The following supplement accompanies the article

Trophic plasticity of the methanotrophic mussel *Bathymodiolus childressi* in the Gulf of Mexico

P. M. Riekenberg, R. S. Carney*, B. Fry

*Corresponding author: rcarne1@lsu.edu

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Supplement.

Extent of and corrections for preservation effects

The use of formaldehyde as a fixative and preservative for natural history specimens has remained a common methodology since the 1890s (Eleftheriou & Moore 2013). Stable isotope analyses of specimen archives can be ecologically informative, but preservation-introduced biases must be determined (Sarakinos et al 2002). While the formaldehyde-preserved tissues used in the current study did provide useful values for all three stable isotopes, that is not the case for all archives. For example, in the case of oceanic copepods and chaetagnaths preserved in formalin for 11 years, $\delta^{13}C$ values had been rendered unreliable but not $\delta^{15}N$ values (Rau et al 2003, Ohman et al 2012). The archived specimens analyzed herein were fixed and stored in 5% seawater formalin (3.7% formaldehyde in sea water), which permanently binds with and cross links proteins, glycoproteins and nucleic acids (Fox et al 1985, Puchtler and Meloan 1985). The addition of the fixative carbon obscures the original tissue $\delta^{13}C$ value.

When $\delta^{13}C$ values have a narrow range the change caused by preservation can be effectively treated as a simple additive effect (i.e. Demopoulos et al 2008). Experimentally determined changes for $\delta^{13}C$ range from -0.8‰ to -2.0‰ and a lesser +0.1 to -0.5‰ for $\delta^{15}N$ with the largest change occurring during initial fixation (Edwards et al 2002, Sweeting et al. 2004, Bicknell et al. 2011, Lau et al. 2012). In the present study of chemosynthetic organisms the $\delta^{13}C$ values of tissue have such a wide range that it should not be treated as a simple effect. Rather the preservation effects will be determined by the amount of formaldehyde carbon bound to the preserved tissue, and the contrast in $\delta^{13}C$ between formaldehyde and tissue.

In this study, live specimens of the commercial mytilid mussel *Perna canalicula* (New Zealand Green Lipped Mussel) were used to experimentally establish the incorporation amount of formalin into mussel tissue. The adductor muscles from 6 mussels were split with half treated with formalin for 60 days and half not. Both treated and untreated samples were run for CNS analysis along with the formalin used. The measured $\delta^{13}C$ value for formalin was -44.1‰ and the calculated incorporation amount based on the treatment effect was 6.1±0.9% (standard error reported throughout). This measured value for formalin agrees with previously measured $\delta^{13}C$ values of between -38‰ and -52.5‰ for formalin from multiple manufacturers (Edwards et al. 2002) and falls between the ranges observed for the muscle tissues in this study (-36.6 to -75‰). A correction for formalin incorporation was applied through the use of a two-source mixing equation:

$$\delta^{13}C_{OBSERVED} = \delta^{13}C_{TRUE}*(1-f) + (\delta^{13}C_F * f)$$

so that

$$\delta^{13}C_{TRUE} = (\delta^{13}C_{OBSERVED} - \delta^{13}C_F * f)/(1 - f)$$

where $\delta^{13}C_{TRUE}$ is the calculated untreated tissue value, $\delta^{13}C_{OBSERVED}$ is the measured or observed treated tissue value, $\delta^{13}C_F$ is the measured value of formalin (-44.1‰), and f is the measured fraction of tissue carbon represented by formalin after the fixation process (0.06). This method resulted in

corrections ranging from -0.5 to +2‰ for the tissues from the sites, with depletion in $\delta^{13}C$ values occurring predominately in the Bush Hill samples and enrichment of $\delta^{13}C$ values occurring for the Brine Pool samples.

Since the $\delta^{13}C$ value of the archival formalin is unknown the magnitude of preservation effects on the preserved seep mussels were estimated utilizing the 14.5‰ published range of manufactured formalin (-38‰ to -52.5‰). The maximum effect was small at about 1‰, i.e., for the most ^{13}C-enriched observed tissue values was a shift from a value of-36.2‰ to -35.2‰, and the maximum effect on the most depleted observed tissue was a shift from a value of -77.4‰ to -76.5‰. Thus with a small $\delta^{13}C$ shift of ~1‰ for the samples expected to have the most significant corrections preservation effect should not be sufficient to invalidate the ecological interpretations presented. Preservation effects on $\delta^{15}N$ and $\delta^{34}S$ were considered minor and consistent with previous studies with the measured values being taken as equal to unpreserved values (Sweeting et al. 2004).

