Polyembryony in Armadillos

An unusual feature of the female nine-banded armadillo's reproductive tract may explain why her litters consist of four genetically identical offspring

W. J. Loughry, Paulo A. Prodöhl, Colleen M. McDonough and John C. Avise

Observant travelers passing through the southern U.S. may be struck by the unusual appearance of the nine-banded armadillo (*Dasypus novemcinctus*). This animal, which is about three-quarters of a meter long, can be identified by the leathery plates of armor that cover it nearly from the tip of its nose to the end of its tail. It also has a series of transverse plates that wrap around its midsection, giving it a banded look. Odd as its appearance may be, though, a nine-banded armadillo is even more unusual in its reproductive habits.

Animals create offspring in a variety of ways. In some species, an adult makes identical copies of itself asexually through budding or *parthenogenesis*. In other animals, including vertebrates, gametes from a male and female fuse in sexual reproduction to produce offspring. A form of reproduction that combines features of both these strategies is called *polyembryony*, in which one sexually produced embryo splits into two or more. Many animals, including humans, are occasionally polyembryonic, as can be seen in the birth of identical twins, triplets and so on. In a review of the occurrence of polyembryony, Sean Craig and his colleagues at the State University of New York at Stony Brook showed that it is the norm in a variety of animals, including parasitic wasps, certain flatworms and various aquatic invertebrates. Among vertebrates, however, only the six species of armadillos in the genus *Dasypus* are always polyembryonic. In the nine-banded armadillo, which is the best studied, females typically give birth to litters of genetically identical (which also means same sex) quadruplets.

As pointed out by Craig and his colleagues, polyembryony is an odd, and seemingly costly, way to produce offspring because it lacks the advantages of sexual and asexual reproduction, and it suffers from some seemingly substantial costs. For example, sexual reproduction creates genetically variable offspring, some of which may be better adapted to a changing environment, but polyembryony generates offspring with only one genotype to confront environmental change. Parthenogenesis allows rapid replication of a successful genotype—the mother's—for a particular environment. Polyembryonic species lose that benefit because their young are produced sexually, which scrambles whatever good genetic combinations might have been passed from the mother. In the words of Craig and his colleagues, it is as if polyembryonic animals are "purchasing multiple tickets of the same lottery number, even though there is no reason to prefer one number over another."

Given those problems, how could such a bizarre system have evolved? Craig and his colleagues identified two main possibilities. First, many polyembryonic species are parasites. In such cases offspring develop in a host's body, where they are not in contact with the mother. Consequently, an embryo can "judge" environmental conditions and then divide into the number of offspring that optimizes resources per individual. So polyembryony might evolve when offspring have more information about optimal clutch size than their parents do.

Although this hypothesis might explain the evolution of polyembryony in many species, it does not account for all of them. In the remaining cases, Craig and his colleagues proposed that polyembryony might be favored as a means of increasing the number of offspring when sperm are limited or there are constraints on the production of eggs or embryos by females. In those cases, polyembryony is viewed as an adaptation by a parent to increase its reproductive success given certain constraints of its reproductive physiology.

Polyembryony might also provide us with insight into an intriguing evolutionary puzzle: altruistic behavior, in which one animal suffers a cost to aid another. For example, an animal might give an alarm call to warn other group members, despite the fact that the noise advertises the caller's position to a predator. Simple Darwinian logic predicts that selfish individuals who remain silent would fare better, in terms of survival and reproduction, than altruists. In the early 1960s William Hamilton, now at Oxford University, proposed a solution to the seeming incompatibility between evolutionary success and altruistic behavior in some species. He pointed out that animals can pass their genes to the next generation in two ways: directly by producing off-

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Figure 1. Nine-banded armadillos inhabit areas from northern Argentina to the southern U.S. These armadillos and five closely related species always reproduce through polyembryony, in which one sexually produced embryo splits into two or more. These armadillos have a uniquely shaped uterus that has such a small implantation site that it can accommodate only one blastocyst—seemingly limiting the production of offspring to one per breeding season. Perhaps polyembryony evolved in the animals to bypass that constraint and increase their reproductive success.

spring, or indirectly by aiding genetic relatives. So an animal that puts itself at risk by emitting an alarm call may benefit if its kin are alerted and avoid a predator. When animals increase their evolutionary fitness by aiding relatives, biologists call that *kin selection*. Hamilton suggested that the probability of altruism should vary with the degree of relatedness between the individuals. So polyembryonic species, which produce genetically identical offspring, seem very likely to be altruistic.

