The sparrow with *four sexes*

Ecologist Elaina Tuttle spent her life trying to understand the bizarre chromosome evolution of a common bird — until tragedy struck.

BY CARRIE ARNOLD

There was one sound that biologist Rusty Gonser always heard at Cranberry Lake — and there was one sound that he would never hear again.

Every summer for more than 25 years, Gonser and his wife, Elaina Tuttle, had made the trip to this field station in the Adirondack Mountains — a 45-minute boat ride from the nearest road. Now, as he moored his boat to the shaky wooden dock, he heard a familiar and short song that sounded like 'oh-sweet-Canada'. The whistle was from a white-throated sparrow calling hopefully for a mate.

What he didn't hear was the voice or laughter of his wife. For the first time, Gonser was at Cranberry Lake alone. Just a few weeks earlier, Tuttle had died of breast cancer.

Her entire career, and most of Gonser's, had been devoted to understanding every aspect of the biology of the white-throated sparrow (*Zonotrichia albicollis*). Less than six months before she died this year at the age of 52, the couple and their team published a paper[1] that was the culmination of that work. It explained how a chance genetic mutation had put the species on an extraordinary evolutionary path.

The mutation had flipped a large section of chromosome 2, leaving it unable to pair up with a partner and exchange genetic information. The more than 1,100 genes in the inversion were inherited together as part of a massive 'supergene' and eventually drove the evolution of two different 'morphs' — subtypes of the bird that are coloured differently, behave differently and mate only with the opposite morph. Tuttle and Gonser's leap was to show that this process is nearly identical to the early evolution of certain sex chromosomes, including the human X and Y. The researchers realized that they were effectively watching the bird evolve two sex chromosomes, on top of the two it already had.

"This bird acts like it has four sexes," says Christopher Balakrishnan, an evolutionary biologist at East Carolina University in Greenville, North Carolina, who worked with Tuttle and Gonser. "One individual can only mate with one-quarter of the population. There are very few sexual systems with more than two sexes."

The work helps to explain a long-standing puzzle for biologists. It shows how two identical chromosomes can evolve into distinct subtypes that can define the sexes of a species and their different behaviours. "These birds are an amazing system," says Catherine Peichel, an evolutionary ecologist at the University of Berne. "The process of sex-chromosome evolution tends to erase much of the evidence of how it happened, so being able to watch the process in action is a huge benefit."

What makes Tuttle and Gonser's project even more unusual is the accumulation of almost 30 years of data, "something that is almost unheard of in biology now", says Melissa Wilson Sayres, a computational biologist at Arizona State University in Tempe. "Most people tend to jump from project to project."

Gonser is determined to carry on the project. He returned to the field station this summer to continue Tuttle's legacy and use this drab little garden bird to understand how sex chromosomes may have evolved. "Who knows — there might be many more species that have weird sex chromosomes and we've just never bothered to look," he says.

A FLOCK OF IDEAS

Gonser's mind moves at a mile a minute, his thoughts racing ahead of his grey Honda Civic. Dressed in basketball shorts and a T-shirt, and sporting days-old stubble, he speeds west on the I-70 interstate towards Indiana State University in Terre Haute, where he and Tuttle shared an office. As ideas tumble out — about new research projects, people to meet, restaurants to avoid — he pauses briefly and apologizes. "Sorry if I'm repeating myself. My brain hasn't been working right since …" he pauses briefly, and clears his throat, "since Elaina passed."

Tuttle and Gonser first met in 1991 at the State University of New York at Albany, where both were pursuing PhDs in ecology. Gonser was investigating the Puerto Rican frog *Eleutherodactylus coqui* and Tuttle was studying fish ecology at New York's Finger Lakes. Long months in the field suited her love for the natural world, and it was there that she got to know the curious lifestyle of the white-throated sparrow.

The bird is relatively common in gardens across the eastern half of North America — and on first glance it is rather plain. All the birds have mostly tan and grey plumage, except for a patch of white under the chin and a bright pop of yellow between the eyes and beak. But closer scrutiny reveals the two types: some have white stripes on their heads, and some have tan stripes. And, as bird spotters and naturalists have long known, the two morphs behave in different ways.

