Chapter 11

Crane Fly Spermatocytes and Spermatids: A System for Studying Cytoskeletal Components

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I. Introduction

This chapter describes methods for rearing crane flies in the laboratory, and for dissecting living testes to study living spermatogenic cells *in vitro*. Why study crane flies? One can rear crane flies in the laboratory and obtain cells at any time, so one is not restricted to a given season. Year-round availability is an advantage compared to seasonally available cells, such as *Haemanthus* endosperm or sea urchin zygotes; why, though, would one choose to work with cells from crane flies rather than other consistently available cells such as tissue culture cells or cells from *Drosophila* or from other animals that can be reared in the laboratory?

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There are two major reasons for choosing to work with crane fly spermatocytes. One reason, of interest to those studying mitosis and the mitotic apparatus, is the favorable *cytology* during cell division: the spermatocytes are large (the spindle pole-to-pole distance is 20–30 μm in flattened cells), and primary spermatocytes contain only five small chromosomes (three autosomal bivalents and two sex-chromosome univalents), each of which can be followed simultaneously *in vivo,* throughout cell division (e.g., Fig. 11). Further, in each half-spindle there are only five chromosomal spindle fibers, each of which is ~ 1 μm in diameter: These large, easily studied chromosomal spindle fibers can be followed throughout cell division (e.g., Fig. 11). Before considering the second reason for studying crane fly spermatogenic cells, I give a brief overview of the kinds of experiments that have been done on the mitotic apparatus in crane fly spermatocytes.

Normal living crane fly spermatocytes have been studied using phase-contrast and polarization microscopy. Information has been obtained about chromosomal orientations (and reorientations) during various stages of meiosis; about velocities of movements during the different stages of meiosis; about the timing (and speeds) of autosomal anaphase relative to the later sex-chromosomal anaphase; about the development and birefringence of individual spindle fibers during various stages of meiosis; about spindle-shape changes during various stages; and about velocities of autosome movements and sex-chromosome movements at different temperatures (e.g., Bauer, 1931; Bauer *et al.,* 1961; Begg and Ellis, 1979b; Behnke and Forer, 1966; Dietz, 1956, 1963, 1969, 1972a,b; Forer, 1965, 1966, 1969, 1976; La Fountain, 1972, 1974a, 1976; Salmon and Begg, 1980; Schaap and Forer, 1979). Living crane fly spermatocytes have been studied after ultraviolet microbeam irradiation of single chromosomal spindle fibers; after flattening so that centrioles are found in the cytoplasm and not at the spindle poles; during pressure treatment or micromanipulation of spindle fibers or chromosomes; after colchicine treatment and irradiation with 360-nm light; and after treatment with hypotonic or hypertonic media (e.g., Begg and Ellis, 1979a,b; Dietz, 1959, 1966; Forer, 1965, 1966, 1969, 1972; Forer and Koch, 1973; Salmon, 1975; Salmon and Ellis, 1975; Sillers and Forer, 1981a,b,c). Finally, electron-microscopic data include study of kinetochore shapes and sizes during cell division; serial-section analysis of microtubule numbers and arrangements in spindles; study of microtubules in association with nonkinetochore ("lamellae") regions of autosomes and sex chromosomes; study of spindle microtubules after tannic-acid staining or after freeze-fracture; and study of presence (or absence) of spindle microfilaments after usual fixations, after tannic-acid fixation, or after glycerination and reaction with heavy meromyosin (HMM) (e.g., Behnke and Forer, 1966; Behnke *et al.,* 1971; Forer and Behnke, 1972a; Forer and Brinkley, 1977; Fuge, 1971, 1973, 1974a,b, 1975, 1977a,b, 1980a,b, 1981; Fuge and Müller, 1972; La Fountain, 1974a,b, 1975, 1976; La Fountain and Thomas, 1975; La Fountain *et al.,* 1977; Müller, 1972). Some of the main conclusions from these data have been summarized elsewhere (Forer, 1980). I think it is fair to say that many details of cell division have been studied quite well in crane fly spermatocytes: some progress has been made in identifying the force producers (for chromosome movement), in describing the organization and properties of spindle fibers, in describing how microtubules are distributed during cell division, in describing changes in birefringence in individual chromosomal spindle fibers during cell division, and in studying how the movements of individual chromosomes can influence the movements of other chromosomes in the same cell.

