf tubes before being

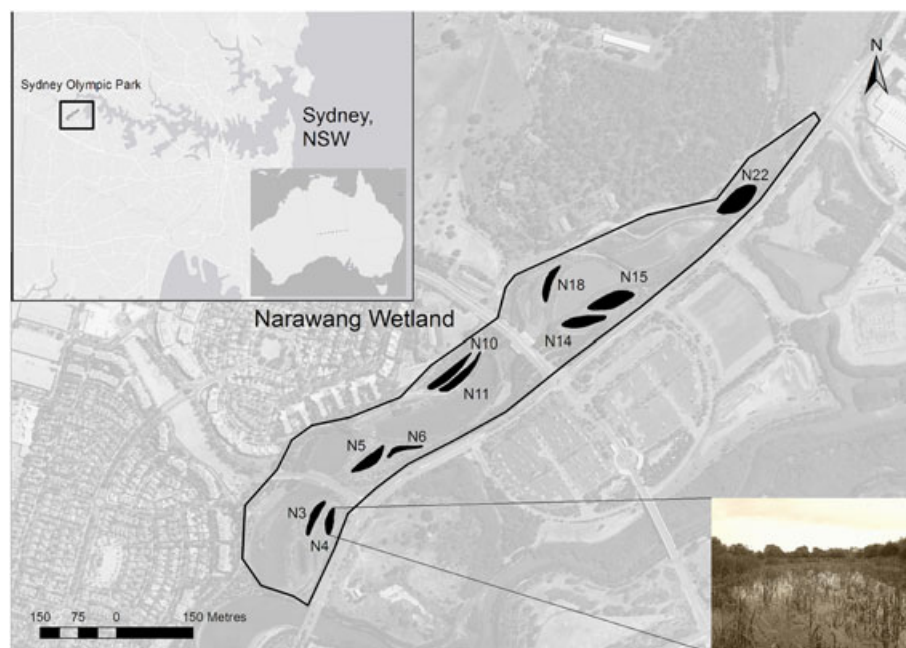


Figure 1. Narawang Wetland study site in Sydney Olympic Park (Australia). Ten ponds were sampled in January, April and May 2013.

frozen at -18°C . *Gambusia holbrooki* and invertebrates were collected, counted and frozen at -18°C . Invertebrates were identified to family level (Gooderham and Tsyrlin, 2002) in a constant temperature room in the Biological Science Department at the University of Newcastle, Callaghan. Three random adult *G. holbrooki* were selected from each pond. They were weighed and measured but the analyses did not discern fish sizes because small and large *Gambusia* sp. are likely to have similar impacts on amphibian larvae (Drake *et al.*, 2014). Fish muscle tissue was extracted using scissors and scalpel. Invertebrates from the orders Hemiptera, Odonata and Coleoptera, which were represented by three or more individuals in each pond, were retained. Three dragonfly larvae (order Odonata, family Telephlebiidae, Megapodagrionidae and Austopetaliidae), three adult beetles (order Coleoptera, family Dytiscidae and Hydrophilidae), and three true bug larvae (order Hemiptera, family Corixidae and Notonectidae) were randomly chosen and washed with distilled water. Trichoptera and the classes Arachnida and Gastropoda were sampled in low abundance and therefore excluded.

Sampling macrophytes and algae

Plants (Pyke, 2005) and algae (Blanco *et al.*, 2004) can be important food sources for *G. holbrooki*. Therefore, three random samples of each of the most abundant macrophyte species (*Baumea articulata* and *Triglochin procerum*) were collected in each pond in April and frozen at -18°C . In May, two samples of algae were taken at random in each pond at 50 cm from the edge and frozen at -18°C . Macrophyte and algal samples were cleaned to eliminate biofilms and other dead organisms and then washed using distilled water.

