PHENOTYPICALLY FLEXIBLE SEX ALLOCATION IN A SIMULTANEOUS HERMAPHRODITE

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Previous studies on sex allocation in simultaneous hermaphrodites have typically focused either on evolutionary or one-time, ontogenetic optimization of sex allocation, ignoring variation within an individual's lifetime. Here, we study whether hermaphrodites also possess facultative sex allocation, that is, a phenotypic flexibility, allowing them to distribute resources to either sex in an opportunistic way during their adult lifetime. We used the simultaneously hermaphroditic free-living flatworm *Macrostomum lignano* and raised individuals in pairs and groups of eight worms (further called octets) until sexual maturity was reached and sex allocation for the current conditions was expected to be set. Treatment groups were subsequently transferred to the alternative group size, that is, from pairs to octets or from octets to pairs, and compared to two control groups, which were transferred without changing group size. The results show that worms in treatment groups responded as expected by the local mate competition theory for simultaneous hermaphrodites: increasing group size resulted in a shift toward a more male-biased sex allocation and vice versa. These findings reveal that sex allocation in these animals is not fixed during ontogeny, but remains flexible after maturation. We argue that phenotypically flexible sex allocation in hermaphroditic animals may help us to understand the evolution and ecology of hermaphroditism.

KEY WORDS: Flatworms, hermaphroditism, mating group size, phenotypic flexibility, phenotypic plasticity, plathyhelminthes, resource allocation.

Sex allocation theory for outcrossing simultaneous hermaphrodites predicts a more female-biased investment of reproductive resources when mating group size decreases (Charnov 1980, 1982; Fischer 1981, 1984). It suggests that in a relatively smaller group individuals need to produce fewer sperm to be successful in sperm competition, leaving more resources for the female function. So far, this local mate competition theory (Charnov 1980) for simultaneous hermaphrodites (from here on simply

called "hermaphrodites") has only focused on the evolutionary adjustment of sex allocation to mating group size. Yet, there are in fact three different levels at which sex allocation can be adjusted: first, the allocation strategy can be the result of selection and evolution, and thus be an adaptation to the average mating group size over many generations (e.g., Charnov 1987; Petersen 1991). In this case sex allocation is genetically fixed. In the second case it is not or not strictly fixed genetically, but it is set during ontogeny and therefore influenced by environmental conditions such as population density (developmental plasticity, Piersma and Drent 2003; Raimondi and Martin 1991; Trouvé et al. 1999; Schärer and Ladurner 2003; Tan et al. 2004; Schärer et al. 2005). In the third case sex allocation is fixed neither during evolution nor ontogeny, but remains flexible throughout adult life and can be adjusted to current environmental conditions (Lorenzi et al. 2005; phenotypic flexibility: Piersma and Drent 2003).

Recent publications on sex ratio and sex allocation have pointed out that little is known about the ability of organisms to change sex allocation during adult lifetime and their speed and efficiency of adjustment (West et al. 2002, 2005). To our knowledge Lorenzi et al. (2005) provide the only study on facultative sex allocation in hermaphrodites under changing mating group sizes, but they mainly found effects in the female function and no clear response was visible in sperm production. In this study we investigated whether adult individuals of the outcrossing, simultaneously hermaphroditic flatworm *Macrostomum lignano* (Ladurner et al. 2005) can adjust sex allocation according to changes in mating group size. The experiment consisted of worms that grew up in pairs or groups of eight worms (further called octets). After having reached maturity, group size was swapped for some of the replicates, resulting in two experimental groups: one in which group size was increased (further called 2to8) and one in which it was decreased (further called 8to2). The remaining replicates were used as two control treatments where individuals were placed in a group of the original size (further called 2to2 and 8to8). Measurements of testis, ovary, body, and seminal vesicle area were taken at four different days after group size manipulation. We expected to see an increase in male allocation and a decrease in female allocation in the 2to8 treatment compared to its control (i.e., 2to2), and the opposite effects in the 8to2 treatment compared to its control (i.e., 8to8).