A second reason for choosing to study crane fly spermatogenic cells, perhaps of interest to those studying cytoskeletal elements other than the mitotic apparatus, is the *synchrony* of the cells inside a testis: 5000–10,000 spermatogenic cells are enclosed within the testis wall (the "wall" is a single layer of cells), and the spermatogenic cells are synchronous—that is, *all* the spermatogenic cells in a given testis are in about the same stage of spermiogenesis at the same time. For example, in *N. abbreviata,* all the cells in one testis enter meiotic S-period at about 1 day after the start of the IV-larval stage, all enter zygotene about 2 days later, all enter pachytene about a day or two later, and all take part in cell division (i.e., prometaphase-I through telophase-II) on days 8–9. (The IV-instar is the *form* of the insect; the time period during which the larvae have the IV-instar form is the IV-larval *stage,* or stadium.) Thus if one removes a testis at day 7, all cells in the testis are *pre*-prometaphase; at day 8, cells in the testis are in various stages between prophase-I and spermatid; at day 10, all cells in the testis are early spermatids; and so forth. Thus, unlike testes from *Drosophila* or mouse or grasshopper, which all contain cells at various stages of meiosis (from spermatogonia through mature sperm), each crane fly testis contains cells at one stage only. Consequently, one can easily follow developmental sequences in cytoskeletal elements (such as time course of synthesis, or of assembly, or of phosphorylation) by choosing testes that contain cells of the appropriate stage. Before describing methods for rearing the flies, I give a brief overview of the kinds of experiments that have been (and can be) done on cytoskeletal components of this system.

Electron microscopy after glycerination and HMM labeling has been used to study the changing arrangements of cortical microfilaments at different stages of meiosis in crane fly spermatocytes (Forer and Behnke, 1972b). Microtubules have been studied in young spermatids (just after meiosis), and four groups of microtubules were defined, based on their relative stabilities to different treatments (Behnke and Forer, 1967). Cytoplasmic microtubules and axoneme microtubules change during spermiogenesis. Cytoplasmic microtubules are relatively "labile" in young spermatids, but become less labile in older spermatids (Behnke and Forer, 1972; Forer and Behnke, 1972c); vinblastine treatment converts cytoplasmic microtubules in older spermatids to "helices" that remain "in place" (Behnke and Forer, 1972). The 9 + 2 (axoneme) microtubules appear hollow in young spermatids (e.g., Behnke and Forer, 1967), but those in older spermatids and in mature sperm are no longer "hollow" but are very densely staining and substructured (see Behnke and Forer, 1972; Forer and Behnke, 1972c). Actin-containing filaments seen electron-microscopically in crane fly spermatids also change their organization during maturation of the spermatids, from single filaments to tightly packed, "semicrystalline" bundles (Forer and Behnke, 1972c). Crane fly testes cells can be analyzed biochemically (e.g., Petzelt, 1970), and indeed there are biochemically detectable changes in the organization of actin during crane fly spermiogenesis (Strauch *et al.*, 1980). Crane fly spermatocytes and spermatids will live *in vitro* in Ringer's solution, and spermatocytes incorporate radioactively labeled precursors (e.g., [^{3}H]thymidine) at the appropriate stages (A. Forer, unpublished data); hence one can use the synchronous system *in vitro* to study cytoskeletal elements chemically.

II. Rearing Crane Flies in the Laboratory

This section first gives a general description of rearing crane flies in the laboratory, then details of various aspects of the procedures.