Stable isotope and resource relationships

Correlations among measured isotope values and proportional source utilization revealed different trophic dependencies at each seep. At Bush Hill utilization of $DSOM_{BH}$ had the greatest overall influence on all three isotope values followed in declining importance by SM_B_{BH}, and approximately small and equal contributions from SM_A_{BH} and DPHY based on magnitude of correlations (Table S1). At Bush Hill more enriched $\delta^{13}C$ values were primarily associated with higher utilization of $DSOM_{BH}$ ($r = 0.82$) and lower utilization of SM_B_{BH} ($r = -0.67$) and to a lesser extent SM_A_{BH} ($r = -0.49$). Utilization of DPHY was only weakly correlated ($r = -0.20$) with enriched $\delta^{13}C$. Bush Hill enriched $\delta\ ^{15}N$ values were associated with decreased use of SM_B_{BH} ($r = -0.86$) coupled with increased use of $DSOM_{BH}$ ($r = 0.74$). Even though SM_A_{BH} contains ^{15}N-enriched nitrogen it plays no significant role in the $\delta\ ^{15}N$ values ($r = 0.04$). Similarly, DPHY is only weakly related to $\delta\ ^{15}N$ values and its increased utilization lead to more depleted values ($r = -0.33$). For Bush Hill $\delta^{34}S$ values $DSOM_{BH}$ has a large effect with increased utilization leading to more depleted values ($r = -0.85$).

Spatial patterns

Post hoc analysis revealed that within-bed homogeneity was more prevalent than the ANOVA results might suggest (Fig. S1). Beds 1, 1a and 2 were not significantly different for utilization of SM_A_{BH} and DPHY. When significant Position effects were found they were actually restricted to only a single bed, Bed 1 in the case of SM_B_{BH}, Bed 2 in the case of $DSOM_{BH}$. Significant Bed:Position interaction for DPHY and $DSOM_{BH}$ indicated inconsistent spatial effects.

The Brine Pool samples contained five specimens from a single scoop sample taken in 1991 at the North Edge that were distinct outliers especially with respect to $\delta\ ^{15}N$ values. ANOVA was carried out with the full data set (Table S2) and then with the outliers omitted. For the full data set at the Brine Pool SM_C_{BP} showed End (N vs S) effects and Position effects (Inner vs Edge) but with significant interactions indicating inconsistency. *Post hoc* analysis revealed that the significant results were primarily caused by high values encountered at the North Edge, the three other combinations of End and Position were homogenous (Fig. S2). SM_D_{BP} showed marginally significant End effects, Position effects and an interaction in the analysis of variance, but in *post hoc* analysis (Table S2) the End effect was not significant and differences were primarily due to the low values at the North Edge. Utilization of DPHY showed significant End effects but insignificant Position effects and no significant interaction. *Post hoc* analysis revealed the same effects but pairwise comparisons indicated few major differences except for a variable range. $DSOM_{BP}$ showed no significant End or Position effects although a significant interaction was found. *Post hoc* analysis indicated a difference in means between Edge and Inner only at the South end. Otherwise the mean $DSOM_{BP}$ utilization was relatively uniform across the sampled locations.

Omission of the outliers had little impact on the ANOVA results. Similar Bed and Position effects were found for $SM\_C_{BP,}$ SM_D_{BP} and DPHY. The North End remained the location of significantly greater SM_C_{BP} utilization with the maximum still found in the North Edge. The major change in results was for $DSOM_{BP}$, which after outlier exclusion did show significant End, Position and interaction effects. It had the greater utilization at the North End and the greatest overall at the North Edge.