Materials and Methods
STUDY ORGANISM

Macrostomum lignano is a marine free-living flatworm and an obligatorily outcrossing simultaneous hermaphrodite. It has paired testes and ovaries situated laterally in the body (see Ladurner et al. 2005 for detailed description). Produced sperm are stored in the seminal vesicle prior to sperm donation. The

size of this organ depends on its fill state, which in turn reflects mating rate (Schärer and Ladurner 2003). Adult animals are about 1.5 mm in length and feed on diatom algae. Under culture conditions young animals hatch five days after egg deposition and reach maturity after another 13 days (Schärer and Ladurner 2003). In well-fed individuals one to two eggs are laid per day. Because individuals are nearly transparent, non-invasive morphometric measurement of many internal structures, such as testis and ovary size, is possible (Schärer and Ladurner 2003; Schärer et al. 2005).

EXPERIMENT

To produce juveniles of the same age, 2000 adult worms were placed in plastic Petri dishes, allowed to lay eggs, and removed two days later. After five days 1248 hatchlings were collected from these plates on two consecutive days. They were randomly assigned to 240 pairs and 96 octets and raised in wells of six well tissue culture plates in 5 ml of f/2 (a nutrient enriched artificial sea water, Andersen et al. 2005). During the course of the experiment worms were transferred to new well-plates with fresh medium about once every six days. On day 26 after egg laying, that is, about one week after worms had reached maturity, all replicates were randomly assigned to one of two treatments and were thereby transferred to fresh well-plates. One half stayed in their original group sizes and served as control groups (2to2 and 8to8). The other half was changed to contrasting group sizes as follows. To produce the 2to8 treatment four pairs were joined into one octet. To produce the 8to2 treatment two worms were randomly drawn from an octet to create a pair. This procedure resulted in four different experimental groups with 48 replicates each: (1) pairs that stayed in pairs (2to2), (2) pairs that were merged into groups (2to8), (3) pairs that were extracted from groups (8to2), and (4) worms that stayed in octets (8to8).

Blind measurements were taken 1, 4, 7, and 10 days after changing group sizes. On each of these days 12 of the 48 replicates within each treatment group were used to record body, testis, ovary, and seminal vesicle size. Replicates were only measured once and then discarded to avoid pseudoreplication. The complete setup resulted in a $2 \times 2 \times 4-$factorial design with 12 independent replicates per cell.

MORPHOMETRY

Morphometric measurements were done as described in Schärer and Ladurner (2003). However, before anesthetization with $MgCl_2$, worms were put in a solution of 50 mM bromodeoxyuridine (BrdU) in f/2-medium for 30 min for another experiment on gonad activity (to be published elsewhere), washed for 15 min in f/2-medium, and transferred to fresh f/2-medium for another 15 min. Each individual was then anesthetized for 10 min in a 1:1 solution of f/2-medium and an isotonic $MgCl_2$ solution $(71.4 g \cdot l^{-1})$.

Images of body area were taken at a 63-fold magnification (Sony DFW-X700 digital video camera connected to an Olympus BH-2 microscope via a c-mount) and acquired using the image capture software BTV Pro (http://www.bensoftware.com). Pictures of the testes, ovaries, and seminal vesicle were taken at 400× and evaluated with the image analysis software Object-Image 2.09 (http://simon.bio.uva.nl/object-image.html). Body area was measured by setting a gray-scale threshold for each picture to silhouette the worm against the background. In a few cases where the threshold was not usable the worm was circumscribed manually. Once calibrated the exact area is automatically determined by the program. For testes and ovaries, all circumferences were drawn manually.

STATISTICAL ANALYSIS

To keep variances of pairs and octets comparable, only two random individuals from octets were measured. Because such two worms are dependent we calculated the mean per replicate for each trait. From the initial 384 worms 48 had to be excluded because of mistakes during the measurements. Effective sample size was reduced to 156 independent replicates with two (dependent) data points and 24 replicates consisting of one data point making up a total of 336 worms in $N = 180$ replicates. For each worm testis area was calculated as the sum of left and right testis areas. When images of only one testis could be made, but the presence of the other testis was confirmed in the microscope, the measured testis was counted double (the sizes of the two testes are highly correlated within a worm). The same was done for ovaries. This extrapolation was necessary in only 13 cases. Testis and ovary areas are reliable estimates for investment in male and female function as has been shown earlier (testis: Schärer et al. 2004, ovaries: Schärer et al. 2005). However, because body area was strongly correlated with ovary area, we used residual ovary area for the statistical analysis ($R^2 = 0.42$, $N = 180$, $P < 0.001$). Though the correlation of body area with testis area was also significant, residual testis area did not yield different results and was therefore not used in the analysis.