The tan-striped birds are poor at singing, monogamous and fiercely protect their hatchlings from predators such as raccoons and snakes. The white-striped ones are aggressive, promiscuous, more cavalier about their offspring, and tuneful: Gonser says that they produce a more operatic refrain of oh-sweet-Canada. White-striped birds seem to mate only with tan-striped ones — a relatively unusual

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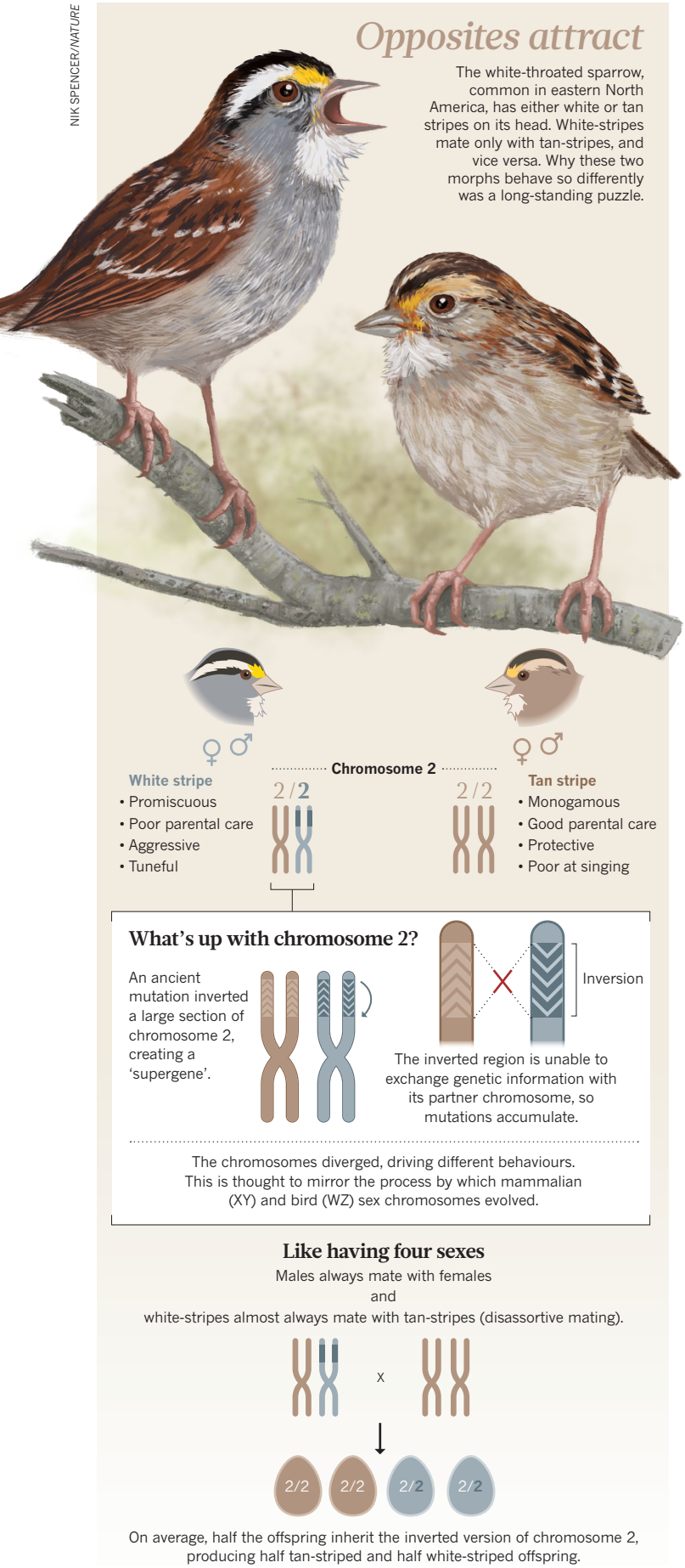

phenomenon called disassortative mating (see 'Opposites attract'). Tuttle became interested; why do the two morphs behave in this way?

