The Pex16p Homolog SSE1 and Storage Organelle Formation in *Arabidopsis* Seeds

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Mature *Arabidopsis* seeds are enriched in storage proteins and lipids, but lack starch. In the *shrunken seed 1* (*sse1*) mutant, however, starch is favored over proteins and lipids as the major storage compound. SSE1 has 26 percent identity with Pex16p in *Yarrowia lipolytica* and complements *pex16* mutants defective in the formation of peroxisomes and the transportation of plasma membrane– and cell wall–associated proteins. In *Arabidopsis* maturing seeds, SSE1 is required for protein and oil body biogenesis, both of which are endoplasmic reticulum–dependent. Starch accumulation in *sse1* suggests that starch formation is a default storage deposition pathway.

To support young seedling growth flowering plants deposit storage compounds (principally composed of carbohydrates, proteins, and lipids) in their seeds. Cereal plants deposit relatively more carbohydrates, whereas legume seeds and oilseeds contain relatively more proteins and lipids, respectively (*1*). Understanding the molecular mechanisms underlying the cellular differentiation programs involved in storage deposition would allow better manipulation of seed quality and the nutritional value of crop seeds.

In *Arabidopsis*, proteins and lipids are the major reserves in mature seeds (*2*) (Fig. 1, A and B). We have isolated a *shrunken seed 1* (*sse1*) mutant that alters this seed storage profile, accumulating starch over proteins and lipids (Fig. 1, C and D). The cotyledon and the hypocotyl cells of *sse1* contain no recognizable protein bodies and few oil bodies. Starch granules, membrane stacks, vesicles, and vacuoles, all of which are absent in wild-type cells, are present in *sse1* cells, and the oil bodies in *sse1* contain higher electron density substances than the wild type. The *sse1* seeds shrink upon desiccation (a likely consequence of insufficient deposition of storage molecules), whereas the wild-type seeds are desiccation tolerant (Fig. 1, E and F).

The *sse1* mutant was identified in a transferred DNA (T-DNA) transgenic line [T line (*3*)] that exhibited the shrunken seed phenotype. Among the T2 seeds on the T1 plant (the primary transgenic plant), 90% of the seeds were shrunken and 10% were normally rounded. The shrunken seeds were not viable, and plants grown from the round seeds produced ~90% shrunken seeds. This pattern of inheritance continued for generations after self-pollination (*4*). However, after backcrossing the T2 line to wild-type plants, *sse1* behaved as a typical single-recessive Mendelian gene (*5*) and, when the segregation patterns of F_3 families were observed, *sse1* cosegregated with the T-DNA (*6*). The *SSE1* gene and its cDNA were cloned (*7*), and the sequences obtained were used to design three primers for determining the genotypes of shrunken and round seeds by single-seed polymerase chain reaction (PCR) (*8*) (Fig. 2A). Sixty-six percent ($n = 6$) of round F_2 seeds were heterozygous and 33% were homozygous for the wild-type allele, whereas 100% ($n = 5$) of shrunken F_2 seeds were homozygous for the T-DNA insertion (Fig. 2B). T4 seeds were also analyzed. All round seeds ($n = 13$) were heterozygous and all shrunken seeds ($n = 21$) were homozygous for the T-DNA insertion (Fig. 2C). Thus, *sse1* is recessive and in the self-pollinated T line, the mutant allele is transmitted at a higher frequency than the wild-type allele (*9*).

The *SSE1* cDNA (GenBank accession number AF085354) encodes a predicted protein of 367 amino acids (Fig. 3A). Expression of *SSE1* cDNA in transgenic *sse1* plants (*10*) complements the shrunken seed phenotype (Fig. 3B). Similar to wild type, seeds are tolerant of desiccation and cells are filled with storage proteins and lipids, but lack starch. The SSE1 sequence showed similarity to Pex16p, a membrane-associated protein required for the assembly and proliferation of peroxisomes (*11*) and for the trafficking of plasma membrane– and cell wall–associated proteins (*12*), in the yeast *Y. lipolytica* (Fig. 3A). Pex16p is glycosylated and transiently localized in the endoplasmic reticulum (ER) (*13*). Despite the limited amino acid sequence similarity (26% identity), the two proteins have similar arrangements of their hydrophobic and hydrophilic regions (Fig. 3A). A predicted glycosylation site was found in SSE1 (Fig. 3A). SSE1 complements the growth of *pex16* mutants on oleic acid as sole carbon source (Fig. 3C), indicating restoration of peroxisomal function (*11*). The restoration of limited growth of the disruption allele *P16KO-8A* (*11*) indicates that SSE1 cannot fully replace Pex16p in peroxisome formation, probably because of the functional difference (or differences) between the two proteins. In addition, SSE1 partially complemented the *pex16-1* mutant for the dimorphic transition from yeast to the mycelia form (Fig. 3D). Pex16p is normally re-