To investigate changes in sex allocation we used testis and ovary areas as well as the ratio of testis area over the sum of testis and ovary areas to obtain a measure of relative male allocation. Proportional male investment is a popular definition of sex allocation (e.g., Charnov 1982; Klinkhamer et al. 1997; Pen and Weissing 2002). It is a useful approach for comparisons between individuals as it separates budget effects from relative allocation decisions, which is important when the correlation between absolute male and female investment is positive due to budget effects, as in this study (Pearson: $r = 0.573$, $N = 180$, $P < 0.001$).

To check whether mating group size had the expected effect on seminal vesicle, testis and ovary areas, and relative male alloca-tion, we performed a fully factorial 3−way-ANOVA with original group size (PRE), final group size (POST), and measurement day (DAY) as fixed factors.

Results

BODY AREA

Body area remained relatively constant during the experiment (Fig. 1). Yet, initial group size had a significant effect, because worms originating from octets were slightly bigger (PRE: $F = 4.6$, df $= 1, 164$, $p = 0.034$). However, this is possibly because 8to2 individuals showed a temporary increase in body size on day 7 whereas the opposite effect was visible in 2to8 worms, which also led to significant interaction terms of both the original and the final group sizes with measurement day (PRE*DAY: $F = 3.1$, df $= 3, 164$, $p = 0.03$; POST*DAY: $F = 3.4$, df $= 3, 164$, $p = 0.02$). Yet, no clear difference is visible between the control groups 2to2 and 8to8, which suggests that the effect of initial group size must in fact have been marginal. No overall temporal effect on body area was detectable (see also Appendix for non-significant values).

SEMINAL VESICLE AREA

Seminal vesicle area was clearly and exclusively determined by final group size (POST: $F = 54.5$, df $= 1, 164$, $P < 0.001$) (Fig. 1). Worms in pairs had significantly larger seminal vesicles than worms from octets, and this difference was independent from the initial group size (see Appendix). The immediate adjustment of seminal vesicle area to the new group size suggests that more sperm are given away in octets. Hence the experimental manipulation clearly had the desired effect of regulating sperm expenditure with group size. Interestingly, the 8to2 group showed a rhythmic pattern with exceptionally big seminal vesicles at time points 2 and 4. Possibly, testis size and thus sperm quantity in these worms was still being down-regulated from the originally large mating group size, resulting in excessive sperm production and overfilled seminal vesicles.