The literature already contained a huge clue. In 1966, ornithologist H. B. Thorneycroft published a paper in *Science*[2] pointing out the bird's unusual chromosome pair. Tan birds carry two identical copies of chromosome 2, but in white birds, one copy contains an inversion. It's as if someone had taken a pair of scissors, snipped out most of the chromosome and placed the DNA back in reverse. A chromosomal inversion this large was rare in vertebrates, Thorneycraft noted. It looked like disassortative mating maintained the two morphs in equal proportions in the population, because Mendelian inheritance ensures that, on average, half the offspring of a white–tan pair will inherit the inverted version of chromosome 2. But it would take more work to prove that this was true.

To Tuttle, it was a fascinating puzzle — a way to shed light on chromosome evolution, as well as the genes underlying social behaviour. In the early 1990s, however, it was too expensive and laborious to find answers by sequencing the bird's genome. So Tuttle initially focused on collecting more detail about their behaviour, such as how they selected mates and where they built nests. The goal was to understand what might affect offspring survival. She caught and tagged birds, drew blood samples and perfected the art of collecting semen. "Elaina was the best bird masturbator I ever met," Gonser says.

Gonser soon became drawn into the work. (The couple married in 1994.) After the birth of their son Caleb in 2000, the family began spending summers at Cranberry Lake, and they slowly learnt more about the sparrow. For a 2003 paper[3], Tuttle used genetic analysis of nestlings to quantify the different reproductive strategies of the two morphs. She showed that nearly one-third of the offspring fathered by white-striped males were not born to females with whom they shared a nest. Tan males, by contrast, spent less of their energy finding extra partners and more time guarding their own, so they were more likely to have fathered the chicks they were raising. Although the two morphs went about reproduction in very different ways, both achieved equal reproductive success.

Six years later, the team secured a grant from the US National Institutes of Health to start genetic analyses. They mapped chromosome 2 in detail[4] and found that the changes were not a single inversion as Thorneycroft had indicated, but actually a series of inversions within inversions that scrambled the order of genes. They identified several genes that seemed to be associated with feather colour and behaviour, and that might explain the differences between the two morphs. "It's really, really rare to find such a direct relationship between a set of genes and behaviours. That's what makes these birds so interesting to study," says Donna Maney, a neuroendocrinologist at Emory University in Atlanta, Georgia, who uses the white-throated sparrow as a model to understand how hormones affect the brain.

But then, in 2011, just as Tuttle and Gonser were ramping up their genome sequencing, a routine mammogram revealed a lump in one of Tuttle's breasts. A biopsy confirmed that it was invasive cancer. The couple was shocked — their son was only 11 — but a mastectomy and the drug tamoxifen seemed to contain the disease. Determined not to let the cancer control her life, Tuttle pressed on with her work.

DOUBLY DIVIDED

By this point, the researchers had come up with the concept that the birds were evolving a second set of sex chromosomes. "It's a bizarre idea, but it just makes sense from the data," said Alan Bergland, a geneticist who studies the molecular basis of evolutionary adaptation at the University of Virginia in Charlottesville. No other species was known to have one set of fully operational sex chromosomes and another pair that subdivided the species again on another aspect of mate choice.

Evolution has led to many weird and wonderful sexes and means of determining them. Some animals, such as reptiles, have two sexes and no sex chromosomes, whereas the freshwater protozoan *Tetrahymena thermophila* has seven sexes, each of which can mate with any type except its own. Two sexes with one set of sex chromosomes is the most

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Elaina Tuttle dedicated her scientific career to studies of the white-throated sparrow.

common arrangement, and has evolved independently many times. But there's no reason a species can't have more sex chromosomes, Wilson Sayres says. "If you've got two genes linked together that can't cross over during meiosis, and one of them plays a role in sex determination, suddenly you can have a new sex chromosome."

Researchers believe that the X and Y chromosomes in many mammals, and the W and Z in birds, emerged from a major inversion on a normal pair of chromosomes that prevented them from swapping genetic material, or 'crossing over'. The suite of genes in the inversion was cemented together as a supergene that was inherited in one large chunk. On both the Y and Z chromosomes, the inversion shifted the location of a gene that determined male or female sexual development, respectively (male birds are ZZ and females are ZW). Over time, the Y and Z chromosomes accumulated mutations, because crossing over wasn't weeding them out. But, because all this happened in the distant evolutionary past, scientists had struggled to identify the precise steps involved.