Table S1. Tests for position effects on mean utilization of four sources

BUSH HILL 1991-1992					
	Sum Sq	df	*F* value	Pr(>*F*)	
SM_A$_{BH}$					
Bed	0.0923	2	13.2810	6.218e-06	***
Position	0.0000	1	0.383	0.9748	
Bed:Position	0.0105	2	1.5101	0.2251	
Residuals	0.4133	119			
SM_B$_{BH}$					
Bed	0.3791	2	31.619	9.71e-12	***
Position	0.0862	1	14.383	0.00236	**
Bed:Position	0.0035	2	0.2878	0.75043	
Residuals	0.7135	119			
DPHY$_{BH}$					
Bed	0.05720	2	19.450	4.91e-08	***
Position	0.00525	1	3.574	0.0611	
Bed:Position	0.02966	2	10.0849	9.000e-05	***
Residuals	0.17497	119			
DSOM$_{BH}$					
Bed	1.0753	2	94.4439	<2.2e-16	***
Position	0.0977	1	17.1591	6.46e-05	***
Bed:Position	0.1046	2	9.1906	0.0001943	***
Residuals	0.6775	119			
BRINE POOL 1989-1991					
	Sum Sq	df	*F* value	Pr(>*F*)	
SM_C$_{BP}$					
End	0.17956	1	11.22	0.0012450	**
Position	0.33114	1	20.703	1.941e-05	***
End:Position	0.24444	1	15.283	0.0001961	***
Residuals	1.24758	78			
SM_D$_{BP}$					
End	0.03497	1	3.8889	0.05215	.
Position	0.26860	1	29.8679	5.358e-07	***
End:Position	0.41237	1	45.8540	2.136e-09	***
Residuals	0.70146	78			
DPHY					
End	0.03589	1	8.2427	0.005265	**
Position	0.00113	1	0.2584	0.612662	
End:Position	0.00000	1	0.0009	0.975885	
Residuals	0.33965	78			
DSOM$_{BP}$					
End	0.00022	1	0.0747	0.78540	
Position	0.00416	1	1.4599	0.23060	
End:Position	0.02243	1	7.8711	0.00634	**
Residuals	0.22223	78			
Signif. codes: 0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1					

Table S2. Temporal and position effects

Bush Hill Year 1991, 1992, Bed 1 Position and Interactions					
	Sum Sq	df	*F* value	Pr(>*F*)	
SM_A_{BH}					
Year	0.0362	1	15.2349	0.0002181	***
Position Bed 1	0.0156	1	6.5690	0.0125615	*
Year:Position	0.0028	1	1.1634	0.2845209	
Residuals	0.1642	69			
SM_B_{BH}					
Year	0.08858	1	25.9948	2.864e-06	***
Position Bed 1	0.01672	1	4.9072	0.03005	*
Year:Position	0.00003	1	0.0096	0.92237	
Residuals	0.23514	69			
Year	0.00001	1	0.0115	0.9151	
Position Bed 1	0.00001	1	0.0081	0.9287	
Year:Position	0.00010	1	0.0828	0.7744	
Residuals	0.08601	69			
$DSOM_{BH}$					
Year	0.2345	1	77.8162	6.311e-13	***
Position Bed 1	0.0000	1	0.0004	0.9836	
Year:Position	0.0013	1	0.4480	0.5055	
Residuals	0.2079	69			
Brine Pool Years 1989, 1991 and 2006, Pool End and Interactions					
	Sum Sq	df	*F* value	Pr(>*F*)	
SM_C_{BP}					
Year	0.02758	2	8.8690	0.0002592	***
End	0.00031	1	0.2001	0.6554899	
Year:End	0.10165	2	32.6879	5.276e-12	***
Residuals	0.18192	117			
SM_D_{BP}					
Year	0.0061	2	0.7003	0.498488	
End	0.0285	1	6.5408	0.011821	*
Year:End	0.0490	2	5.6207	0.004669	**
Residuals	0.5104	117			
DPHY					
Year	0.0479	2	8.9247	0.000247	***
End	0.0139	1	5.1909	0.024520	*
Year:End	0.2016	2	37.5927	2.462e-13	***
Residuals	0.3137	117			
$DSOM_{BP}$					
Year	0.0001	2	0.0388	0.96198	
End	0.0047	1	3.3771	0.06864	.
Year:End	0.0076	2	2.7166	0.07027	.
Residuals	0.1629				
Signif. codes: 0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1					