The obligate polyembryony of the nine-banded armadillo spurred our interest in its ecology and evolution. After six years of work, we believe that an unusual characteristic of the female's reproductive tract may have promoted the evolution of polyembryony in armadillos.

Natural History

Today, 20 living species of armadillos are known, and 19 of them are confined to South and Central America. Nine-banded armadillos, however, are found from northern Argentina to the southern U.S. Armadillos have been expanding their range in the U.S. during the past 200 years. They were originally reported in southern Texas in the early 1800s, but since then they have been moving north and eastward, currently reaching southern Missouri and Arkansas. In addition, some captive armadillos were released in central Florida during the 1920s, and those animals spread throughout Florida and southern Georgia. The population of armadillos that we study—located on the Tall Timbers Research Station just north of Tallahassee, Florida—probably came from those captive armadillos. Populations that can be traced back to Florida and others that can be traced to Texas recently came into contact, probably somewhere in western Georgia or eastern Alabama.

Nine-banded armadillos are primarily nocturnal, but they become more active during the day as temperatures cool. As a relative of the anteater, the armadillo feeds on ants as well as other invertebrates that it finds while digging in the soil, but armadillos also eat some vegetable matter—including persimmons and other fruits—and will even scavenge on vertebrate carcasses. Armadillos seem to require access to fresh water, so they are typically found in swamps and other wet or waterside areas. Wherever armadillos live, they burrow. Adults are typically solitary, except during the breeding season, and

Figure 2. Litter of nine-banded armadillos consists of four offspring that are all of the same-sex and genetically identical. (Photograph courtesy of Colleen M. McDonough.)

usually do not share the same burrow with other adults. Juvenile littermates are more social: They forage together for at least some of their first summer above ground and continue to share the same burrows. In different populations, home-range sizes can vary greatly, from 1 to 20 hectares. In most cases, home ranges overlap considerably between sexes and between adult females, but not between reproductively active adult males.

In the U.S., armadillos typically breed during the summer, when males avidly follow females. Males are often observed paired with more than one female during a breeding season, but females typically are seen with a single male. After copulation, pairs split up and adults resume a solitary existence. Females can implant a fertilized egg sometime in the fall—usually long after copulation—and a litter of genetically identical offspring is born the following March or April. (Incidentally, a newborn armadillo has armor that is softer and more pliable, which must be a relief to a mother giving birth.) Juveniles emerge from their natal burrows between May and July, at which point they seem to be on their own. Juveniles usually are active earlier in the day than adults, and it is rare to see a juvenile near an adult. Littermates forage together and continue to share burrows, but litters appear to break up from dispersal or mortality by late summer or early fall.

Kin Selection

When animals increase their evolutionary fitness by aiding relatives, biologists call that *kin selection*. The operation of kin selection depends on a specific behavioral prerequisite: Individuals must be able to distinguish kin from nonkin, because an altruist's genes receive no benefit if the recipients are not relatives. Consequently, we needed to determine whether armadillos can distinguish relatives and nonrelatives.

As we shall discuss below, juvenile armadillos are most likely to encounter kin in our population. To test whether they could distinguish kin from nonkin, we ran two sets of experiments. In the first, we caught litters of juveniles in the wild and brought them in the laboratory. We housed each juvenile individually in a plastic tub for no more than 48 hours. We placed cotton pads in the tub to obtain odor samples from the urine, feces and scent glands of each juvenile. Then we tested the juveniles in a circular arena that had tape lines dividing it into quadrants. In one quadrant we placed a pad containing the scent of a littermate, and in the opposite quadrant we placed a pad containing the scent of a "strange" juvenile—one captured at least 1 kilometer away, making it unlikely to be closely related to the test subject. The pads were taped to the sides of the arena so that the animals had to lift their snouts to investigate, which gave us an easy measurement of their interest in each pad. We also measured the time a juvenile spent in each quadrant. We found that juveniles spent significantly more time near and investigated more often the pads that contained the scents of littermates as compared to stranger-scented pads—suggesting that armadillos can discriminate kin from nonkin.