A. Procedures

Adults are placed in cages. Copulating pairs (e.g., Fig. 1) are captured in glass dishes that have moist paper (papier-mâché) in the bottom and that are covered with plastic petri dish lids. Within a few days after copulation the adult females lay eggs in the moist paper that lines the bottom of the dishes. Egg laying also can be induced by cutting the heads off the females: see Bodenheimer (1924).

After eggs are laid in the paper pad at the bottom of the dish (there are generally 150–300 eggs per female), the pad (plus eggs) is removed, placed in the bottom of a 90-mm diameter plastic petri dish, and covered with a lid. The eggs hatch about 6–8 days after they are laid; at this time dried nettle leaves are sprinkled on top, and the surface of the paper is broken in several places to allow the larvae to burrow. The nettle leaves are eaten by the larvae, and to help these early larvae eat the nettle I generally crush the leaves between my fingers, to make a fine powder that is sprinkled over the paper. For older larvae ($\gtrsim$ 2 weeks old), I use the nettle leaves as they come (i.e., as small pieces, 2–4 mm on a side).

The small pad of paper (with contained larvae) is transferred to a larger ($\sim$ 150-mm diameter) plastic petri dish at about 9–10 days after hatching: the small

FIG. 1. Photograph of a male and female crane fly *in copulo*. The male is the partner with open wings. (This photograph and the rest of the photographs in Figs. 1–11 are of *Nephrotoma abbreviata* animals or cells.) The photograph is about 1.5× magnification.

pad of paper is torn into four or five pieces and interspersed in clean new paper that fills the larger dish. The larvae are kept in these large plastic petri dishes and are fed every 2–3 days until they become pupae. I do not powder the nettle leaves for larvae in these larger petri dishes.

Pupae begin to appear about 24–30 days after hatching, for the species I keep; pupae are removed from the petri dishes and are placed on moist paper at the bottom of a glass dish that is then covered with a plastic petri dish lid. (Pupae have external sex characteristics that enable one to separate male pupae from females, if this is required.) Adults emerge about 5–6 days later, and are placed in cages for mating.

Having described the procedure in overview, I now give some of the details.

Special cages are not required; I have used various sizes and designs. When no alternatives were available I even used cardboard boxes with arm holes and a transparent plastic sheet for the top! At present we use as cages transparent Plexiglas cubes about 50 cm on a side. One side is removable, for cleaning the insides. Holes (about 15 cm in diameter) are cut in two opposite sides and these are lined with cheesecloth or discarded panty hose and used as arm holes. The only "requirement" in designing a cage is that the arm holes be large enough for the glass dishes (which contain pupae and adults) to pass through. Our cages often contain orange peels and pieces of moist tissue containing sucrose. Fresh orange peels seem to promote mating: quiescent adults become very active when fresh orange peels are placed in the cages, and the "frantic" flying around often results in mating. The sucrose-containing moist tissue seems to attract the adults. Our subjective impression is that males and females that suck on the sucrose live longer than they otherwise would, and that, for some species anyway, females lay more eggs than they otherwise would.

Glass dishes used for pupae and for copulating adults are nominally 90 mm diameter × 50 mm high. I select them so that lids to plastic 90 × 15 mm plastic petri dishes fit over the top of the glass. Then, when I transfer the paper plus eggs to a plastic petri dish, I use the same lid for both the glass dish and the 15-mm-high petri dish bottom. I use 150-mm (diameter) × 25-mm plastic petri dishes for the larger larvae. The plastic petri dishes are recycled until they break; dirty ones are soaked and cleaned *without soap,* using tap water only, and are left to dry on a dish rack before the next use.

When feeding the larvae, I try to give enough food to last them for 2–3 days. That is to say, I try to add nettles so that after 2–3 days no uneaten food remains, and the paper has a mottled appearance, from being dug up by the larvae. Then I feed them again. If too much food is added, food that remains after 2–3 days tends to become moldy, requiring the animals to be removed from the paper and placed in fresh paper (as described below).