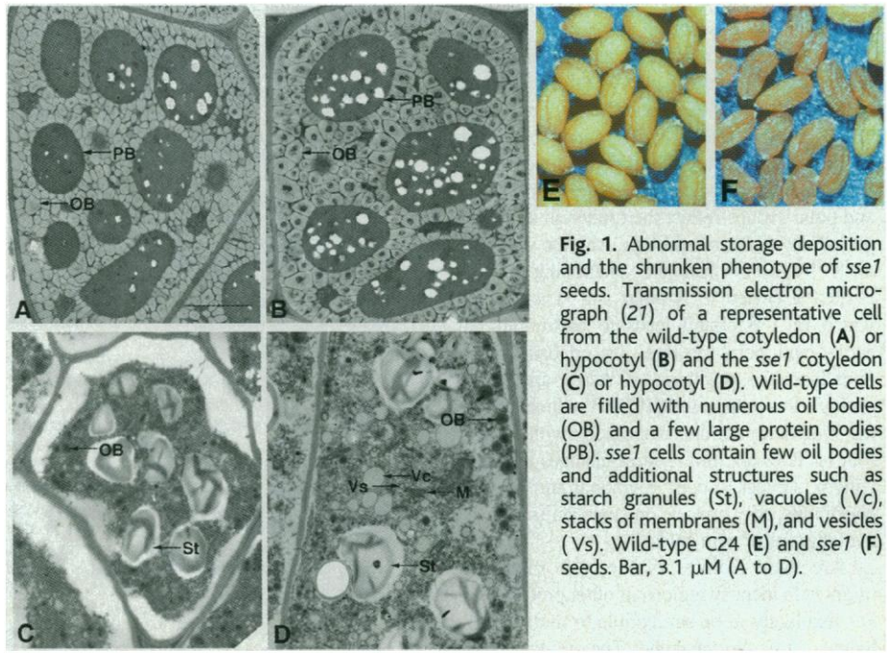

Fig. 1. Abnormal storage deposition and the shrunken phenotype of *sse1* seeds. Transmission electron micrograph (*21*) of a representative cell from the wild-type cotyledon (**A**) or hypocotyl (**B**) and the *sse1* cotyledon (**C**) or hypocotyl (**D**). Wild-type cells are filled with numerous oil bodies (OB) and a few large protein bodies (PB). *sse1* cells contain few oil bodies and additional structures such as starch granules (St), vacuoles (Vc), stacks of membranes (M), and vesicles (Vs). Wild-type C24 (**E**) and *sse1* (**F**) seeds. Bar, 3.1 μM (A to D).

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quired for mycelia phase–specific cell surface protein transport.

Peroxisomes are not generally found in dry seeds (*14*) (Fig. 1). Protein and oil bodies are the most abundant organelles in mature *Arabidopsis* seeds, and the formation of both is ER-dependent (*2*, *15*, *16*). By analogy to the function of Pex16p in peroxisome assembly and cell surface protein transport, SSE1 could participate in oil body formation and storage protein delivery. The vesicles and stacks of membranes in the *sse1* cells (Fig. 1D) resemble the subcellular structures in the *pex16-1* mutant of *Y. lipolytica* (*11*). The similarities between oil body and peroxisome biogenesis are consistent with their related functions in germinating seedlings of fat-metabolizing plants, where oil bodies are broken down by glyoxysomes (*14*).