Stable isotope preparation

All samples (tadpole tails, fish muscle tissue, whole invertebrates, algae and macrophytes) were dried in an oven (Thermoline Scientific, Sydney, Australia) at 60°C for 24–48 h. Dried material was ground into a homogeneous fine powder using a mortar and pestle and then weighed to 10^{-5}g using a Shimadzu AUW220D precision balance (Shimadzu Corporation, Kyoto, Japan). 6–8 mg of primary

producer material (macrophytes and algae) and 1–2 mg of animal tissues were weighed into $8 \times 5\text{ mm}$ tin capsules (Sercon Ltd - UK). Samples were analysed at the Isotope Analytical Facility of Griffith University (Australia). Natural abundance of ^{15}N and ^{13}C was measured using an automated Isoprime Isotope-Ratio Mass Spectrometer (precision $\pm 0.2\%$). Values are presented as differences between sample isotope ratio and international laboratory standards (^{13}C : Vienna Pee Dee Belemnite; ^{15}N : atmospheric N_2). Results refer to delta (δ) in units of per mil (‰) using the following equation: $\delta^{\text{H}}X = [R_{\text{sample}}/R_{\text{standard}} - 1] \times 1000$ with $R = {}^{\text{H}}X/{}^{\text{L}}X$. Where X refers to the elements C or N, and H and L indicate heavy and light isotope components. (δ) values reflect energy transfers in food chains and allow the identification of food sources by the consumer, as well as organism trophic level.

Data analysis

The contribution of different food sources was estimated for *G. holbrooki* using the Bayesian stable isotope mixing model, Stable Isotope Analysis in R (SIAR) (Parnell *et al.*, 2010). The model incorporated the variation of stable isotope values from consumers (*G. holbrooki* in this case) and its potential food sources as well as variation in the isotopic discriminant factor known as the trophic enrichment factor (TEF), representing the difference in isotopic composition between a consumer and its diet. Information incorporated in the SIAR model were the consumer isotopic data ($\delta^{13}\text{C}$ and $\delta^{15}\text{N}$), means and standard deviations of food source isotopic data ($\delta^{13}\text{C}$ and $\delta^{15}\text{N}$), and means and standard deviations of TEF ($\Delta^{13}\text{C}$ and $\Delta^{15}\text{C}$; see SIAR package for more details). Three groups were analysed for the consumer data (*G. holbrooki*), based on the month of sampling: January, April and May. Five main food sources were considered for *G. holbrooki*: tadpole, invertebrate, macrophyte, algae and *G. holbrooki*. Within each food source, species (or order for invertebrates) were grouped if their isotopic signatures were similar. When signatures differed significantly, they were considered as a specific food source. Accordingly, tadpole species were grouped and considered as one food source because of similar isotopic signatures among tadpole

species (see Results and Figure 2). Two food sources were considered for invertebrates: Coleoptera and Odonata+Hemiptera because Odonata and Hemiptera had similar isotopic signatures (see Results and Figure 2). *Baumea articulata* and *T. procerum* were considered as two distinct food sources because of different isotopic signatures (see results and Figure 2). The other food sources considered were algae and *G. holbrooki* (all data grouped). Successful determination of an animal diet depends on an accurate estimation of TEF (Hussey *et al.*, 2014). Trophic enrichment factors for prey versus consumers are usually low for carbon ($0.75 \pm 0.11\text{‰}$) and higher for nitrogen ($2.75 \pm 0.10\text{‰}$) (Caut *et al.*, 2009). In the present study, different TEF values were considered depending on whether the prey was an animal or a primary producer, as suggested by McCutchan *et al.* (2003). TEF incorporated in the model for $\Delta^{13}\text{C}$ was $1.3 \pm 0.30\text{‰}$ for each food source. For $\Delta^{15}\text{N}$, TEF values of $1.4 \pm 0.20\text{‰}$, $3.3 \pm 0.26\text{‰}$ or $2.2 \pm 0.30\text{‰}$ were used depending on whether the food sources were invertebrates, vertebrates (fish and tadpole) or primary producers, respectively (McCutchan *et al.*, 2003). All models were performed with 500 000 iterations and the first 50 000 iterations were discarded.

