

## Trophic relationships of the platypus: insights from stable isotope and cheek pouch dietary analyses

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**Abstract.** The unique Australian monotreme, the platypus (*Ornithorhynchus anatinus*) potentially exerts a strong top-down influence on riverine food webs in eastern Australia. However, despite considerable interest in the evolutionary history and physiology of the platypus, little is known of its trophic relationships. To address this lack of knowledge we used stable isotope analysis, in combination with the analysis of food items stored in cheek pouches, to determine its position in a typical riverine food web. This was the essential first step in the process of designing a larger study to investigate the relative importance of top-down and bottom-up effects in rivers where the platypus occurs. We found that platypuses were feeding on a wide range of benthic invertebrates, particularly insect larvae. The similarity of  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  values recorded for the platypus, a native fish (*Galaxias* sp.) and the exotic mosquitofish (*Gambusia holbrooki*) indicated dietary overlap and potential competition for the same resources. Although cheek pouch studies identify most of the major groups of prey organisms, the potential for contribution of the soft-bodied organisms such as larval dipterans, is suggested by stable isotope analysis, indicating that the use of both techniques will be important in future ecological investigations.

**Additional keywords:** diet,  $\delta^{13}\text{C}$ ,  $\delta^{15}\text{N}$ , macroinvertebrate, *Ornithorhynchus anatinus*.

Received 11 November 2013, accepted 28 March 2015, published online 4 September 2015

### Introduction

Fish have been long recognised as important predators in riverine food webs (Diehl 1992; Sih *et al.* 1998) and a range of effects has been described. These include: suppressing the abundance of prey species, altering the behaviours and distribution of other species and redistribution of resources within the immediate environment (Power 1992a; Peckarsky and McIntosh 1998; Power *et al.* 2008). Although the influence of fish and birds (e.g. Steinmetz *et al.* 2003) on stream food webs is well described, much less is known about the influence of other aquatic and semiaquatic vertebrate predators. Generally, there are both top-down and bottom-up processes in play, as predators can exert top-down effects on standing stocks, but prey abundances can influence the density, distribution, production and fitness of vertebrates (e.g. Ormerod *et al.* 1991). Water shrews are known to feed on lotic macroinvertebrates (Churchfield and Rychlik 2006; Churchfield *et al.* 2006). However, their influence on stream food webs is likely to be mediated by the fact that their diet also includes terrestrial invertebrates. Riverine birds that have high energy requirements (e.g. the dippers, *Cinclus* spp.), are recognised as being

quantitatively significant predators of stream invertebrates and potentially play an important, but overlooked, ecological role in riverine ecosystems (Ormerod and Tyler 1991; Harvey and Marti 1993). In Australian rivers, the platypus (*Ornithorhynchus anatinus* Shaw, 1799) is an iconic, semiaquatic vertebrate predator that potentially exerts a considerable influence on lotic food webs because of the importance of benthic invertebrates in its diet (Grant 2007). The platypus spends  $\sim 11\text{--}12$  h  $\text{day}^{-1}$  feeding almost exclusively on these organisms (Bethge *et al.* 2003). However, the ecological role of the platypus in riverine ecosystems has been largely ignored, with only a single study to date investigating its trophic interactions (McLachlan-Troup 2007).

Dietary studies of the platypus have been limited because observational studies are difficult to conduct on an animal that feeds underwater and primarily at night. Food material is not accumulated in the very small stomach (Griffiths 1978) and food items in the gut contents and faeces are largely unidentifiable. Traditionally, information on the platypus' diet has been obtained through the investigation of the contents of its cheek pouches where it stores food items while foraging (Jones 1923;

Grant 2007). These pouches are located next to keratinous grinding pads in the mouth that replace the deciduous teeth of early juveniles (Griffiths 1978). Cheek pouch investigations have suggested that platypuses feed widely and opportunistically on stream benthic macroinvertebrates (Faragher *et al.* 1979; Grant 1982; McLachlan-Troup *et al.* 2010). Non-breeding platypuses have been reported to consume food at a rate equivalent to 15–30% of their wet bodyweight per day (Krueger *et al.* 1992; Munks *et al.* 2000) and the consumption by lactating females is considerably higher (Holland and Jackson 2002). The findings of these studies suggest that platypuses could be expected to have an important direct or indirect, top-down effect on benthic food webs in rivers and streams.

Understanding the relative importance of top-down (predators) and bottom-up (resource supply) effects on food webs is an important aspect of community ecology (e.g. Power 1992b; Nyström *et al.* 2003). We hypothesise that platypuses have a major top-down effect on riverine food webs. The first step in the investigation of this hypothesis was to accurately determine the platypus' diet. We sought to do this using both an analysis of cheek pouch contents and stable isotope analysis of their fur. Stable isotope analysis, where isotopes of carbon and nitrogen are used to study material flow in freshwater ecosystems, has proven to be a powerful dietary analysis technique for food web studies (Peterson and Fry 1987; Bunn and Boon 1993; Syväranta *et al.* 2006), particularly when combined with conventional dietary analyses. It is an ideal technique for determining the diet of cryptic species like the platypus, with fur being easy to obtain in a non-invasive manner. Analysis of the fur provides an integrated measure of the diet over time because platypus fur is gradually moulted rather than lost in a single seasonal event. The turnover time of the fur is not known, but the presence of gradual moulting suggests that it occurs over several months (Grant and Carrick 1978).