SSE1 gene expression was analyzed by competitive reverse transcription–polymerase chain reaction (RT-PCR) (Fig. 4). The amount of *SSE1* mRNA is reflected in the target-to-competitor cDNA ratio (*17*). *SSE1* steady-state mRNA level in the siliques increases during seed maturation to a maximum in mature 19- and 21-day-old brown siliques. The level of mRNA is also high in cotyledons of germinating seedlings and flowers, but low in expanding leaves and roots. Glyoxysomes are assembled in germinating seedlings (*14*); therefore, SSE1 is likely to be required in this process. The low expression in expanding leaves, where leaf peroxisomes are formed, may be due to low peroxisome abundance. Alternatively, SSE1 may not normally be involved in peroxisome and glyoxysome formation; rather, expression in germinating seedlings may be required for maintenance of the remaining oil bodies. The high expression levels in flowers suggests additional functions of SSE1, possibly the formation of oil body–like organelles in tapetum and pollen (*16*).

Efficient use of limited amounts of assimilates for seed storage deposition requires coordinated metabolic pathways and organelle assembly. In *sse1* mature embryos, cotyledon and hypocotyl cells accumulate excess starch (Fig. 1). The functional similarity of SSE1 and Pex16p argues against SSE1 being a direct inhibitor of starch synthesis; rather, it implies that protein and oil body proliferation repress starch accumulation. Starch accumulation may also be a secondary effect of the *lec* mutations (*18*). Consistent with the observations in *Arabidopsis*, simultaneous reduction in storage proteins and increase in starch content were also observed in a soybean shriveled seed mutant (*19*). Thus, in at least some species of flowering plants, starch accumulation maybe a default storage deposition pathway during seed development.

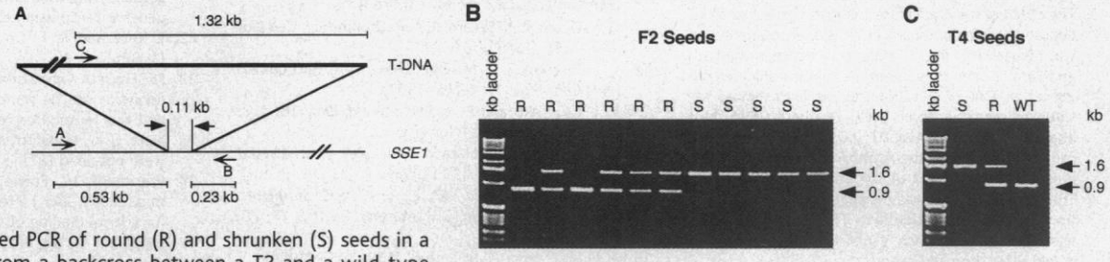

Fig. 2. Genotype determination by single-seed PCR. (**A**) PCR diagram. Primers A and B amplify a 0.9-kb fragment from the wild-type *SSE1* allele. Primers C and B amplify a 1.6-kb fragment from the T-DNA interrupted *sse1* allele. (**B**) Single-seed PCR of round (R) and shrunken (S) seeds in a F_2 population derived from a backcross between a T2 and a wild-type plant. (**C**) Representative single-seed PCR results of round (n = 13) and shrunken (n = 21) T4 seeds. A control reaction from a wild-type (WT) seed is also shown.