General Linear Mixed Models (GLMMs) were used to compare stable isotope values ($\delta^{13}\text{C}$ and $\delta^{15}\text{N}$) of *G. holbrooki* among months. 'Month' was defined as a categorical fixed factor and 'pond' as a random categorical factor. In order to determine the minimum number of food sources to improve

the estimation of *G. holbrooki* diet in the SIAR mixing model, $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values of tadpole species (GLMM), invertebrate orders (GLMM) and macrophyte species (t-tests) were compared. Tadpole species and invertebrate orders were the fixed categorical factors, respectively, and month of sampling was included as a random categorical factor in both GLMM analyses. Macrophyte species differences in $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ were compared using a t-test and a Welch two sample t-test (because variance was unequal), respectively. Software developed by R Development Core Team v3.0 (2013) was used to run all the statistical analysis. All data were tested for normality and homogeneity of variance using graphical tools, in addition to Shapiro tests. For all GLMMs, best models were selected based on the AIC criterion indices. F-values were calculated using Satterthwaite approximation for degree of freedom. Post hoc analyses were completed using the package 'lsmeans'. Results were considered significant when $P < 0.05$. Results are given with standard errors except for food source percentages in *G. holbrooki* diet (SIAR outputs), which are given with standard deviations because calculation of standard errors is not relevant to iterations.

RESULTS

Isotopic signatures of food sources and consumers

The two species of macrophytes had distinct isotopic signatures (Figure 2, Table 1). *Baumea*

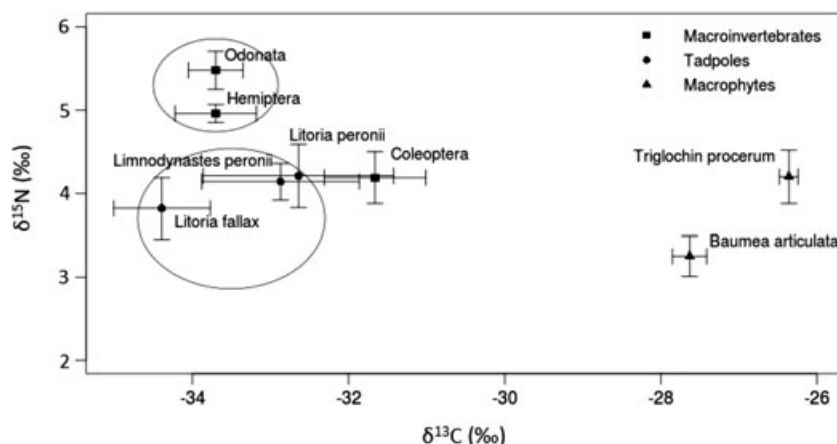


Figure 2. Stable isotope values ($\delta^{13}\text{C}$ and $\delta^{15}\text{N}$, mean \pm se) for macroinvertebrate orders, tadpoles and macrophyte species. The circles represent organisms that are pooled together in the analysis because of similar isotope signatures as determined by GLMM ($P > 0.05$).

Table 1. Mean \pm SE isotopic signatures of the taxa studied in ponds in Sydney Olympic Park. Organisms sampled were algae, macrophytes (*Baumea articulata* and *Triglochin procerum*), tadpoles (*Litoria fallax*, *Limnodynastes peronii* and *Litoria peronii*) and the invasive fish, *Gambusia holbrooki*. Higher level of $\delta^{15}\text{N}$ represents organisms higher in the food chain. Sample size is represented by 'n'

Species	$\Delta^{15}\text{N}$ (‰)	$\Delta^{13}\text{C}$ (‰)	n
Algae	2.2 \pm 0.3	-28.7 \pm 0.2	16
<i>Baumea articulata</i>	3.2 \pm 0.2	-27.6 \pm 0.2	30
<i>Triglochin procerum</i>	4.2 \pm 0.3	-26.4 \pm 0.1	30
Coleoptera sp.	4.2 \pm 0.3	-31.7 \pm 0.6	18
Hemiptera sp.	5.0 \pm 0.1	-33.7 \pm 0.5	21
Odonata sp.	5.5 \pm 0.2	-33.7 \pm 0.4	23
<i>Litoria fallax</i>	3.8 \pm 0.4	-34.4 \pm 0.6	17
<i>Limnodynastes peronii</i>	4.1 \pm 0.2	-32.9 \pm 1.0	9
<i>Litoria peronii</i>	4.2 \pm 0.4	-32.6 \pm 1.2	8
<i>Gambusia holbrooki</i>	8.3 \pm 0.2	-32.2 \pm 0.2	58