RELATIVE TESTIS AREA

All three main factors had clear effects on relative testis area (PRE: $F = 33.7$, df $= 1, 164$, $P < 0.001$; POST: $F = 14.9$, df $= 1, 164$, $p < 0.001$; DAY: $F = 4.4$, df $= 3, 164$, $p = 0.005$) (Fig. 1). Both initial and final group sizes had the effect that relative testis area was smaller in worms from pairs than in worms from octets, meaning that this trait generally increased in size from 2to2 individuals, to 2to8, then to 8to2, and finally to 8to8 worms, which had the largest relative testis area. Worms from experimental groups gradually increased (2to8) or decreased (8to2) their relative male allocation over time, whereas no temporal change was visible in the control groups (Fig. 1; see also Appendix). Yet, the interaction of time with initial group size or final group size was not significant due
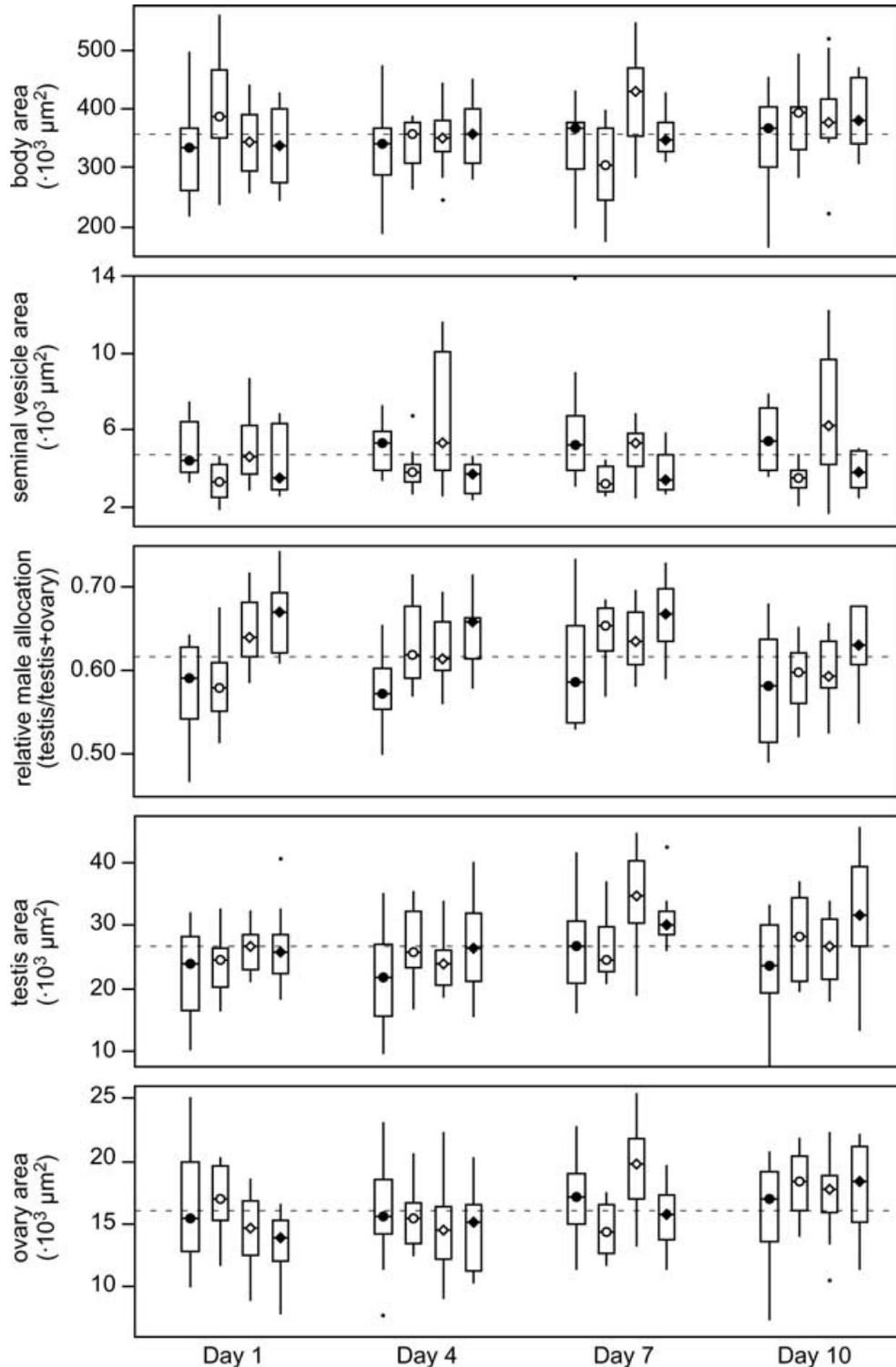
Figure 1. Effect of initial and final group size (2to2, closed circles; 2to8, open circles; 8to2, open diamonds; 8to8, closed diamonds), and sampling day on body area, seminal vesicle area, relative male allocation, testis area, and ovary area. Stippled lines indicate the grand mean of the response. The box plots indicate the medians and the quartiles, and the whiskers indicate the range that includes 1.5 times the interquartile range (points outside these whiskers could be outliers). Compare closed symbols to visualize the effects between control treatments, compare circles to visualize the effects of increased mating group size, and compare diamonds to visualize the effects of decreased mating group size. Measurements were taken 1, 4, 7, and 10 days after transfer into new group sizes. Note that the figure presents raw data, whereas it was necessary to use residual ovary area in the statistical analysis due to a strong correlation of ovary area with body area.

to a sudden drop of groups 2to8, 8to2, and 8to8 from day 7 to day 10. This drop was also the reason for the overall time effect.

Note that in 2to8 worms the increase in relative male allocation goes along with a reduction of body size, whereas in 8to2 worms the decrease in relative male allocation goes along with an increase in body size (Fig. 1).