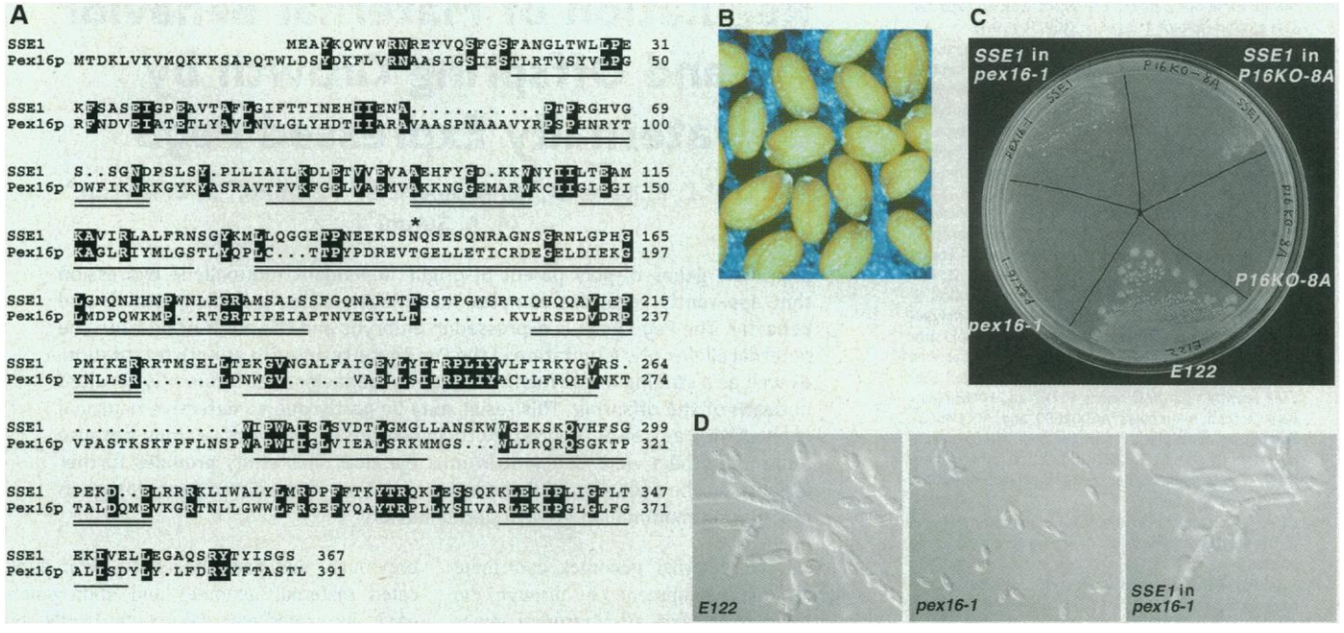

Fig. 3. SSE1 amino acid sequence analysis and *SSE1* complementation of the *Arabidopsis sse1* and *Y. lipolytica pex16* mutants. (**A**) Alignment of SSE1 and Pex16p. Dots indicate gaps. Identical residues are boxed. Hydrophobic (single line) and hydrophilic (double line) regions for both proteins are underlined (*22*). The predicted glycosylation site of SSE1 is indicated with an asterisk. Single-letter abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr. (**B**) *sse1* seeds complemented by the *SSE1* transgene (*10*). (**C**) *SSE1* complementation of *pex16* mutants *pex16-1* and *P16KO-8A* (*11*) for growth on oleic acid as sole carbon source (*23*). *E122* is the wild-type strain. (**D**) *SSE1* complementation of *pex16-1* mutant for the dimorphic transition from yeast to mycelia form. Cells were grown at 30°C in YND liquid medium (*11*). The *SSE1* transformant underwent dimorphic transition at a lower frequency than the wild-type strain *E122*.

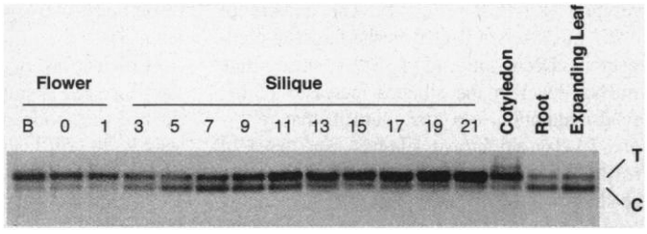

Fig. 4. Competitive RT-PCR analyses of *SSE1* expression profiles. RNA was isolated from flowers before (B), on the day of (0), or 1 day after pollination (1); from siliques 3 to 21 days after pollination; from cotyledons of 2-day-old seedlings; and from expanding rosette leaves and roots. An equal amount of competitor cDNA template was included in each reaction. The *SSE1* target (T)-to-competitor (C) cDNA ratios reflect the relative expression levels of the *SSE1* gene (*17*).