articulata had lower $\delta^{15}\text{N}$ ($t_{58} = 2.36$, $P = 0.022$) and $\delta^{13}\text{C}$ ($t_{44.5} = 5.01$, $P < 0.0001$) than *T. procerum*.

The $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ isotopic signature for invertebrates were significantly affected by the taxa of invertebrate ($\delta^{15}\text{N}$: $F_{2,55.2} = 10.57$, $P = 0.0001$; $\delta^{13}\text{C}$: $F_{2,54.1} = 5.94$, $P = 0.005$). More precisely, Coleoptera had significantly lower $\delta^{15}\text{N}$ than Hemiptera ($P = 0.032$) and Odonata ($P < 0.001$), but there were no significant differences in $\delta^{15}\text{N}$ signature between Hemiptera and Odonata ($P = 0.21$). Similarly, Coleoptera had significantly higher $\delta^{13}\text{C}$ than Hemiptera ($P = 0.032$) and Odonata ($P = 0.008$). Hemiptera and Odonata were not significantly different in $\delta^{13}\text{C}$ ($P = 0.90$). Therefore, Hemiptera and Odonata were considered as a single source in the SIAR model (Figure 2).

Tadpoles were detected in each pond except pond N11. Three species of tadpoles were sampled: *Litoria fallax* (n = 17), *Limnodynastes peronii* (n = 9) and *Litoria peronii* (n = 8). Tadpoles had significantly different $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ among species ($\delta^{15}\text{N}$: $F_{2,27.0} = 5.08$, $P = 0.013$; $\delta^{13}\text{C}$: $F_{2,15.7} = 8.99$, $P = 0.002$). However, post hoc tests showed that $\delta^{13}\text{C}$ was not significantly different among *L. fallax*, *Limn. peronii* and *L. peronii* (all $P > 0.05$). Similarly, $\delta^{15}\text{N}$ was similar among species (post hoc tests, all $P > 0.05$). Therefore, all amphibian species were considered as a single food source for the diet of *G. holbrooki* in the SIAR model (Figure 2).

Mean algal values of $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ were 2.2 \pm 0.3‰ and -28.7 \pm 0.2‰, respectively (Table 1). Algae were therefore the organisms with the lowest trophic position sampled (Figure 3).

Gambusia holbrooki were detected in seven ponds (N3, N4, N5, N6, N10, N11 and N18) among the 10 sampled. Mean size of fish was 35.5 \pm 0.6 mm and weight was 506 \pm 31 mg (n = 58). *Gambusia holbrooki* were high in the food chain in Narawang Wetland (Figure 3 and Table 1). The $\delta^{15}\text{N}$ value of *Gambusia holbrooki* was significantly different among months ($F_{2,53.2} = 32.43$, $P < 0.0001$, Table 2). Post hoc tests revealed that *G. holbrooki* had significantly higher $\delta^{15}\text{N}$ in May than in January ($P < 0.0001$) and April ($P < 0.0001$). In January and April, *G. holbrooki* did not differ significantly in $\delta^{15}\text{N}$ ($P = 0.85$). $\delta^{13}\text{C}$ of *G. holbrooki* was not significantly different among months ($P = 0.20$, Table 2).