The aim of this study was to describe the diet of the platypus in more detail than previous studies, enabling progression to further experimental investigations of the ecological role of the platypus, especially any top-down effects on riverine food webs.

## Materials and methods

Platypuses were sampled from two pools on the upper Shoalhaven River, New South Wales (35°38'31"S, 149°36'49"E), at the junction of Jerrabattgulla Creek and the Shoalhaven River and ~800 m upstream on the main river on 18–19 May, 8–9 August, 18–19 December 2009 and 13 March 2010. Pool substrates consisted mainly of cobbles, separated by a single long bedrock riffle. The stream is perennial with little seasonality in flows. Tape grass (*Vallisneria spiralis*), floating pondweed (*Potamogeton tricarlinatus*), water milfoil (*Myriophyllum* sp.) stonewarts (*Chara* sp.) and benthic algae were present within the stream. A sparse riparian zone contained a mix of introduced plant species, predominantly willows (*Salix* sp.) and African love grass (*Eragrostis curvula*), and natives, predominantly tea tree (*Leptospermum obovatum*). The river banks were badly eroded, with visible evidence of disturbance by livestock and common wombats (*Vombatus ursinus*).

Platypuses were caught in unweighted mesh ('gill') nets (80-mm mesh) as described by Grant and Carrick (1974). On the

first night of each sampling period, two unweighted nets (50 × 2 m) were placed end to end in the centre of the upstream sampling pool before dusk. On the second night, two shorter nets (25 × 1.5 m and 25 × 2 m) were laid parallel to each other in the downstream sampling pool.

Nets were set 1 h before dark, checked for the following 5 h, and then removed. Captured platypuses were held in a cloth bag, processed and then released at the point of capture, once nets had been removed from the river. A small (1 cm<sup>2</sup>) sample of dorsal fur and tail fur was clipped for stable isotope analysis.

### Preparation of platypus fur

Fur samples were collected from 21 platypuses (6 male and 12 female adults and 3 juvenile females) (Table 1). The fur was prepared for stable isotope analysis using a combination of the methods previously described for marsupials (McIlwee and Johnson 1998) and brown bears (Hilderbrand *et al.* 1999) to remove any surface oils and dust. Fur samples were rinsed with warm distilled water (40°C) followed by rinsing in chloroform–methanol (2 : 1). Samples were dried for 48 h and then ground (using a mortar and pestle) while immersed in liquid nitrogen, followed by air drying for 24 h to remove liquid nitrogen residues. Grinding and air drying was repeated until a fine powder was obtained. Samples were then oven-dried at 60°C for 24 h. Fur samples from individual platypuses were analysed separately.

### Cheek pouch contents

Cheek pouch sampling was undertaken on platypuses captured every two months at the Shoalhaven River pools, between November 2008 and December 2009. A total of 62 cheek pouch samples were collected for analysis (Table 2) during this and an associated study (Marchant and Grant 2015). These were extracted from animals lightly restrained on a sponge rubber block by inserting a small long-handled stainless steel spoon into the buccal cavity via the bill (Faragher *et al.* 1979; Grant 2007). The cheek pouch contents were stored in 70% ethanol until subsequent identification. Cheek pouch contents were examined under a low magnification (<40×). Volumes or counts of specimens were not made because the material was often damaged.

### Sampling and processing of resources

Aquatic invertebrates were collected with a 250-µm-mesh long-handled net. In-stream sediments immediately upstream of the net were disturbed by kicking for a period of 1 min. In May 2009, five invertebrate samples were taken at haphazardly chosen sites in the pools sampled for platypuses and the reach between them.

Aquatic invertebrates were held in river water at a minimum of 4°C for at least 24 h to void gut contents and to ensure minimal contamination of the isotope values. All invertebrates were identified to the level of family (to ensure an adequate sample weight was obtained for stable isotope analysis) and frozen whole. Of the invertebrates collected from the kick net samples (comprising 36 families), only specimens representative of those ever seen in the cheek pouches were used for the analysis (23 families) (McLachlan-Troup *et al.* 2010; Marchant and Grant 2015). Tissue samples were removed from freshwater

**Table 1.** Stable isotope values ( $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$ ) of dorsal body fur and morphometric measurements of platypuses captured from the upper Shoalhaven River, New South Wales from May 2009 to March 2010