TESTIS AREA

The effects of the main factors on testis area were qualitatively similar to those of relative testis area, though less pronounced (PRE: $F = 13.2$, df $= 1$, 164, $P < 0.001$; POST: $F = 4.7$, df $= 1$, 164, $p = 0.032$; DAY: $F = 6.3$, df $= 1$, 164; $P < 0.001$). When separately considering initial as well as final group size as factors, testis area was bigger in octets than in pairs and changes proceeded slowly over time (Fig. 1). However, the experimental groups showed a sudden reverse response on day 7, which had the effect that no interaction was visible between two factors. However, the overall interaction of all three factors was close to significant (PRE*POST*DAY: $F = 2.6$, df $= 3$, 164, $p = 0.054$) (App. 1).

OVARY AREA

Ovary area showed the opposite effect to testis and relative testis area, with biggest trait size in 2to2 and 8to2, intermediate sizes 2to8, and smallest in 8to8 (PRE: $F = 10.0$, df $= 1$, 164, $p = 0.002$; POST: $F = 4.0$, df $= 1$, 164, $p = 0.048$; DAY: $F = 6.3$, df $= 1$, 164; $P < 0.001$) (Fig. 1). Changes also occurred slowly between day 1 and day 10, but interaction effects of time with initial or final group size were not significant (see also Appendix). This is probably because the overall increase in ovary area in all treatment groups masked the less pronounced reverse trends between pairs and octets.

Discussion

The data for relative testis area strongly suggest that *M. lignano* can, even after having reached maturity, adjust its sex allocation to current mating group size. The alternative explanation that relative male allocation was determined by differences in resource budgets seems unlikely even though feeding was not controlled. The control groups clearly show that this trait does not correlate with body size (Fig. 1). Also density-related differences between the treatment groups do not explain sex allocation in *M. lignano*, as has been shown in a previous study (Schärer and Ladurner 2003).

In fact the data also show that the direction of change of phenotypically flexible sex allocation in *M. lignano* is the same as predicted by sex allocation theory for evolutionary adjustment of sex allocation, which means that it is more female-biased in smaller mating groups, or more male-biased in larger mating groups, respectively (Charnov 1980, 1982; Fischer 1981, 1984). When looking at the absolute measures, the effects on testis area are

clearer than those on ovary area. One reason for this might be that sperm competition acts on the male function first, with the result that the response in testis size is faster and stronger than the response in ovary size, which might only be the consequence of a change in the male function. Another reason might be that the trade-off between male and female function is masked by a budget effect (Schärer et al. 2005). Due to ad libitium, feeding resource budgets in the worms might be so high that an increase in, for instance, the male function does not need to be compensated by a decrease in the female function, but that an increased investment can be paid using additional reserves. Nevertheless, by looking at the relative investment in male versus female function, we can make the expected changes in sex allocation visible.

Interestingly, the sex allocation adjustment in the experiment seems to cover the whole time span of 10 days, which appears to be rather slow. On the other hand *M. lignano* reacts directly to changing mating group size by adjusting the mating rate, as can be inferred from the seminal vesicle data. Together, this suggests that the worms are physiologically constrained to act faster, and not that they are hampered by their perception.

Because facultative sex allocation in *M. lignano* is slow, it might be rather costly. Depending on the frequency of environmental fluctuation, a too slow response might be highly inefficient because it results in a too long period with suboptimal sex allocation (Piersma and Drent 2003; DeWitt et al. 1998). Other costly aspects of phenotypic flexibility in general might include the risk of wrong allocation decisions, or the maintenance of genetic and physiologic adaptations that are necessary to perceive external changes and to translate them into a physiological response. Moreover, flexibility may increase the susceptibility to manipulation. In separate-sexed species (gonochorists) a large number of sex ratio distorters are known that produce male- or female-biased sex ratios by very different mechanisms (Stouthamer et al. 2002). In hermaphrodites sex allocation manipulators may also encompass mating partners, which want to enhance the opponent's female allocation to increase their own paternal reproductive success (Michiels 1998).