Gambusia holbrooki diet

The most important dietary components of *G. holbrooki* in all time periods were tadpoles, invertebrates and conspecifics. Tadpoles comprised ~30% of the fish diet with little variation among months (Figure 3 and Table 3). The consumption of Hemiptera and Odonata was higher in April than in January but decreased in May. Coleoptera were much less consumed than the other invertebrates, comprising ~10% of the fish diet in January, decreasing throughout sampling times and representing less than 5% of the fish diet in May. Pooled together, the consumption of invertebrates varied from about 24% (May) to 39% (April) in the fish diet. By the end of autumn, *G. holbrooki* consumed fewer tadpoles and invertebrates but consumed a much larger amount of conspecifics. Forty-five per cent of its diet consisted of other *G. holbrooki* in May, which represented about twice its consumption in January (24%) and April (20%). Primary producers were a negligible part of the diet of *G. holbrooki* with macrophytes and algae representing ~11% of the diet of the fish in January. This percentage decreased in April and in May to 6% (Figure 3 and Table 3).

DISCUSSION

Results reveal three findings: (1) *Gambusia holbrooki* are likely to have a large impact on the amphibian community because ~30% of fish diet was consistent

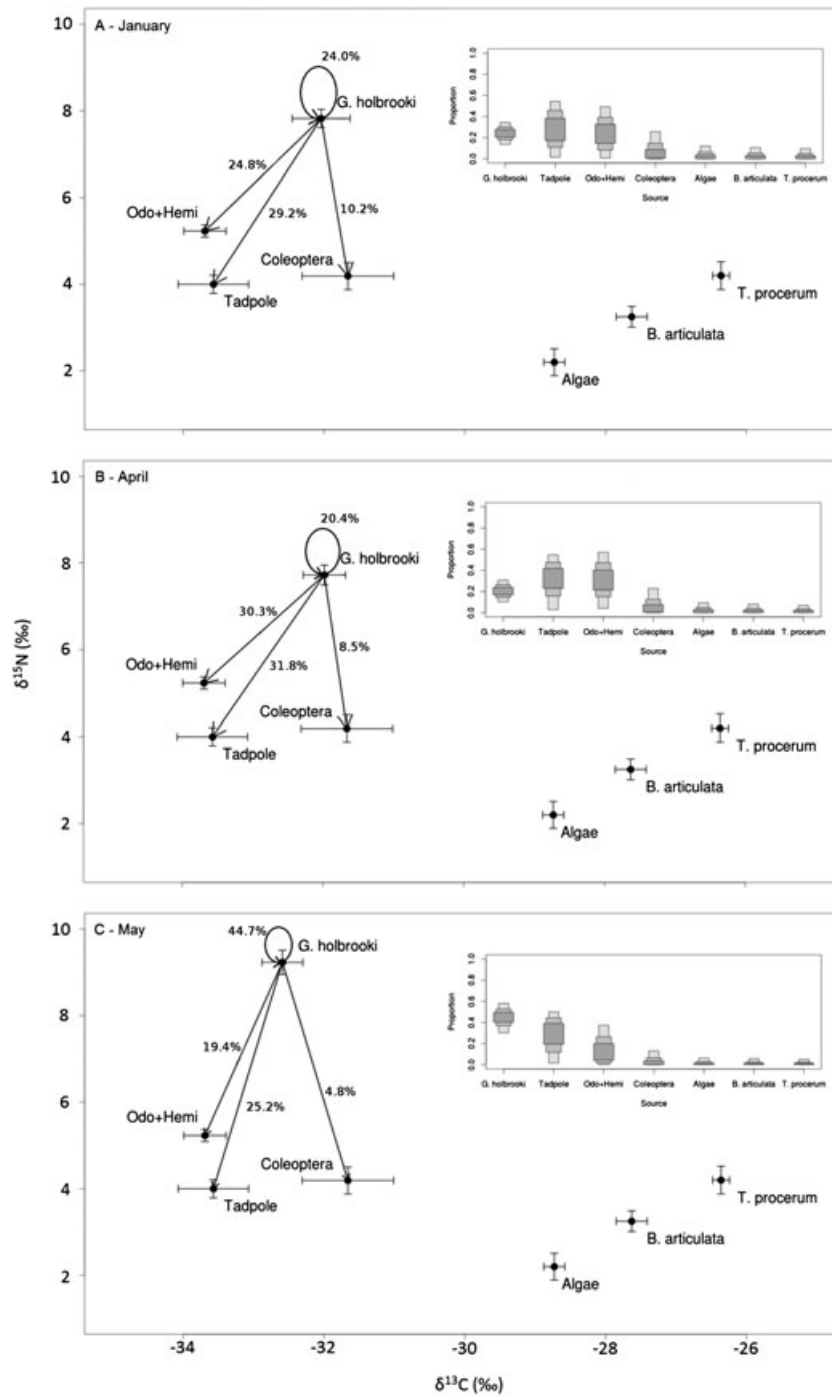


Figure 3. Stable isotope values ($\delta^{13}\text{C}$ and $\delta^{15}\text{N}$, mean \pm se) of the organisms analysed in Narawang Wetland (Sydney Olympic Park, Australia) during (a) January, (b) April and (c) May. Primary producers in this food chain are algae and macrophytes (*Baumea articulata* and *Triglochin procerum*). Macroinvertebrates are Coleoptera, Odo+Hemi (Odonata and Hemiptera, respectively), which are pooled together because of similar isotope signatures). 'Tadpole' includes data from three amphibian species that have similar trophic signatures (*Litoria fallax*, *Limnodynastes peronii*, and *Litoria peronii*). Proportion of prey consumed by *Gambusia holbrooki* is represented as the percentage of *G. holbrooki* diet in each time period (January, April and May) based on results from SIAR mixing models (boxplots with 95, 75 and 25% probability intervals at the top right of figures). Percentages are represented only for tadpoles, macroinvertebrates and conspecifics because primary producers (algae, *B. articulata* and *T. procerum*) are poorly represented in the diet of fish.