Individual	Season captured	Age	Sex	Weight (g)	Length (cm)	Dorsal fur $\delta^{13}\text{C}$	Dorsal fur $\delta^{15}\text{N}$
FA660	May 2009	Adult	Female	750	42.5	-22.5	7
FA676	May 2009	Adult	Female	690	42	-22.1	7.4
FA677	May 2009	Adult	Female	780	42.5	-23.4	7.2
FA679	May 2009	Adult	Female	690	42	-21.9	7.2
FJ642	May 2009	Adult	Female	840	42.5	-22.9	7.1
MA476	May 2009	Adult	Male	1000	46	-22.4	7.4
MA487	May 2009	Adult	Male	940	44	-22.3	7.8
FA662	Aug. 2009	Adult	Female	780	42	-23.0	7.2
FA675	Aug. 2009	Adult	Female	740	42.5	-22.8	6.9
FA678	Aug. 2009	Adult	Female	580	42.5	-23.0	7.4
FJ689	Aug. 2009	Juvenile	Female	580	38	-26.4	8.3
MA474	Aug. 2009	Adult	Male	1250	48	-21.9	7
MA476 <sup>A</sup>	Aug. 2009	Adult	Male	910	46	-22.6	7.9
MA498	Aug. 2009	Adult	Male	1290	n.a.	-22.7	7.1
FA677 <sup>A</sup>	Dec. 2009	Adult	Female	970	42.5	-23.2	6.6
FA678 <sup>A</sup>	Dec. 2009	Adult	Female	840	42.5	-22.1	6.7
FA679	Dec. 2009	Adult	Female	760	42	-22.1	6.7
FA692	Dec. 2009	Adult	Female	800	41	-21.9	7.2
MA476 <sup>A</sup>	Dec. 2009	Adult	Male	1060	n.a.	-22.6	6.5
FA693	Mar. 2010	Adult	Female	830	40.5	-21.1	6.2
FJ694	Mar. 2010	Juvenile	Female	500	36	-21.9	7.2

<sup>A</sup>Recaptured individual. To avoid bias, samples from recaptured individuals were not used for analysis of stable isotope data.

**Table 2.** The percentage of platypus individuals from the upper Shoalhaven River, New South Wales, Australia with cheek pouches containing each family and order of macroinvertebrates

Frequency of occurrence was calculated as the percentage of platypuses, out of the total of 62 sampled, in which a particular taxon was found. Percentage dominance of each order, their ranked percentage (out of 100%) contribution to the platypus diet, is given in parentheses

Order	% occurrence of order	Family	% occurrence of family
Ephemeroptera	61 (16)	Leptophlebiidae	55
		Baetidae	5
		Caenidae	6
Trichoptera	74 (32)	Leptoceridae	56
		Ecnomidae	34
		Odontoceridae	10
		Hydroptilidae	11
Diptera	42 (10)	Chironomidae	37
		Ceratopogonidae	7
		Tipulidae	3
Odonata	60 (26)	Gomphidae	15
		other Anisoptera <sup>A</sup>	24
		Lestidae	13
Coleoptera	36 (10)	other Zygotera <sup>B</sup>	16
		Dytiscidae	32
		Psephenidae	6
Hemiptera	8	Corixidae	8
Bivalvia	26 (6)	Sphaeriidae	19
		Hyriidae	8
Ostracoda	11		
Hydracarina	2		

<sup>A</sup>Aeshnidae, Hemicordulidae, Telephlebiidae, Libellulidae, Cordulephylidae.

<sup>B</sup>Coenagrionidae, Isostictidae.

mussels (as per Post 2002) by dissection and frozen before preparation for stable isotope analysis.

Galaxiids (*Galaxias* sp.) and mosquitofish (*Gambusia holbrooki*) were captured in May 2009 with a long-handled net during invertebrate sampling. Samples of muscle tissue were removed from one galaxiid and 10 mosquitofish by dissection and frozen before preparation for stable isotope analysis.

All species of riparian and aquatic vegetation present were sampled to determine the basal resources to the food web. This included canopy, understorey and ground-cover species in the riparian zone, submerged and emergent macrophytes in the river and benthic algal biofilms (scraped from cobbles with a knife and brush). All samples were rinsed in distilled water and oven-dried at 60°C for one week. Plant samples (leaves only) were ground using a coffee grinder or mill grinder, depending on the toughness of the plant, to obtain a fine powder. Repeat sampling of some plants through time found no evidence of baseline shifts (data not shown), consistent with previous studies of these basal sources in similar streams in the region (Reid *et al.* 2008).