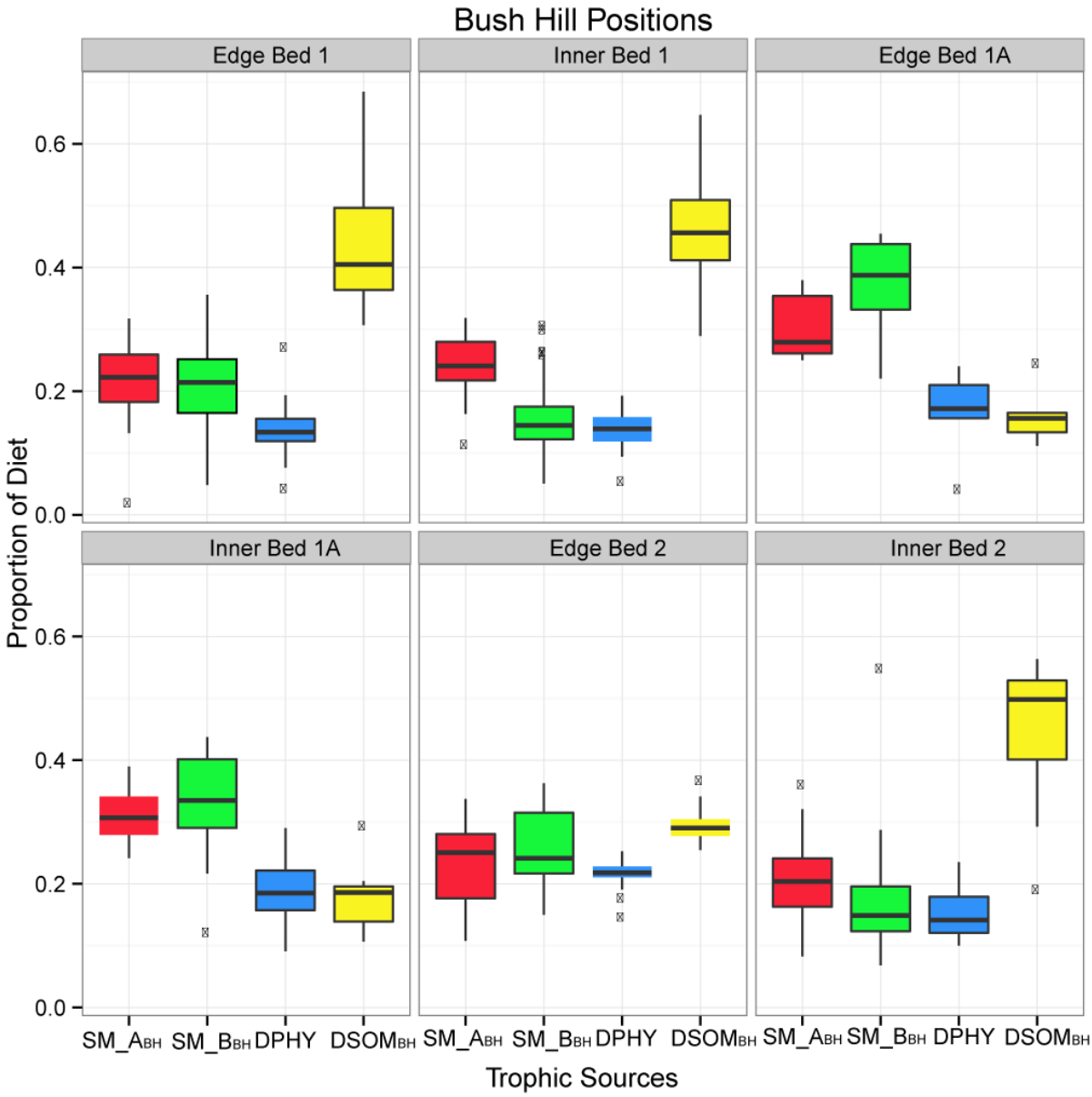

Fig. S1. Box and whisker plots of Bush Hill trophic-resources spatial pattern presented with the bed sampled and position within those beds as factors. Boxes represent the 25th and 75th quartiles around the means (horizontal lines) with the 90th quantiles indicate by vertical lines. Outlier points (squares) are also indicated.