Table 2. Mean \pm SE isotopic signatures of the invasive mosquitofish, *Gambusia holbrooki* in January, April and May 2013 in ponds in Sydney Olympic Park. Sample size is represented by 'n'

Month	$\Delta^{15}\text{N}$ (‰)	$\Delta^{13}\text{C}$ (‰)	n
January	7.8 \pm 0.2	-32.0 \pm 0.4	16
April	7.7 \pm 0.2	-32.0 \pm 0.3	21
May	9.2 \pm 0.3	-32.6 \pm 0.3	21

with the isotopic signature of tadpoles; (2) *Gambusia holbrooki* were primarily carnivorous in urban ponds at Sydney Olympic Park and; (3) fish diet varied seasonally with a shift in late autumn towards more cannibalism.

The percentage of amphibians consumed by *G. holbrooki* was high at Sydney Olympic Park suggesting that the impact of predation may contribute to the challenges faced by remaining amphibian populations. Tadpoles were one of the principal food sources for the introduced fish and comprised ~30% of the total taxa consumed consistently from summer to late autumn (25 to 32% depending on the month of sampling; Table 3). These results are of particular interest when considering the relative absence of amphibian larvae consumption reported in the wild by *G. holbrooki* (Pyke, 2005). To our knowledge, there is unfortunately no comparison available in the literature concerning the percentage of amphibian larvae eaten by invasive fish using a similar method. The results presented here contrast with a previous study showing that *G. holbrooki* is a weak per capita predator on *Hyla cinerea* (Gunzburger and Travis, 2004), but support the conclusions of Stanback (2010) on the high predation impact of *G. holbrooki* on anuran larvae. The data were unable to determine whether the percentage of tadpoles eaten by *G. holbrooki*

reflects the availability of tadpoles in ponds or represents a preference for tadpole consumption by the fish (that is, targeting tadpoles is a priority even if other food sources are present). Goodsell and Kats (1999) found that even when mosquito larvae are present, *G. holbrooki* prey on amphibian larvae to the same extent as when mosquito larvae are absent. This suggests that *G. holbrooki* follow, to some extent, the optimal foraging model (Werner and Hall, 1974; Kumar *et al.*, 2015) which predicts that predators choose prey that minimize the energy used in foraging and optimize energy intake. Combining a stable isotope study with an estimation of abundance of each prey item in ponds would help to address this question.