For kin selection to work, however, animals must use their kin-recognizing ability to bias their behavior in favor of relatives. To see if that happens, we ran a second experiment in which two juveniles—either littermates or strangers—were placed in another arena and videotaped to record their interactions. We expected that littermates would interact amicably and remain in close proximity to one another and that strangers would

Dave Norris (Photo Researchers, Inc.)

Figure 3. Nine-banded armadillos dig in the soil in search of food, predominantly invertebrates. In addition these armadillos dig burrows, in which they live.

avoid each other and interact hostilely when they came in contact, but that did not happen. Instead, juveniles interacted amicably in all tests, and there were no significant differences in the behavior of juveniles toward littermates or strangers. The results from the behavioral-interaction tests led us to conclude that kin selection is unlikely to have shaped the behavior of juvenile armadillos.

There are some alternative explanations for those findings. Perhaps the test situation was so artificial that juveniles did not exhibit normal behavior. Or juveniles might possess an ability to discriminate kin from nonkin that is not used until later in life. That proposal may have some merit. For juveniles, there may be little advantage in treating littermates and nonlittermates differently. As juveniles age and establish home ranges and engage in reproductive behavior, however, such distinctions might become more important. Compared to juveniles, adults are much more likely to behave aggressively toward other individuals, and that aggression might be targeted preferentially at nonkin. So we turned our attention to the possibility that kin selection operates on adult armadillos.

For kin selection to be an important influence on adult behavior, adults must interact with kin. Each time we captured an armadillo, we determined its precise spatial location with a satellite-based global positioning system (GPS). We marked each animal with tags to allow future identification. We also obtained GPS readings of these marked animals whenever they were sighted again. Over the course of five field seasons (1992–1995 and 1997), we amassed over 750 sightings of nearly 400 animals. The animals typically moved less than 200 meters between sightings, regardless of the interval between observations or whether the animal was an adult or a juvenile. If armadillos stay put over time, as our sighting data suggest, that could promote the operation of kin selection because such limited movements should generate spatial clustering of littermates.

Data on spatial locations could be interpreted differently. Of the nearly 400 animals captured during our study, only about one-third were seen again. So most animals disappeared from our view, presumably through death or emigration. Of particular interest regarding kin selection is the subsequent fate of juveniles. Resightings of animals marked as juveniles indicate whether they get recruited into their natal population. Only 20 of 106 animals caught as juveniles between 1992 and 1995 were recaptured in a subsequent year. Interestingly, 15 of those animals were male, suggesting that male armadillos may be more likely to remain in their natal population than are females, in contrast to the typical mammalian pattern. Of the 20 recaptures, we found three cases, all male, where more than one member of the same litter was recruited into our population.

Where had all the juveniles gone? Some may have dispersed off the study site. After all, nine-banded armadillos have expanded their geographic range dramatically during this century, so

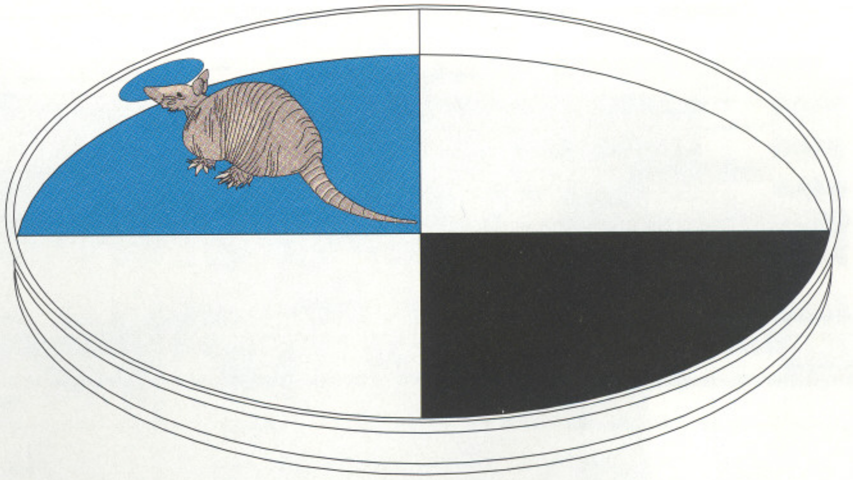

Figure 4. Kin selection, or one relative helping another to pass common genes to future generations, might exist in genetically identical quadruplets. The authors wondered if juvenile nine-banded armadillos could distinguish kin from nonkin. To find out, the authors placed juveniles in an arena that contained a cotton pad scented by a littermate *(blue)* and another pad scented by a stranger *(black)*. In these tests, the juveniles spent more time near the littermate-scented pads, indicating that they can recognize a sibling's odor. Nevertheless, further tests showed that juveniles interacted with strangers just as readily as they did with littermates, which suggests that kin selection might not affect the behavior of these animals.