The sparrows offered such a chance. The inversion on chromosome 2 doesn't include genes that determine sexual development — but it does contain some that affect the birds' reproductive behaviour. Over time, those genes diverged and drove the different characteristics of the two morphs. "Whatever genes control these behavioural differences will ultimately be traced back to this inversion," says Maney.

But to truly show that chromosome 2 was evolving like a sex chromosome, Tuttle and Gonser would need to demonstrate that the genes in the inversion were acquiring mutations much faster than those on other chromosomes. This would prove that the white and tan morphs were using true disassortative mating, so that all pairs were white–tan. Even a tiny percentage of white–white matings would allow the inverted chromosome 2 to undergo crossing over that would help to cleanse it of mutations, and undermine the idea that it was acting like a sex chromosome. Tuttle and Gonser would need to sequence the genomes of many birds, to show parentage and to compare mutation rates in the inversion with the rest of the genome.

Finding DNA to sequence was easy. By now, Tuttle had several freezers full of blood samples collected over the years from thousands of birds. But other biology was getting in the way.

In autumn 2013, a chronic cough sent Tuttle back to the doctor with what she thought was bronchitis. She learned that her cancer had returned and spread to her lungs. Genome sequencing of her tumour revealed that her original cancer was a mosaic of cell types; the tamoxifen had tamed the hormone-sensitive cancer cells, but the insensitive ones had survived. She underwent more chemotherapy, which held the cancer in check.

THE LAST LAKE TRIP

By the summer of 2015, the team had gathered both the ecological and the genetic data that they needed and were putting the finishing touches to their big paper — when a routine scan revealed that Tuttle's tumours were growing again. Tuttle made the 13-hour drive from Terre Haute to Cranberry Lake between chemo treatments. "We knew she was sick, but we didn't realize just how sick she really was," says graduate student Lindsay Forrette. Soon, Tuttle was getting increasingly grim predictions about how much time she had left. "We were all thinking she was going to beat the cancer, and when we finally understood she wasn't…" Gonser's voice trails off. When Tuttle realized she would never return to Cranberry Lake, she broke down and cried.

In January 2016, when the paper was published in *Current Biology*, it showed unequivocally that chromosome 2 was evolving like a sex chromosome. White–white and tan–tan matings were exceedingly rare. Using the whole-genome sequences of 50 birds, the team demonstrated that the genes in the inversion were acquiring mutations much more quickly than elsewhere in the genome, a pattern that echoed the evolution of sex chromosomes in humans and birds.

In a press release, Tuttle said: "This is probably my pinnacle paper." It would also be her last. By spring, her health had worsened. Just five days before she died, Tuttle busied herself from her hospital bed, writing more papers and helping graduate students to analyse data. She died on 15 June. When Gonser announced the news, he received condolences from around the world. "Everyone who knew her, loved her. She was just that kind of person," Gonser says.

> "These birds are an amazing system."

Tuttle leaves a rich research legacy that raises further questions, including whether this chromosomal system is ultimately destined to disappear. Balakrishnan says it is unsustainable. "That we never see systems with four sexes says that they're evolutionarily unstable and one of these alleles will ultimately go extinct." That's because in a four-sex system, each bird must work that much harder to find a mate — a white female is not just looking for a male, she needs a tan one — so selection will favour a more advantageous two-sex system instead. Balakrishnan intends to use the white-throated sparrow to further tease apart the genetic and environmental factors that drove its evolution in the first place.

Gonser will keep going back to Cranberry Lake. He and his group want to better understand which genes control the sparrow's behaviour — from mate selection to parenting — and how those characteristics were affected by the inversion. They are using digital maps and satellite data to chart nesting sites, track tagged birds and build a richer set of behavioural data.

"There's a lot more information left in these birds," Gonser says. "And I think Elaina would like that we're trying to uncover their secrets." ■

Carrie Arnold *is a writer based near Richmond, Virginia.*

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