Water is added to the paper pads while waiting for adults to lay eggs or for eggs to hatch, or while feeding the larvae—in general, at all steps in the procedure. The water is added to the paper (about two or three times a week *when necessary*) in order to keep it at the original moisture level. Tap water is generally good enough: I have used tap water in Toronto, Canada; in New Hampshire; in France; and in various places in Denmark and England. Some tap water can be lethal, however: in southern California, for example, contaminants in the laboratory "industrial water" kill the larvae.

Nettle leaves ("cut and sifted") can generally be obtained at health food shops in both Europe and North America. (They are said to be good for the spleen, and are used to make tea, beer, soup, and hair rinses.) The nettle leaves can be used as they come, but we generally put them in 2-liter beakers and heat them at 160°C for 1–2 hours, in case some parasites otherwise might be brought into the culture. One can also substitute lettuce for the nettle leaves (also see Section IV).

The "pads" of paper are made from the cheapest possible paper that readily becomes papier mâché. For this, the paper must be *without* "wet strength." I previously used tissue, but we now use cheap toilet paper. The pads are made as follows. Water is added to a roll of toilet paper, in a plastic bucket. The paper is then converted to pulp (papier mâché) by strong agitation. The water is then squeezed out of the pulp, to form a round "pad" of paper, as follows. A plastic

or glass dish is filled about three quarters full with the pulp. (I use a 150 mm diameter × 75 mm high glass crystallization dish; my present students prefer a plastic dish.) Then the water is squeezed out using a plastic disk that has holes in it. (The holey plastic disk is made from a piece of 3- to 5-mm-thick methacrylate—Plexiglas in North America or Perspex in the United Kingdom—that is cut to fit snugly on the inside of the glass dish. Many holes—about 2-3 mm diameter—are drilled in this disk. When the holey disk is pushed to the bottom of the papier mâché-filled dish the water comes to the top and the paper is pushed to the bottom, as in Fig. 2.) The water is poured out, the holey disk is removed, and the pad of paper is transferred to the bottom of a 150 × 25 mm plastic petri dish (Fig. 2). A lid is *not* placed immediately over the moist pad of paper. Rather, the paper is generally left open (e.g., overnight) to dry out somewhat: the "proper" moisture level for adding larvae is when water is not visible (the paper should not be "sopping wet"), but when water appears at the surface when the paper is pressed with a finger. (Some crane fly species do better when the paper is more moist, and others when the paper is less moist.) We generally use tap water for this procedure, since in most locations tap water is not harmful to the larvae.

Larvae sometimes have to be removed from the paper in a petri dish and placed in new paper. This is necessary when extra food has allowed too much mold to grow, when the paper has been eaten up, or to time the larvae (see below). In all cases, the larvae can be removed from the old paper quite easily and rapidly by flotation on a $MgSO_4$ solution. I buy 50-lb bags of technical-grade Epsom salts; using a graduated 1-liter beaker to measure volume of $MgSO_4$ powder, I dissolve 2200 ml of this powder in 5 gallons of tap water. A dish of larvae-plus-paper is placed in this crude $MgSO_4$ solution, and the paper is dispersed (i.e., broken up into small pieces), after which the larvae float and the paper and debris sink. The larvae are scooped up from the surface using a tea strainer or curved forceps, the larvae are rinsed by immersion in tap water (except in southern California!), and—except when we are timing the appearance of the IV-instar—the larvae are then placed in clean paper in a petri dish.

One need not worry that lengthy immersion in water or flotation on Epsom salts will harm the larvae. I have seen no effect at all after several hours in water or after several hours floating in $MgSO_4$; nor, on several accidental occasions, has overnight immersion seemed harmful to the larvae. [Ecologists do not report harmful effects either, after flotation on $MgSO_4$ (Coulson, 1962; Freeman, 1967), or after flotation on "cattle salt" (Laughlin, 1967; Meats, 1967), or after 10 days' immersion in aerated water (Hadley, 1971a).] Indeed, larvae have crawled away after a 1-hour immersion in 2% glutaraldehyde (O. Behnke and A. Forer, unpublished data), and have survived for at least a few days thereafter.