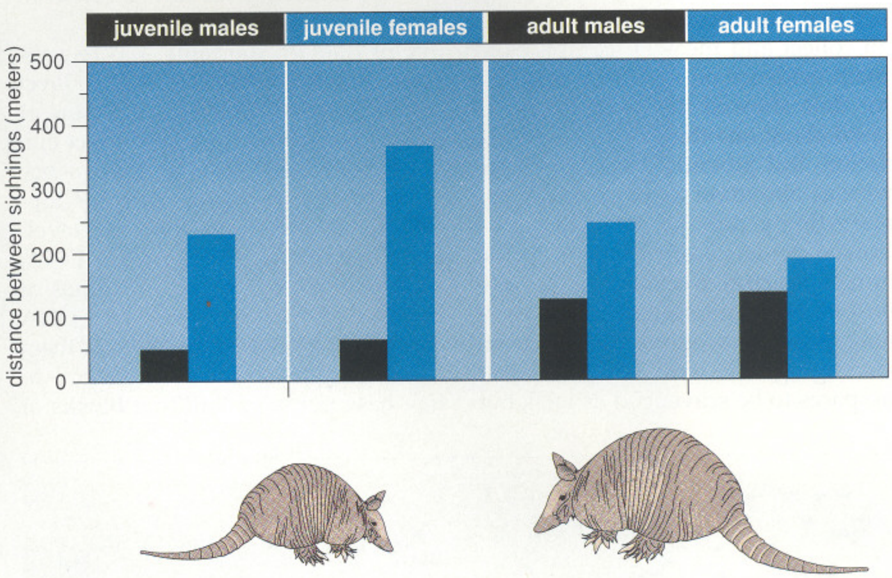

Figure 5. Nine-banded armadillos do not venture far from home. The authors captured, marked and determined the position of many animals, and then marked their position if they were seen again. If an animal was seen again in the same year *(black)*, it was usually within 150 meters of the original sighting. Animals seen in subsequent years *(blue)* were usually within 350 meters of the original sighting. These results apply across both age and sex. If armadillos stay put over time, that could promote the operation of kin selection because such limited movements should generate spatial clustering of littermates. (Adapted from Loughry and McDonough 1998.)

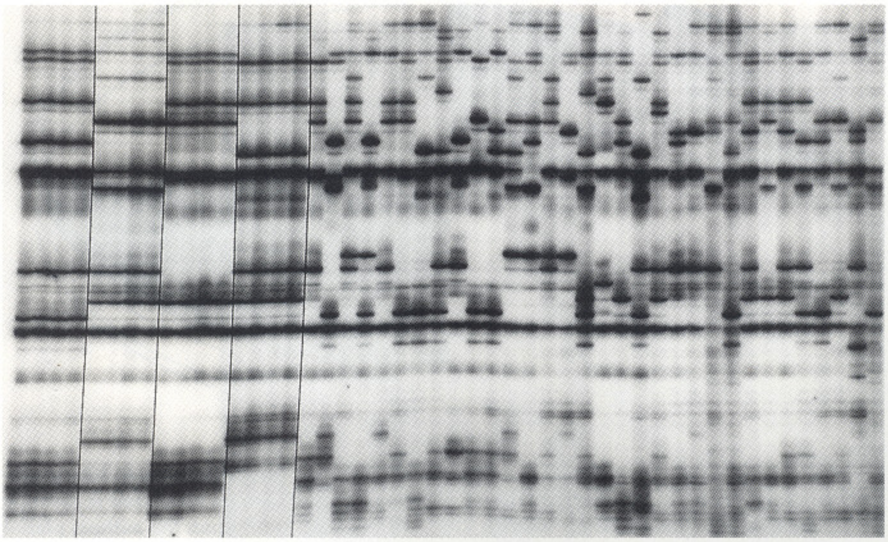

Figure 6. DNA fingerprinting of nine-banded armadillos using microsatellite markers shows that littermates are genetically identical. Randomly selected individuals in the population, however, differ from one another. Using such fingerprinting techniques, the authors found that few adults have littermates in the population, and, when they do, littermates do not live close together—providing little opportunity for behavioral interactions between adults that could lead to kin selection. (Reprinted with permission from Prodöhl *et al.* 1996.)