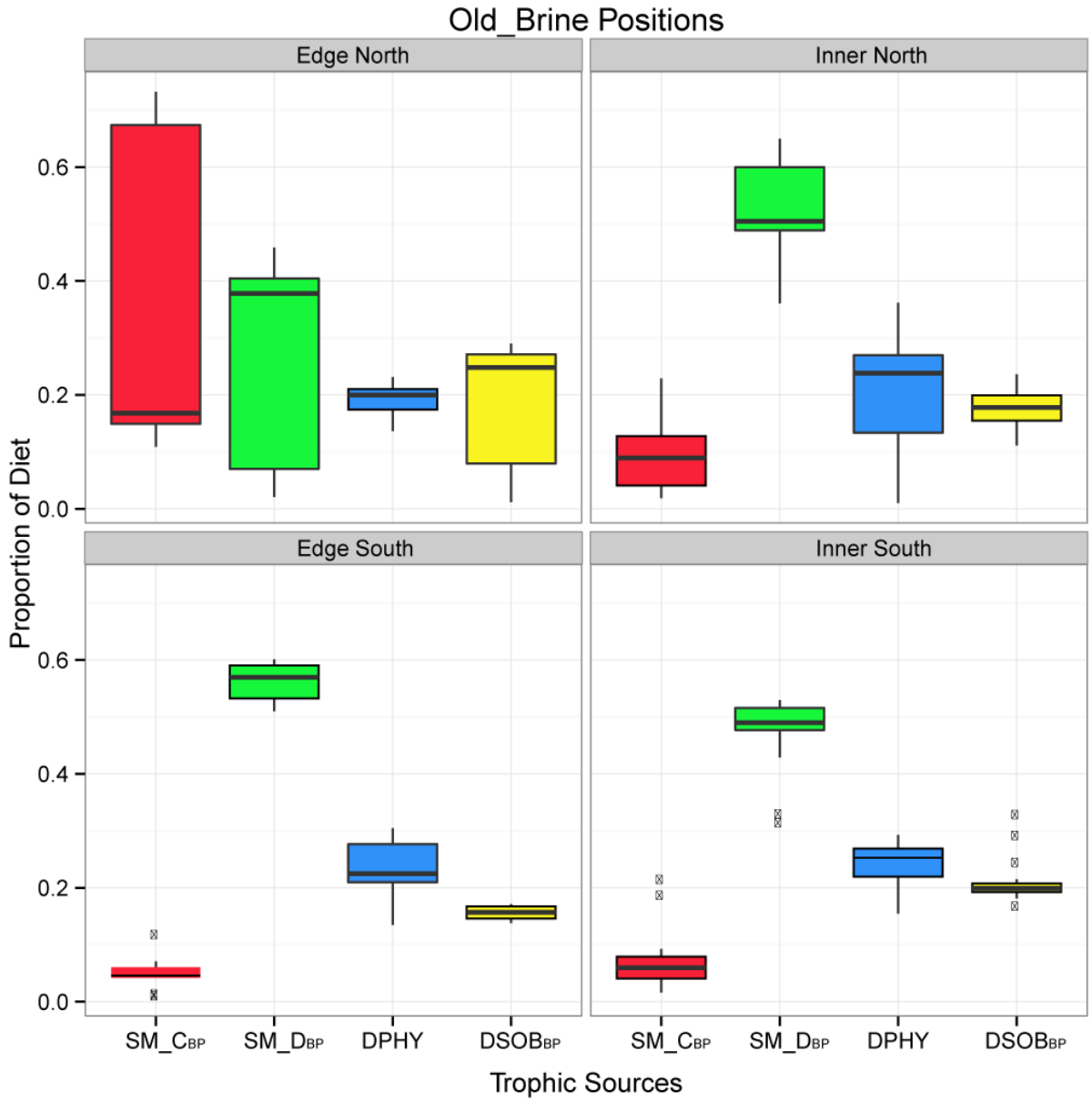

Fig. S2. Box and whisker plots of Brine Pool trophic-resources spatial pattern for 1989 and 1991 samples combined presented with pool end and position at those ends as factors. Boxes represent the 25th and 75th quartiles around the means (horizontal lines) with the 90th quantiles indicate by vertical lines. Outlier points (squares) are also indicated.