#### Stable isotope analysis

All samples were processed using an elemental analyser (EuroEA 3000) and a GV Isoprime (Manchester, UK) isotope ratio mass spectrometer at the Australian Rivers Institute laboratory at Griffith University, Brisbane. PeeDee Belemnite for carbon and atmospheric air for nitrogen (Peterson and Fry 1987) were used as isotopic standards. Ammonium sulfate and Australia National University sucrose were used as secondary standards for each run. To ensure that the carbon and nitrogen values calculated were within a reliable range, working standards were also used: 'flour' for plant samples and 'prawn' for

platypus fur, cheek pouch and invertebrate samples. These ratios were reported using standard notation,  $\delta^{13}\text{C}$  ( $\pm 0.1\text{‰}$ ) and  $\delta^{15}\text{N}$  ( $\pm 0.2\text{‰}$ ).

#### Data analysis

Paired *t*-tests (Quinn and Keough 2002) were used to investigate the variation in  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  between tail and dorsal fur for each individual platypus. Variation in mean  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  values of dorsal fur between individuals were compared using Welch's *t*-tests. Variation between the sexes and between sampling seasons was examined using Welch's *t*-test and single-factor ANOVA respectively (Quinn and Keough 2002). Dual stable isotope plots of mean  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  values were used to summarise potential feeding relationships and construct a food web. Nitrogen values were corrected for trophic fractionation using the value  $2.75\text{‰}$  ( $\pm 0.10$ , s.e.) (Caut *et al.* 2009) and used to determine trophic position using the formula of Zeug and Winemiller (2008). Carbon values were corrected for trophic fractionation using the value  $0.75\text{‰}$  (s.e. = 0.11) (Caut *et al.* 2009). A Bayesian mixing model, which utilised mean values (plus standard deviations) for the platypus and invertebrates, was used to indicate the most likely contribution that each invertebrate group made to platypus diet using the MixSIAR Graphical User Interface (GUI) (Stock and Semmens 2013). Owing to the similarity in isotopic signatures between the invertebrate families, families with similar isotopic signatures were grouped as follows for analysis: (1) Hemiptera, Ephemeroptera and Coleoptera; (2) Odonata and Diptera; (3) Trichoptera; and (4) Mollusca (Phillips *et al.* 2014). All tests were conducted in R statistical and graphical environment (R ver. 3.1.0: R Development Core Team 2008) with statistical significance set at 0.05.

## Results

### Isotope values of the platypus

Of the 21 platypus fur samples, four were from recaptured individuals. Similar stable isotope values suggested minimal change in recaptured individuals over the sampling period (Table 1). To avoid bias, only the initial fur samples from the recaptured animals were included in our analyses. Results for one of the juvenile females (born in the immediate past breeding season) appeared as outliers, causing the data to be non-normal, and so were removed from subsequent analyses. The juvenile had a tail fat index indicative of having depleted fat storage (Grant and Carrick 1978) and the difference in  $\delta^{13}\text{C}$  values could be due to the animal having drawn on its own stored fat reserves. Utilisation of tail fat reserves usually occurs during winter and is found to be significantly greater in juveniles than in adult platypuses (Grant and Carrick 1978; Hulbert and Grant 1983). This left 16 individual fur samples for analysis.

Duplicate fur samples (from the same individual) produced very similar results (Table 1). The mean  $\delta^{13}\text{C}$  of tail fur ( $-22.0 \pm 0.5$ ) was statistically significantly higher than the  $\delta^{13}\text{C}$  of the dorsal fur ( $-22.3 \pm 0.5$ ) ( $t = 2.919$ , d.f. = 15,  $P = 0.011$ ), although unlikely to be biologically significant, whereas no differences were recorded in  $\delta^{15}\text{N}$  ( $7.2 \pm 0.4$ ) in individuals ( $t = 1.831$ , d.f. = 15,  $P = 0.087$ ). No significant

differences were detected in the  $\delta^{13}\text{C}$  or  $\delta^{15}\text{N}$  values of dorsal fur ( $P > 0.05$ ) between sampling occasions. Thus dorsal fur values were used in all subsequent analyses. Large numbers of platypuses are rarely caught at any site or on any sampling occasion. To address this issue, samples collected on all four sampling occasions were included in the analysis. No significant differences due to sex were detected in either  $\delta^{15}\text{N}$  ( $t = -1.38$ , d.f. = 5.10,  $P = 0.23$ ) or  $\delta^{13}\text{C}$  ( $t = -0.209$ , d.f. = 10.22,  $P = 0.84$ ).

### Isotope values of prey and basal resources

Shoalhaven River platypuses were feeding on a wide range of aquatic invertebrate taxa with  $\delta^{13}\text{C}$  values between  $-28$  and  $-22\text{‰}$  (Fig. 1). The galaxiid (*Galaxias* sp.) and the mosquitofish or plague minnow (*Gambusia holbrooki*) were feeding within similar isotopic space to the platypus (Fig. 1).