some animals certainly leave their natal areas. Mortality may be another factor. One difficulty of doing field studies is that mortality is rarely observed and must be inferred from indirect lines of evidence. That problem is exacerbated by the generally solitary and nocturnal behavior of nine-banded armadillos. They do, however, have a tough carapace that decomposes slowly, and one can collect and measure carapaces to obtain evidence on mortality patterns. Our data showed that juveniles comprised about one-third of a living population, but juvenile carapaces were twice as abundant as those of adults. Interestingly, only juvenile carapaces showed evidence of predation in the form of puncture wounds from teeth—most likely from bobcats or coyotes. If those puncture wounds resulted from scavenging, one would expect adult carapaces to be scavenged as well, but no adult carapaces exhibited such markings. Those findings suggest that predation on juveniles during their first summer above ground may be a major reason for their low recruitment into their natal population. Such high mortality leading to limited local-population recruitment would severely restrict the opportunities for kin selection to operate among adult armadillos.

Our data on mortality and recruitment, however, provided only indirect insight into the population structure of adult armadillos. To obtain direct evidence, we needed data on the frequency of littermates among adults in our population. To determine that, we developed markers for microsatellite DNA, which exists in the chromosomes of most eukaryotic organisms. That DNA consists of short sections—two of them repeated in tandem—in which two to five base pairs, or building blocks of DNA, are repeated. A given segment of microsatellite DNA tends to be distinct enough to be identified, and such segments get passed along to offspring. In nine-banded armadillos, microsatellite DNA can be used to unambiguously identify littermates. We collected a small tissue sample from the ears of each animal captured between 1992 and 1995 and developed a genetic profile—a DNA fingerprint—for each animal in the population. First, we confirmed that littermates were in fact genetically identical. Despite a number of embryological studies showing that armadillo litters come from a single fertilized egg that splits into multiple zygotes, no one had confirmed that the resulting offspring were genetic clones. We showed that they are. In addition, we found that no two randomly sampled nonlittermates in our population have the same genetic profile. Consequently, any genetically identical animals almost certainly came from the same litter.

With our microsatellite probes, we examined the population for the presence of littermates among adult animals. Among nearly 200 adults screened, we found only 8 groups of siblings. Interestingly, all these sibships were male. In addition, these littermates were not particularly close to one another at the time of capture; the mean distance between them was more than 450 meters. These data suggest that, for most adults in our population, little opportunity exists for behavioral interactions between adults that could lead to kin selection. Few adults have littermates present, and those that do are unlikely to interact with their siblings frequently because they are so far apart.

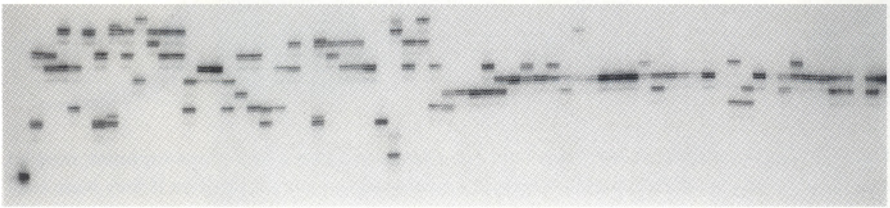

Figure 7. Microsatellite-DNA analyses show that nine-banded armadillos from Brazil are genetically more variable than their North American cousins. Although those differences might suggest an opportunity for kin selection to differ as well, the authors found no evidence of related adults living near each other in Brazil.