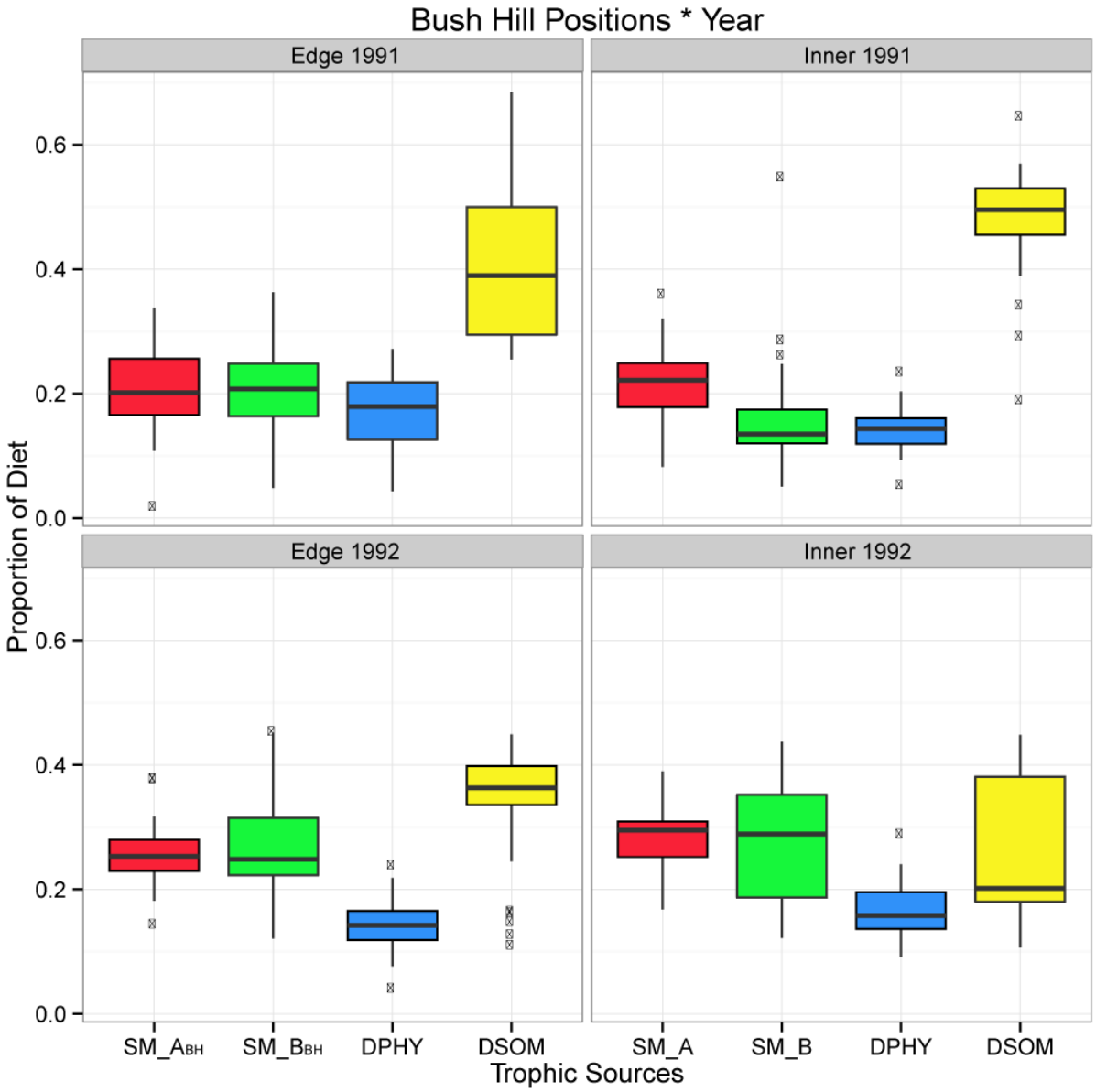

Fig. S3. Box and whisker plots of Bush Hill trophic-resources temporal effects for bed 1 samples in 1991 and 1992 presented with year and position within that single bed as factors. Boxes represent the 25th and 75th quartiles around the means (horizontal lines) with the 90th quantiles indicate by vertical lines. Outlier points (squares) are also indicated.