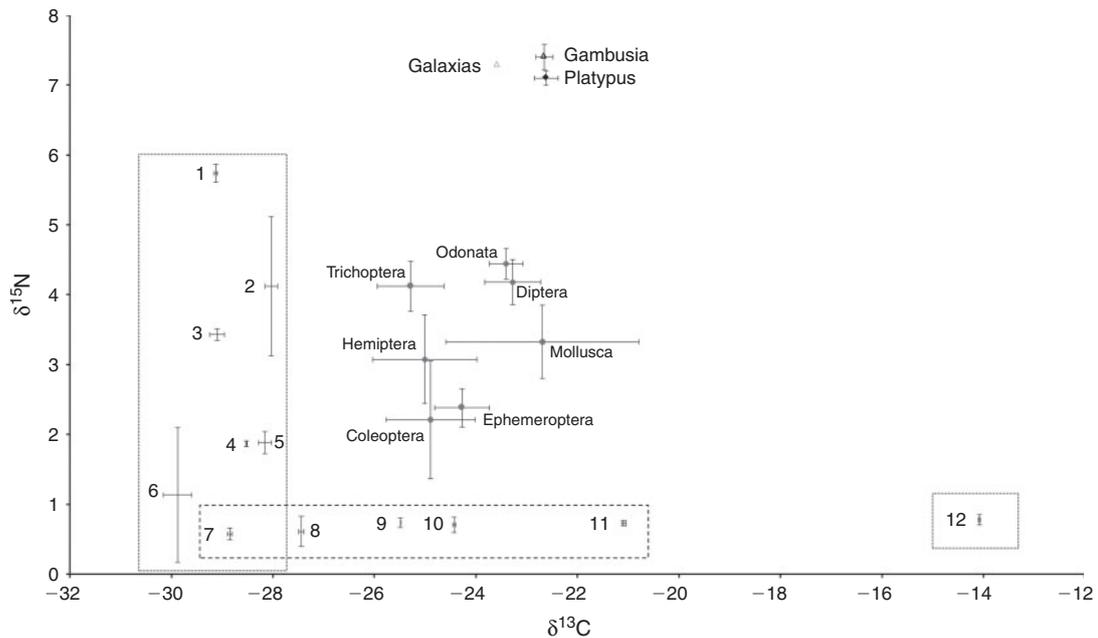
The  $\delta^{13}\text{C}$  values of the aquatic plants *Vallisneria spiralis* ( $-21.085$ ), *Potamogeton tricarlinatus* ( $-25.488$ ), *Chara* sp. ( $-24.431$ ) and benthic algae ( $-27.439$ ) are similar to those of the platypus and its prey and indicate that aquatic and not riparian plants were the predominant basal carbon sources within the Shoalhaven River food web (Fig. 1).

The Bayesian mixing model (MixSIAR GUI) indicated that a wide range of invertebrates are potentially assimilated by the platypus (Fig. 2). Based on means ( $\pm$ s.d., 95% credible interval) from the mixing model, the grouping of Odonata and Diptera ( $0.911 \pm 0.068$ ,  $0.979$ ) appear to be contributing most to the platypus diet, with all other taxon groupings contributing to platypus diet in similar proportions (Hemiptera, Ephemeroptera and Coleoptera ( $0.029 \pm 0.033$ ,  $0.088$ ), Trichoptera ( $0.044 \pm 0.053$ ,  $0.138$ ), and Mollusca ( $0.017 \pm 0.020$ ,  $0.051$ ), see Fig. 2). Ephemeroptera, Trichoptera, Diptera, Coleoptera and Odonata included most taxa identified as present in the cheek pouches (Table 2); each occurred in more than 30% of individuals and in 60% for Odonata. The invertebrate families most frequently observed as present in the cheek pouches of platypuses were Leptophlebiidae, Leptoceridae, Ecnomidae, and Chironomidae (Table 2).