References and Notes

1. D. J. Bewley and M. Black, *Seeds: Physiology of Development and Germination* (Plenum, New York, ed. 2, 1994), chap. 1; A. Vitale and R. Bollini, in *Seed Development and Germination*, J. Kigel and G. Galili, Eds. (Dekker, New York, 1995), pp. 73–102; M. Miquel and J. Browse, in *ibid.*, pp. 169–193.
2. S. G. Mansfield and L. G. Briarty, *Can. J. Bot.* **70**, 151 (1992).
3. The cDNA of the *Arabidopsis* prohibitin gene *Atphb1* (L. Sun and H. M. Goodman) in an antisense orientation was inserted into pBI121 (Clontech) between the Sac I and Bam HI sites to replaced the β-glucuronidase coding region. One of 49 C24 transgenic lines showed the shrunken seed phenotype (*sse1*). Northern (RNA) blot analysis with an *Atphb1* cDNA bottom-strand probe showed that the *Atphb1* mRNA level in this line is similar to that of the wild type.
4. The transgenic line was propagated by self-pollination for four generations (to T5). Ten round seeds were grown from each generation, and each produced 90% shrunken seeds.
5. T2 plants derived from round seeds were crossed reciprocally to wild-type C24 plants. All F_1 seeds were round. The numbers of shrunken/round F_2 seeds in six single siliques were 11/39, 15/36, 12/39, 10/42, 11/36, and 10/40; these numbers are consistent with an expected segregation ratio of 1:3 ($\chi^2 = 0.64$, $P > 0.1$).
6. Two hundred and sixty F_3 seed families were obtained from individual F_2 round seeds; 180 families segregated for both the shrunken and kanamycin-resistant (conferred by the T-DNA) phenotypes, whereas the other 80 showed neither phenotype.
7. A 136–base pair (bp) genomic DNA fragment flanking the T-DNA was isolated by the thermal asymmetric interlaced–polymerase chain reaction (*20*). A 17-kb genomic fragment isolated from a λ-FIXII *Arabidopsis* C24 genomic library (L. Sun and H. M. Goodman) was used as a probe to screen an *Arabidopsis* cDNA library [M. Minet, M.-E. Dufour, F. Lacroute, *Plant J.* **2**, 417 (1992)]. The two *SSE1* cDNA clones have identical 5′ ends, and both include the stop codon. The 3′ polyadenylation site was determined by 3′ RACE (rapid amplification of cDNA ends) PCR. Newly released bacterial artificial chromosome (BAC) sequences revealed that *SSE1* is within the BAC clones F17K2 and F4I18 (GenBank accession numbers AC003680 and AC004665). The SSE1 protein predicted by the open reading frame differs from the F17K2.22 hypothetical protein as a result of discrepancies between the predicted and the actual splicing sites.
8. DNA was isolated from single embryos after removal of the seed coat, which has the same genotype as the parent.
9. F_1 seeds were obtained from reciprocal crosses between a round seed–derived T3 plant and a wild-type plant. Their genotypes were examined to determine the genotypes of the gametes from the T3 plant. The wild-type allele was present in 3 out of 39 eggs and 0 out of 51 sperms; the rest of the gametes carried the mutant allele. Therefore, the T4 progeny would be either homozygous *sse1* (36/39 = 92%) or heterozygous (3/39 = 8%).
10. The *SSE1* cDNA was fused with the 35S promoter (*35SP*) and the nopaline synthase 3′ region (*NOS* 3′). The *35SP-SSE1-NOS*3′ cassette was subcloned into the Kpn I site of the pLVN19R binary vector to make the pLVN19R-*SSE1* construct. T3 plants from round seeds were vacuum-infiltrated with *Agrobacterium tumaficiens* strain GV3101 [N. Bechtold, J. Ellis, G. Pelletier, *C. R. Acad. Sci. Paris Life Sci.* **316**, 1194 (1993)] carrying pLVN19R-*SSE1*. Genotypes of seven methotrexate-resistant transgenic plants were determined by PCR, and six were homozygous *sse1*. Four transgenic *sse1* plants were fertile and produced complemented T2 seeds at 67 to 87%.
11. G. A. Eitzen, R. K. Szilard, R. A. Rachubinski, *J. Cell Biol.* **137**, 1265 (1997).
12. V. I. Titorenko, D. M. Ogrydziak, R. A. Rachubinski, *Mol. Cell. Biol.* **17**, 5210 (1997).
13. V. I. Titorenko and R. A. Rachubinski, *ibid.* **18**, 2789 (1998).
14. L. J. Olsen and J. J. Harada, *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **46**, 123 (1995).
15. M. J. Chrispeels, *ibid.* **42**, 21 (1991); C. Sarmiento, J. H. Ross, E. Herman, D. J. Murphy, *Plant J.* **11**, 783 (1997).
16. A. H. C. Huang, *Plant Physiol.* **110**, 1055 (1996).
17. After deoxyribonuclease treatment, 1 μg of RNA was reverse transcribed in a 20-μl reaction, with 0.4 μM of the *SSE1*-specific primer FP15R (5′-GGCAATATTCTTCCGTTGC-3′). Subsequently, 1 μl of the reverse-transcription mixture and 5×10^{-21} mol of competitor cDNA were used in each 20-μl PCR reaction. The competitor cDNA is identical to the *SSE1* cDNA (designated target cDNA) except for a 95-bp internal deletion from the Eco RI to the Nco I site. The primers FP7 (5′-AAAAATGGAACTACATTATTCTC-3′) and FP14R (5′-ATAAGTAAAACGCTTAACCTHC-3′) amplify 814- and 719-bp fragments, respectively, from the target and the competitor cDNAs. The ratio of the two PCR products reflects the relative amount of *SSE1* cDNA (or mRNA) in each sample [P. D. Siebert and J. W. Larrick, *Nature* **359**, 557 (1992)].
18. D. W. Meinke, L. H. Franzmann, T. C. Nickle, E. C. Yeung, *Plant Cell* **6**, 1049 (1994).
19. Z. Chen, H. Ilarslan, R. G. Palmer, R. C. Shoemaker, *Am. J. Bot.* **85**, 492 (1998).
20. Y.-G. Liu, N. Mitsukawa, T. Oosumi, R. F. Whittier, *Plant J.* **8**, 457 (1995).
21. Mature wild-type C24 and once-backcrossed *sse1* seeds were used. *sse1* seeds were imbibed in water for 20 min before processing. Seeds were cut into halves and fixed in 2.5% glutaraldehyde–0.1 M cacodylate (pH 7.2), post-fixed in 1% osmium tetroxide, dehydrated in an ethanol series, and embedded in Spurr's resin. Thin sections were stained with uranyl acetate and observed under a transmission electron microscope.
22. J. Kyte and R. F. Doolittle, *J. Mol. Biol.* **157**, 105 (1982).
23. *SSE1* cDNA was cloned into the Eco RI site of a *Y. lipolytica* shuttle vector pTc3 between the promoter and the terminator regions of *Y. lipolytica* thiolase gene. Ura^+ transformants of *pex16-1* and *P16KO-8A* were obtained (*11*).