Adaptive Value of Polyembryony

If kin selection had not been a major factor in nine-banded armadillos, why are they polyembryonic? Our best guess comes from ideas developed in 1985 by Gary Galbreath, then at the University of Chicago. He noted that armadillos have a uniquely shaped uterus in which the implantation site is so small that it can only accommodate a single blastocyst, which leads to just one juvenile. Galbreath argued that the uterine shape was the ancestral condition in armadillos and that polyembryony evolved as a way for females to bypass that constraint and increase their reproductive success. His hypothesis is bolstered by the fact that species in other genera of armadillos typically have litter sizes of one and possess similar uterine morphology, suggesting that these are indeed ancestral characteristics.

Odd as it may seem, the "uterine-constraint" hypothesis for polyembryony in *Dasypus* armadillos might bear some analogy to evolutionary conditions that apparently promoted polyembryony in parasitic wasps. Some female wasps lay a single egg inside a host-species egg that later develops into a larger caterpillar, which becomes a food source for the parasite. The parasite's reproductive bottleneck apparently arises at the egg-laying stage, when the small host egg might hold only one parasitic egg. Polyembryony allows the parasite's egg to split as the developing host caterpillar grows large enough to feed more parasitic larvae.

In armadillos, the initial reproductive constraint that may have promoted the evolution of polyembryony appears to be the oddly shaped uterus with its single implantation site. If so, future work should examine how and why such a uterine characteristic arose. Although the uterine-constraint explanation for the evolution of polyembryony in nine-banded armandillos does not preclude the operation of kin selection on the subsequently produced young, our findings indicate that kin selection has not been exploited by them.

Reasons for Caution

Our data on population structure and the inferences that these data allow us to draw about the importance of kin selection in nine-banded armadillos must be regarded cautiously for at least two reasons. First, kin selection is not restricted to interactions among littermates and may occur between more distantly related individuals. Interactions among littermates represent the extreme case where kin selection is most likely, but we cannot exclude the possibility that kin-selected benefits accrue to individuals interacting with more distant relatives. Moreover, our genetic data suggest that our population may be subdivided into clusters of closely related individuals. Development of additional DNA markers is required to confirm that, but if kin clusters occur we will need to determine if more subtle effects of extended kin selection exist.

Second, as stated earlier, armadillos are recent arrivals in the U.S., with most populations having been established for no more than 150 to 200 years. Armadillos were first detected at Tall Timbers in 1974, so our study population is less than 25 years old. Nearly all data on nine-banded armadillos came from U.S. populations, and their behavior and population biology might represent adaptations to unusual conditions. Perhaps a different picture would emerge if animals from their South American homeland were observed.

In 1996, we went to the Poço das Antas Biological Reserve, located in the Atlantic coastal rainforest of Brazil, about 100 kilometers north of Rio de Janeiro, to see whether armadillos there differ in behavior or genetics from their northern cousins. Armadillos in Brazil are genetically more variable, smaller in body size and less abundant. They dug more burrows and were active later at night than armadillos at Tall Timbers. Given those differences, it seems possible that the opportunity for kin selection might also differ. We collected DNA samples from the animals in Brazil, but found no littermates among the animals we captured. On the surface that might indicate that, like in the U.S., kin selection is unlikely to be important in Brazilian armadillos. Nevertheless, that conclusion would be based on a small sample of animals—22 adults and 15 juveniles—and that small sample is due, at least in part, to the fact that people hunt armadillos extensively for food in Brazil. So the impact of human hunting, which is much more common in Brazil, confounds any comparison between our Floridian and Brazilian populations. A valid comparison requires finding a nonhunted South American population, which may be difficult because armadillos are a favored food item in many countries.

Conclusions

Recent advances in biotechnology raise the possibility of producing clonal offspring in a variety of vertebrates, and there has been considerable discussion about the ramifications of animal cloning. One way of assessing the consequences of such artificial manipulations is to examine species that make clones naturally. The data we have collected suggest that the production of clonal offspring by nine-banded armadillos has had little impact on their behavior or ecology. Our data also cast doubt on simplistic models of kin selection that use genetic relatedness as the primary predictor of the incidence of altruism. Similar findings have been reported in other species. For instance, Joe Newsome of San Diego State University showed that individuals of a parthenogenetic lizard, *Lepidodactylus lugubris*, are more aggressive toward one another than are individuals in a related species that does not produce clones. Such findings do not invalidate kin-selection theory as applied to clonal systems, but they do point out that the ecological and evolutionary situations can be more complicated than originally supposed.

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