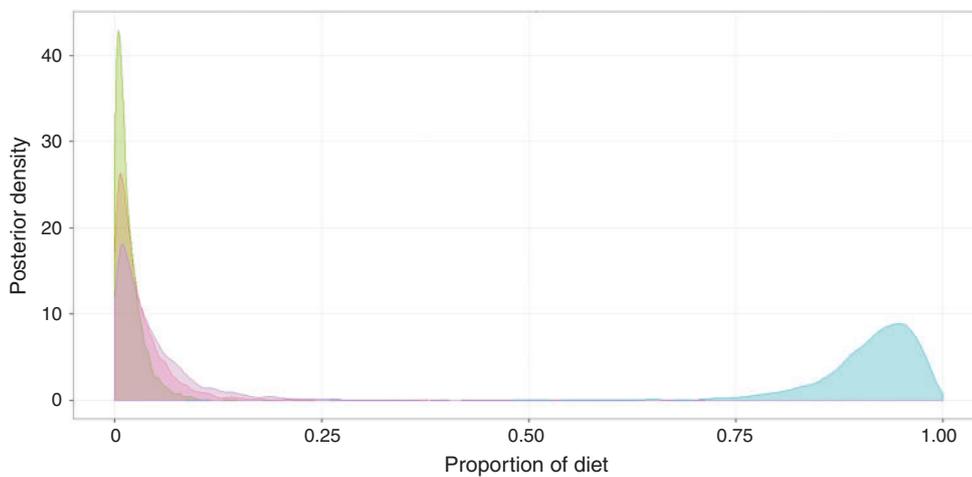
## Discussion

### What resources sustain the platypus?

Aquatic plants (*Vallisneria spiralis*, *Potamogeton tricarlinatus*, *Chara* sp. and benthic algae) are the major basal resources of the Shoalhaven food web. Enriched  $\delta^{15}\text{N}$  values for emergent plants (*Phragmites australis*, *Scirpus polystachyus* and *Juncus subsecundus*), *Salix* sp. and *Rubus* sp. have been related to cleared agricultural catchments, due to a differing source of nitrogen being utilised, rates of microbial decomposition and nutrient cycling (Udy and Bunn 2001). There was very little overhanging vegetation within the riparian zone, so allochthonous inputs to the river would be limited, particularly during the drought conditions present at the time of this study. *Eragrostis curvula* (African lovegrass), a C4 plant, was the dominant pasture grass at the site, and differed greatly from the  $\delta^{13}\text{C}$  values of the terrestrial C3 plants present. Although *E. curvula* was abundant at the site, the difference in  $\delta^{13}\text{C}$  values indicated that it was not an important basal resource. A recent macroinvertebrate feeding study, using stable isotopes and feeding-preference trials, found that aquatic macrophytes may make a greater contribution to



**Fig. 1.** Stable isotope biplot showing mean ( $\pm$ s.e.)  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  values of platypus (*O. anatinus*) dorsal fur, potential invertebrate and vertebrate food resources, and aquatic (dashed line) and riparian (dotted line) carbon resources at the Shoalhaven River, New South Wales, Australia. Figure data are uncorrected for trophic fractionation. Carbon resources: (1) *Phragmites australis*, (2) *Scirpus polystachyus*, (3) *Salix* sp., (4) *Rubus* sp., (5) *Juncus subsecundus*, (6) *Leptospermum obovatum*, (7) *Myriophyllum verrucosum*, (8) benthic algae, (9) *Potamogeton tricarlinatus*, (10) *Chara* sp., (11) *Vallisneria spiralis*, (12) *Eragrostis curvula*.



**Fig. 2.** Stable isotope Bayesian mixing model (MixSIAR Graphical User Interface) results showing the probable contribution of each invertebrate group to the Shoalhaven River platypus population's diet. The maximum density (peak) for each group (Hemiptera, Ephemeroptera and Coleoptera – red, Mollusca – green, Odonata and Diptera – blue, Trichoptera – purple) indicates the most likely contribution to assimilation that group has within the sampled platypuses.

lotic food webs than previously recognised (Watson and Bar-muta 2011) and larger waterbodies (such as the Shoalhaven River) may also receive more energy from microalgae (Finlay 2001) and phytoplankton (Hamilton *et al.* 1992). In some cases, the contribution of algae exceeds its relative availability within the environment when compared to other, more abundant,

carbon sources (Lewis *et al.* 2000; McCutchan and Lewis 2002). Whereas primary production is the dominant resource base for many food webs, riverine food webs are also subsidised by allochthonous inputs (Jefferies 2000; Sabo and Power 2002; Nyström *et al.* 2003). However, this did not appear to be the case in our study.

Platypuses appeared to be feeding on a range of invertebrate taxa (Table 2, Fig. 1). Our results do not conclusively support previous findings that platypuses feed non-selectively by consuming prey in proportion to prey items encountered while foraging (Faragher *et al.* 1979; Grant and Temple-Smith 1998) although a concurrent study by Marchant and Grant (2015) does support lack of selection. The mixing model suggested a large dietary contribution from the combined source of Diptera and Odonata, with Diptera also being abundant at the site. Marchant and Grant (2015) showed that the production by Odonata in the Shoalhaven River system was unlikely to provide enough energy per day to sustain the metabolic demand of even a single platypus but that chironomids were responsible for 75% of invertebrate production in 2009, suggesting that the Diptera are likely to be the main contributor to the diet in this grouping. As it was not possible to separate these two invertebrate taxa isotopically, further investigations are required to establish their relative importance. McLachlan-Troup *et al.* (2010) found that foraging effort was not applied equally across all habitats, and that seasonal differences in dietary items occurred, despite little change in the macroinvertebrate groups present in their study area.