The fact that phenotypically flexible sex allocation in *M. lignano* is still existent in spite of the many possible disadvantages named above suggests that below the line there must be significant advantages (DeWitt et al. 1998). Generally, individuals that can permanently and reversibly adjust certain traits to a rapidly and unpredictably changing environment might experience a selective advantage (Piersma and Drent 2003). Yet, it is important to note that nothing is really known about natural densities and mating group sizes in *M. lignano*, let alone its sex allocation strategy in the field. Nevertheless, it is interesting to speculate about the possible relevance of our findings. One important question is whether phenotypically flexible sex allocation is the consequence of evolutionary competition between

conspecific hermaphrodites, or whether it might also represent the mechanism by which hermaphrodites can outcompete gonochorists. The role of reversible sex allocation in the evolution of hermaphroditism is an interesting idea, because it helps to explain hermaphroditism in situations where existing theories do not seem to hold. Typically, hermaphroditism is explained to be favored over gonochorism when male gain curves are saturating (Charnov 1976, 1979, 1982), which is the case at low densities or in small populations (Ghiselin 1969; Heath 1977; Puurtinen and Kaitala 2002). Yet, empirical studies indicate that at least seasons of high population densities, likely to lead to increased sperm competition, are common in a number of species (e.g., Fischer 1980, 1981; Vreys and Michiels 1997; Michiels 1998; Michiels and Bakovski 2000; Schärer and Wedekind 2001; N. Anthes and N. K. Michiels, pers. obs. 2003). Sperm competition linearizes the male gain curve (Yund 1998), creating a situation where established theories for hermaphroditism do not hold (Michiels 1998). Facultative sex allocation could allow hermaphrodites to outcompete gonochorists also when sperm competition is high, because it is advantageous in habitats where mating rates fluctuate frequently and unpredictably. As a first important step to clarify the role of opportunistic sex allocation for the evolution of hermaphroditism, one would have to find out whether it is a basic characteristic of outcrossing hermaphroditic species.

Conclusions

The experiment we present here provides two important results. First, the simultaneous hermaphrodite *Macrostomum lignano* shows phenotypically flexible sex allocation in response to changes in mating group size during its adult life. Second, the findings are consistent with theory for the evolutionary adjustment of sex allocation in simultaneous hermaphrodites, because also in the case of fully mature individuals the increase in group size resulted in a change to more male-biased sex allocation, the decrease in more female-biased sex allocation. It seems less likely that these results are caused by differences in resource budgets, or other possible factors. In the context of separate versus combined sexes, we argue that opportunistic sex allocation provides a possible explanation for hermaphroditism in large and dense populations, if organisms experience frequent changes in mating group size. Yet, because little is known about the field situation of these worms and of hermaphrodites in general, it remains to be shown that facultative sex allocation plays an important role in nature.

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Appendix 1. Three-way ANOVA for body area, seminal vesicle area, relative male allocation, and testis and residual ovary areas with initial group size (PRE), final group size (POST), and measurement day (DAY) as fixed factors. Significant values are mentioned and discussed in the results and are indicated in the table as * (* for $0.05 > p \geq 0.01$; ** for $0.01 > p \geq 0.001$; * for $p < 0.001$).**

	Body area			Seminal vesicle area			Relative testis area			Testis area			Residual ovary area		
	F	df	*p*	*F*	df	*p*	*F*	df	*p*	*F*	df	*p*	*F*	df	*p*
PRE	4.6	1,164	*	1.9	1,164	0.17	33.7	1,164	***	13.2	1,164	***	10.0	1,164	**
POST	0.1	1,164	0.81	54.5	1,164	***	14.9	1,164	***	4.7	1,164	*	4.0	1,164	*
DAY	1.7	3,164	0.16	0.8	3,164	0.49	4.4	3,164	**	6.3	3,164	***	3.9	3,164	**
PRE*POST	2.8	1,164	0.10	0.0	1,164	0.89	0.1	1,164	0.71	0.8	1,164	0.38	0.1	1,164	0.77
PRE*DAY	3.1	3,164	*	0.9	3,164	0.44	2.5	3,164	0.07	1.2	3,164	0.31	1.3	3,164	0.27
POST*DAY	3.4	3,164	*	0.67	3,164	0.57	0.9	3,164	0.41	2.6	3,164	0.05	1.7	3,164	0.17
PRE*POST*DAY	0.5	3,164	0.67	1.7	3,164	0.16	1.0	3,164	0.41	0.2	3,164	0.86	0.2	3,164	0.91