Although *Gambusia holbrooki* may prefer amphibian larvae instead of other food sources, the introduced fish do not usually show preferences when provided with different tadpole species (Pyke and White, 2000; Pyke, 2005), suggesting that all tadpole species may be exposed to similar predation pressure from *G. holbrooki*. The high abundance that *G. holbrooki* reaches in ponds, the conservation status of native amphibians (Hussain and Pandit, 2012) and the presence of one of the last remaining populations of *L. aurea* (Pyke and White, 2001) in Sydney Olympic Park (Darcovich and O'Meara, 2008), emphasizes the catastrophic impact that *G. holbrooki* can have on native amphibians. A recent field study in urban ponds in southern Australia drew similar conclusions (Hamer and Parris, 2011), showing a positive relationship between the presence of *G. holbrooki* and the low abundance of amphibian larvae in ponds. Furthermore, the present study neglected the possibility of egg consumption by *G. holbrooki*, which may form an

Table 3. Diet of the mosquitofish, *Gambusia holbrooki* at three time periods from summer to late autumn in urban ponds (Australia). Food sources are presented as percentage of the diet with mean and standard deviation (SD)

	January		April		May	
	Mean	SD	mean	SD	mean	SD
Algae	4.4	3.8	3.5	2.9	2.2	2.1
<i>Baumea articulata</i>	3.9	3.1	3.0	2.6	2.0	1.9
<i>Triglochin procerum</i>	3.4	3.5	2.5	2.2	1.8	1.7
Coleoptera sp.	10.2	8.1	8.5	6.7	4.8	4.5
Odonata sp. + Hemiptera sp.	24.8	13.0	30.3	13.7	19.4	11.7
Tadpole sp.	29.2	14.5	31.8	13.6	25.2	13.3
<i>Gambusia holbrooki</i>	24.0	5.4	20.4	5.2	44.7	6.9

important additional component of *G. holbrooki* diet (Komak and Crossland, 2000). Thus, the impact on amphibians by this invasive fish may be underestimated.

Gambusia holbrooki is high in the food chain at Sydney Olympic Park compared with other organisms sampled (Figure 3). Depending on location and type of habitat (pond, river), the diet of *Gambusia holbrooki* may vary significantly. For example, isotopic signatures of *G. holbrooki* were close to those measured by Abbey-Lee *et al.* (2013) in freshwater sites in Florida ($\delta^{15}\text{N}$ and -28.59 to -28.89 ‰ for $\delta^{13}\text{C}$) but differed considerably from those measured in the Hutt River of south-western Australia ($\delta^{15}\text{N}$: ~ 15 ‰ and $\delta^{13}\text{C}$: ~ -24 ‰; Beatty, 2006). The diet of *Gambusia holbrooki* in other studies using gut samples and stable isotopes has been represented by up to 80% of primary producers (Blanco *et al.*, 2004; Specziár, 2004, Capps *et al.*, 2009). Yet, in our study, algae and macrophytes (*B. articulata* and *T. procerum*) were a negligible part of adult *G. holbrooki* diet in Sydney Olympic Park (6% to 11% depending on the month). The primary producers in Narawang Wetland are too enriched in ^{13}C in comparison with *G. holbrooki* and too low in the food chain to represent a significant food source for the introduced fish (Figure 3, Table 3). Invertebrates, tadpoles and conspecifics were, however, consumed in high proportions, even in late autumn, when an increase in the consumption of primary producer by *G. holbrooki* was expected because of the depletion of other food sources (metamorphosis of tadpoles and invertebrate larvae before winter). This suggests that *G. holbrooki* has a strict carnivorous diet in Sydney Olympic Park which corresponds with previous studies (Pen and Potter, 1991; Gkenas *et al.*, 2012; Erguden, 2013). This supports the finding that fish may prefer certain food sources regardless of their availability. For example, *G. holbrooki* could intentionally avoid the consumption of primary producers (owing to toxicity or to their low energy to mass ratio compared with other food sources). Alternatively, it is possible that *G. holbrooki* fed on primary producers but that fish metabolism is inefficient at digesting cellulose (Buddington, 1979), which composes the cell walls

of algae and macrophytes, making it hard to detect primary producer signatures in the fish tissues.