The most comprehensive platypus dietary study to date (McLachlan-Troup *et al.* 2010) conducted in the Kangaroo Valley, New South Wales, identified 55 benthic invertebrate families from 16 orders within the cheek pouch contents, revealing that invertebrates from the edge and pool habitats dominated the platypus' diet. Diptera, Ephemeroptera and Trichoptera were the dominant invertebrates in the pool habitat, whereas Trichoptera and Ephemeroptera were most dominant in the edge habitat. There was a difference between invertebrate richness in summer and winter, which was driven mainly by the stonefly Gripopterygidae, then the elmid beetle, worms and chironomid larvae. The presence of Diptera in the pools where platypuses prefer to forage, as well as the chironomids driving the changes in richness between seasons, indicate that they are potentially one of the most important dietary items for the platypus, despite the fact that they were not among the taxa most frequently found in the cheek pouch samples in this study. Our cheek pouch analysis indicated that Ephemeroptera (Leptophlebiidae) and Trichoptera (Leptoceridae), in addition to the Odonata, were observed in the highest proportion of individuals (Table 2). These taxa have been identified as the most frequently observed constituents of platypus diet in several locations (Grant 1982), including a previous study in the upper Shoalhaven River (Faragher *et al.* 1979). McLachlan-Troup *et al.* (2010) identified larval trichopterans and coleopterans (Psephenidae) as occurring in the highest proportion of individuals (90%), whereas Ephemeroptera (Leptophlebiidae), Megaloptera (Corydalidae) and Odonata (Gomphidae) occurred in the cheek pouches of more than 30% of individuals in their study in streams in the lower Shoalhaven River catchment.

The difference in results between these dietary analysis techniques may have occurred because cheek pouch samples are likely to be biased towards invertebrates with hard exoskeletons. These species remain while soft-bodied organisms are quickly broken down (Sheppard and Harwood 2005). Cheek pouch analysis assumes that each food item is equally likely to be visible within a sample and that each food item is broken down at the same rate. This, however, is unlikely to be the case

for soft-bodied invertebrates such as larval dipterans. Chironomidae were detected in the cheek pouches but not as commonly as Odonata, Ephemeroptera or Trichoptera (Table 2).

Chironomid head capsules are identifiable in the gut contents of fish (e.g. Lobinske *et al.* 2002) and are found preserved in lake cores (e.g. Brodersen and Lindegaard 1999) and so would be expected to persist in platypus cheek pouch samples. Chironomids were found in 37% of the cheek pouches examined (Table 2). It is possible that the small size of larval chironomids, including sclerotised head capsules, means that they move more quickly than larger masticated food remnants back to the water or into the gut and therefore are not always found within the cheek pouches. Differences in relative sizes of the invertebrates also means that the platypus would need to consume more of the smaller invertebrates (e.g. chironomids) to achieve the same effect on isotopic ratios as consuming a larger invertebrate (e.g. Odonata). Although prey body size has not been found to correlate with relative contribution to production, the density of chironomids in the Shoalhaven River was two orders of magnitude greater than that of the Odonata (in both 2009 and 2011) (Marchant and Grant 2015).

#### *Utility of stable isotope analysis for platypus dietary analysis*

This is the first study to investigate the diet of the platypus using stable isotope analysis and one of few such studies conducted on Australian mammals (e.g. Murphy *et al.* 2007; Pate and Anson 2008). Some variation occurred in the stable isotope values of platypus fur depending on the part of the body sampled. This suggested that the site of fur removal needs to be consistent to ensure valid comparisons of diets determined by stable isotope analysis. Further investigation of the turnover time of the different types of fur (body and tail) is needed to ascertain which specific periods of time in the platypus' diet they reflect. The lack of variation in dorsal fur values over time suggested that the diet did not vary significantly between the seasons over the sampling period. However, use of stable isotopes for seasonal comparisons has some limitations, as there can be baseline shifts in isotopic values (Post 2002). Whereas other studies have suggested that baseline shifts are not significant in similar streams in the same region (Reid *et al.* 2008), we have deliberately been conservative in our interpretation of these results, particularly with respect to  $\delta^{15}\text{N}$  values (Post 2002). Neither our study, nor those of Faragher *et al.* (1979) and McLachlan-Troup *et al.* (2010) detected any dietary differences between the sexes.

Caution must be used in applying stable isotope mixing models to the study of animal diets. This is particularly true where there are large numbers of dietary items with similar signatures, and where many possible mixtures of sources are possible (Phillips *et al.* 2014). Mixing models provide the most parsimonious explanation of how a mixture of prey items could produce the isotopic ratio of the consumer (the platypus). Although our mixing model results suggest that Odonata and Diptera are predominant in the platypus diet, this is only one possibility. Platypuses do eat a wide variety of benthic invertebrates and it has been shown that prey items with the highest levels of production are the most common in the diet (Marchant and Grant 2015). Stable isotope analysis does provide additional information on likely energetic importance of taxa, and in this

case suggests that a traditional dietary analysis approach may have underestimated the contribution of dipteran prey items, and could in other instances also underrate the importance of other soft-bodied macroinvertebrates, such as oligochaetes (Marchant and Grant 2015). The use of cheek pouch analysis (and gut analysis in other taxa) together with stable isotope analysis is a particularly powerful approach to determining diet (Ho 2011). We recommend that future studies obtain relative invertebrate biomass information to further determine the contributions of each prey grouping to the platypus diet.