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Regulation of Maternal Behavior and Offspring Growth by Paternally Expressed *Peg3*

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Imprinted genes display parent-of-origin–dependent monoallelic expression that apparently regulates complex mammalian traits, including growth and behavior. The *Peg3* gene is expressed in embryos and the adult brain from the paternal allele only. A mutation in the *Peg3* gene resulted in growth retardation, as well as a striking impairment of maternal behavior that frequently resulted in death of the offspring. This result may be partly due to defective neuronal connectivity, as well as reduced oxytocin neurons in the hypothalamus, because mutant mothers were deficient in milk ejection. This study provides further insights on the evolution of epigenetic regulation of imprinted gene dosage in modulating mammalian growth and behavior.

Maternal and paternal genomes contribute unequally to development (*1*) through the monoallelic expression of imprinted genes that affect embryonic and placental development, as well as behavior in mice (*2*). It is possible that complex behavioral output of the central nervous system (CNS) might be a common function of a disparate group of neurally expressed imprinted genes such as *Mest* and *Peg3*. In this context, we showed previously that parthenogenetic (PG: duplicated maternal genome) and androgenetic (AG: duplicated paternal genome) cells contribute unequally to the formation of the CNS in chimeric mice (*3*). PG cells contribute more to the cortex and striatum, whereas AG cells contribute to the hypothalamus. The imprinted gene *Mest* of paternal origin is expressed in the hypothalamus and functions in regulating growth and maternal behavior