Stable isotope values of *G. holbrooki* differed seasonally. The large increase of $\delta^{15}\text{N}$ in May (Figure 3) suggests that *G. holbrooki* feeds on prey higher in the food chain in late autumn. The SIAR mixing model confirmed that the diet of *G. holbrooki* shifted in late autumn towards a high consumption of conspecifics (45%). This drastic and rapid increase (20% in April) could be explained by the high density of *G. holbrooki* observed in ponds and the low proportion of other food sources in winter (Morton *et al.*, 1988). In fact, the proportion of invertebrates eaten in May decreased to 24% (39% in April). The diminution of invertebrates in the diet of *G. holbrooki* may be directly linked to the drastic diminution of invertebrate abundance in ponds before winter. Although *G. holbrooki* may prefer animals instead of primary producers, when resources become very scarce the fish may shift its diet toward what is left: conspecifics. The high proportion of conspecifics consumed contrasts with current knowledge that *G. holbrooki* shows cannibalism patterns between 1 and 13% (Walters and Legner, 1980; Specziár, 2004). However, *Gambusia holbrooki* was the only fish species sampled for stable isotope analysis and it is also possible that gudgeons, which were present at the site, had similar isotopic signatures to *Gambusia holbrooki* and account for the ingested prey. It is interesting that the increase in the $\delta^{15}\text{N}$ signature of *G. holbrooki* observed in May contrasts with another Australian study that found exactly the reverse pattern ($\delta^{15}\text{N}$ of fish was higher in summer than in winter; Beatty, 2006). This difference could be due to the presence of crayfish in the food chain (Beatty, 2006). Crayfish prey on *G. holbrooki* and forces fish abundance lower than in the present study. Conspecific consumption (resulting in higher $\delta^{15}\text{N}$) may be lower because of less chance to encounter conspecifics in the ponds.

All analyses of stable isotope mixed models are restricted by the input of food sources. In order to obtain perfect estimation of consumer diets, all potential food sources should be sampled. In this study some other potential food sources were not sampled that may be an important part of *G.*

holbrooki diet such as zooplankton (García-Berthou, 1999; Gkenas *et al.*, 2012), Diptera, Trichoptera or detritus (Blanco *et al.*, 2004; Erguden, 2013). This may overestimate the percentage of sampled prey in the *G. holbrooki* diet. Future studies should aim to incorporate other possible food sources, especially zooplankton, using small mesh-size dip nets (<300 µm) for sampling.

In conclusion, it has been shown that *G. holbrooki* has a varied diet with some preference for animals instead of primary producers. However, when resources become scarce (in winter), *G. holbrooki* shifts to more highly cannibalistic behaviour and feeds less on invertebrates. The results provide evidence of high rates of predation on tadpoles by *G. holbrooki*, demonstrating a direct impact of this invasive species on native wildlife in an amphibian community that includes threatened amphibians (*Litoria aurea*). It highlights the usefulness of stable isotopes in identifying such threats. In amphibians, the lack of parental care (in most cases) exposes their larvae to direct environmental conditions. The introduction of non-native species in wetlands is an additional threat to amphibian larvae that suffer already from native predators, disease, competition and desiccation. These results have direct application to the management of threatened frogs in areas occupied by invasive predators by showing that predation by invasive species should be factored into conservation actions and the impact mitigated where possible. For example, the success of releases of captive-bred tadpoles can be improved by head-starting and rearing tadpoles to metamorphosis to obtain larger sizes, or additional soft releases where tadpoles are placed in baskets, free of predators. This study was centred on amphibian conservation, but native invertebrates suffer similarly from invasive fish. Removal of invasive fish by draining ponds (O'Meara and Darcovich, 2008) or excluding recolonization by invasive fish is likely to benefit amphibians and invertebrates. Many emerging methods are now available for conservation biologists and managers at reasonable cost. Stable isotopes are one such example and will help to improve understanding of changes in food webs and the impacts of predation by invasive species on native organisms. We further encourage scientists to use this method to

reveal direct predation impact from invasive fish but also from other invasive species throughout the world.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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