One needs to know when the IV-stage started in order to get testes that contain cells at the "proper" stage of meiosis. For timing the onset of the IV-stage, all

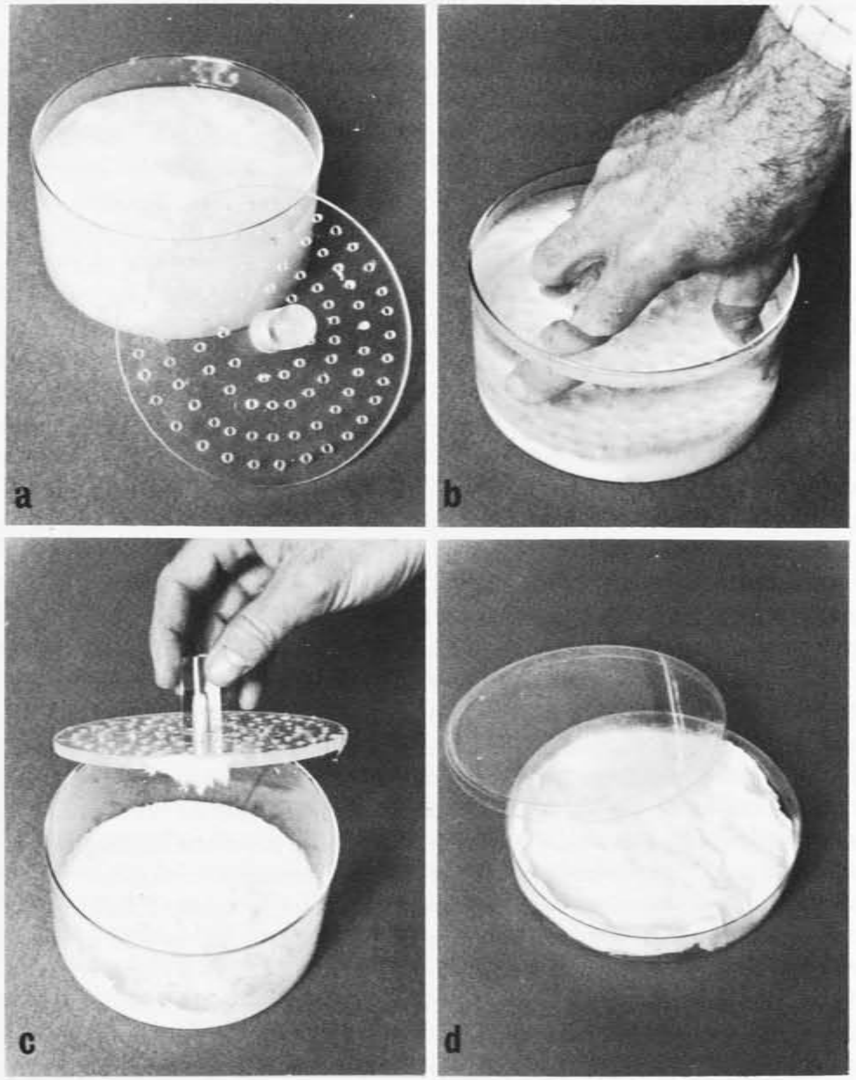

FIG. 2. Series of photographs illustrating how we make paper pads from papier-mâché. (a) Photograph of a 150 × 75 mm high crystallizing dish filled with papier-mâché, in front of which is the "holey disk." (b) Water layer at the top and the paper at the bottom, after the disk is pushed to the bottom. In the next step (c), the water is poured off, and then the disk is removed. Then the pad of paper is removed from the crystallization dish and placed in the bottom of a 150 × 25 mm petri dish (d).