#### *Dietary comparisons with fish*

Although some platypuses have been recorded as eating small fish of various species, including mosquitofish (Grant *et al.* 1977; Grant 2007) in both captivity and in the wild, the similarity of the stable isotope signature of the platypus to that of the mosquitofish (*Gambusia holbrooki*) and the common galaxiid (*Galaxias* sp.), suggests that platypuses are not consuming these species, and that these species are relying on the same resources as the platypus. Care is required, however, when comparing  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  from different tissues of organisms, differing taxa and with differing quality diets, as there are likely different diet-tissue fractionation values between taxa, which will generate misleading results (Stenroth *et al.* 2006; Caut *et al.* 2009). We conclude that there is potential for competition between the platypus and these fish species based on the stable isotope analysis results. However, a greater sampling effort for these species, direct observational fish feeding studies, gut content analysis and targeted sampling of comparable tissues (such as blood or muscle) for stable isotope analysis would be required to ascertain this with confidence.

Faragher *et al.* (1979) found free-swimming freshwater shrimps (*Paratya australiensis*) and Hemiptera (Corixidae and Notonectidae) were more common in the stomachs of introduced brown trout (*Salmo trutta*) than in cheek pouches of platypuses in the upper Shoalhaven River, suggesting greater utilisation of nektonic invertebrates by trout (McDowall 1996). However, the general patterns of resource utilisation reported in this and the previous study from the same location (Faragher *et al.* 1979), suggest that the platypus and all three fish species are utilising similar carbon sources. Brown trout (*Salmo trutta*) were regularly captured in low numbers in the upper Shoalhaven Study area until ~1996. The extended drought of 1979–83, sand accumulation and the short but severe drought in the mid 1990s reduced the number of deep and cool refuge pools favoured by trout (T. R. Grant, unpubl. data). The impacts of the subsequent decadal-length ‘millennium’ drought, which started in 1997 (Thomson *et al.* 2012), probably explains the absence of trout in our study.

Given the higher energy requirement associated with regulating its body temperature (Grant and Dawson 1978), it would be expected that the platypus would exert a stronger top-down pressure on invertebrate prey than fish; however, this hypothesis needs to be tested experimentally. Competition under stress may also be an important factor, with a recent study showing dietary overlap between introduced carp (*Cyprinus carpio*) and an indigenous carp gudgeon (*Hypseleotris* sp.) in an Australian wetland during low water conditions (Mazumder *et al.* 2012).

Although our study was restricted to one river system, during drought conditions, the site was fairly typical of the agricultural habitat present throughout the platypus’ range. For this reason we believe that these results have a reasonable degree of generality, but this will need to be confirmed with further research across a range of habitat types.

#### Conclusions

The combined results of the cheek pouch and stable isotope dietary analysis techniques confirmed earlier findings that platypuses feed exclusively on benthic macroinvertebrates. Differing results between the techniques, particularly with regard to the contribution of soft-bodied Diptera, suggest that dietary studies that combine the two techniques will be the most powerful. Care must be taken when using stable isotope mixing models as many aquatic invertebrates have similar carbon and nitrogen values and so cannot be modelled accurately. This suggests that it will be most beneficial to utilise stable isotope analysis in combination with conventional dietary analysis techniques and in situations where the diet of the platypus varies with site or weather conditions. This study has provided greater insight into the platypus’ diet and the findings will be used to design a larger study to determine the top-down effects of platypuses in rivers in contrasting forested and urban catchments. Further studies are also required to elucidate the trophic interactions between the platypus and other common aquatic and semiaquatic vertebrates, including fishes (both introduced and indigenous), turtles (e.g. *Chelodina longicollis*), water dragons (*Physignathus lesueurii*) and water rats (*Hydromys chrysogaster*), under drought and non-drought conditions.

#### Acknowledgements

This study was undertaken with the following permits: NSW Department of Primary Industries Director-General’s Animal Care and Ethics Certificate of Approval (09/3535) and Scientific Research Permit (F84/1245); NSW Department of Environment, Climate Change and Water Scientific Licence (S10478) Department of Sustainability and Environment (10004130), Department of Primary Industries – Animal Ethics (09.07) and Fisheries (907) and Monash University Animal Ethics Committee Permit (BSCI/2009/02). The authors thank Di Izzard for property access at the Shoalhaven River, James Cassidy, Sheila Hamilton-Brown, Tom Heath, Susie Ho, Stuart Kerr and Tanya McLachlan-Troup for their assistance and advice, and Beth Gott for plant identifications. The stable isotope analyses were undertaken by Rene Diocares at the Australian Rivers Institute, Griffith University. The constructive comments of anonymous reviewers are also gratefully acknowledged. R. M. Thompson was supported by an Australian Research Council Future Fellowship (FT110100957).

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