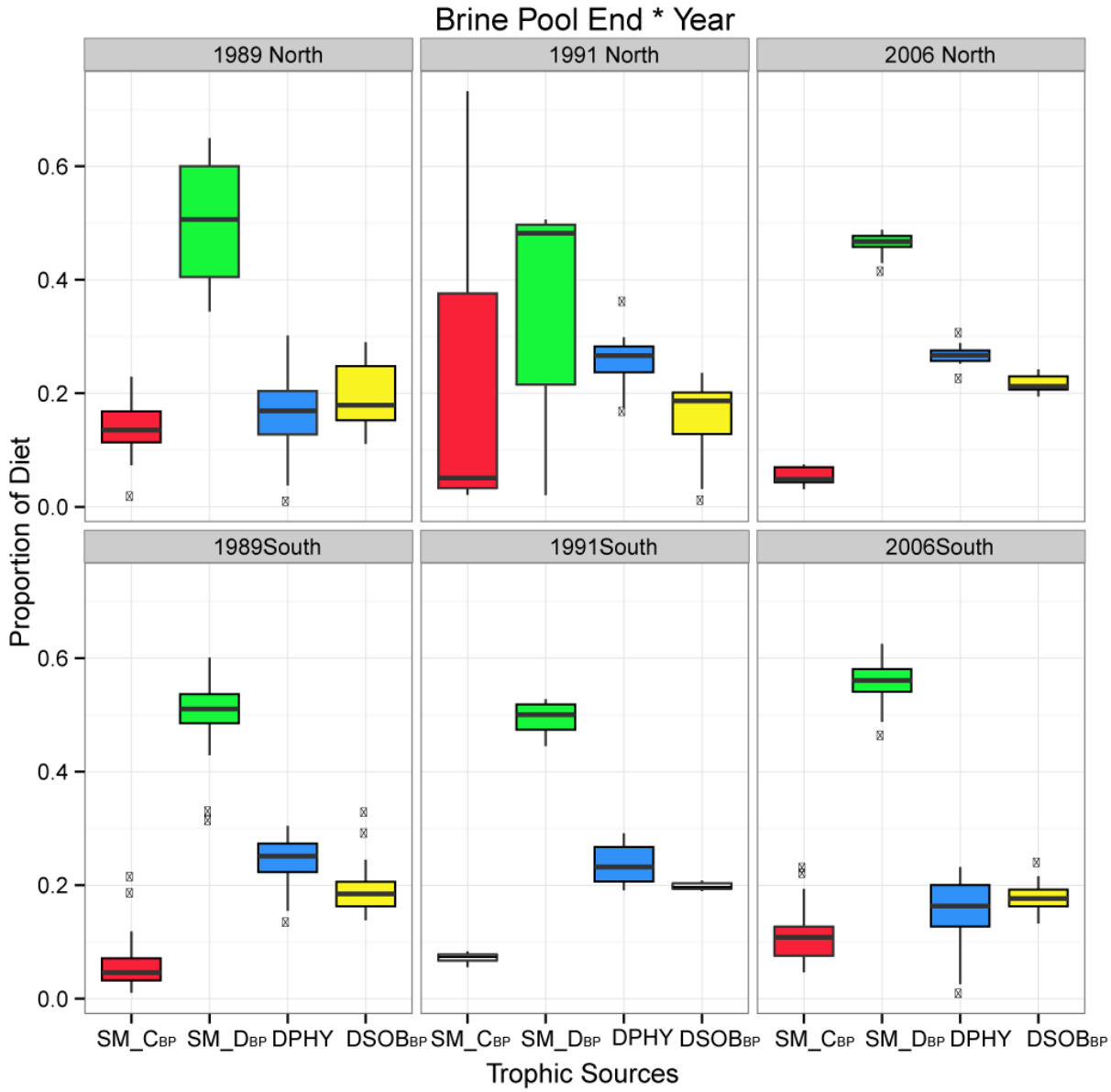

Fig. S4. Box and whisker plots of Brine Pool trophic-resources temporal effects for years 1989,1991 and 2006 with year and end of pool presented as factors. Boxes represent the 25th and 75th quartiles around the means (horizontal lines) with the 90th quantiles indicate by vertical lines. Outlier points (squares) are also indicated.

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