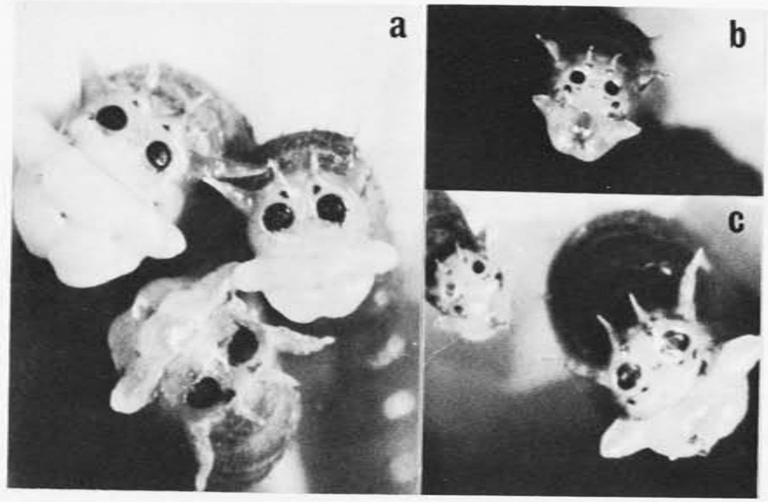

FIG. 3. Spiracles at the rear ends of III-instar and IV-instar *N. abbreviata* larvae. The larvae were killed by immersion in 70°C water; shortly thereafter they were photographed through a dissection microscope. (a) Spiracles in three IV-instar larvae; (b) spiracles in a III-instar larva. The spiracles (the more or less solid black circles) are distinctly larger in the IV-instar larvae than in the III-instar larva, as is also seen in (c), illustrating a III-instar larva and a IV-instar larva in the same photograph. All three photographs are at 11× magnification.

the animals are removed from a dish containing 14- to 18-day-old larvae, using $MgSO_4$, as described previously. Then IV-instar larvae are separated from III-instar larvae and the two groups are placed in paper in separate petri dishes. Those IV-instar larvae found the next day in the dish that previously contained only III-instar larvae must, necessarily, have molted in that 1-day period.

The IV-instar larvae are distinguishable from III-instar larvae by the size of the spiracles at the rear end of the larvae (e.g., Hemmingsen, 1965; Hadley, 1971a; Pritchard and Hall, 1971; Hofsvang, 1972). In the species we rear, the spiracles appear as black dots about 0.3 mm in diameter, whereas those in III-instar larvae are black dots about 0.15 mm in diameter, barely visible to the naked eye (Fig. 3). It is relatively simple to keep all the larvae in water in a white-bottomed dish and to sort them into two groups, based on size of spiracles. There are only two possible sources of confusion: (1) Brand-new IV-instar larvae have pinkish-grey spots rather than black spots; it takes a few hours for the black to develop. (2) Very late III-instar larvae have the old skin on top of the new ones; superficially this might look like a IV-instar animal, but close inspection shows a smaller black spot on the outside skin, with a larger pinkish spot on the inside skin. It is relevant to point out that larval size is not a good marker for IV-instar: some

III-instar larvae, with small spiracles, are larger than other IV-instar larvae, with larger spiracles.

B. Cost

We rear four different species of crane flies, and we have animals of all stages at all times, so that we can do experiments whenever we want. One or two species could be reared, easily, by an undergraduate spending 6–8 hours per week. Other than the initial expenditure for small items (cages, 5-gallon jugs, tea strainer, plastic photographic dishes for sorting out larvae, etc.), the only recurring costs are for toilet paper, $MgSO_4$, and nettle leaves. In rearing four species we use perhaps six cases of toilet paper, a dozen 50-lb bags of Epsom salts, and 25 lbs of nettle leaves per year; our total cost in 1979 was less than $550 for recurring items. Others would probably rear fewer species, so the costs would be reduced correspondingly. Thus keeping the stocks of animals is cheap and really quite easy. The most difficult part of the procedure is explaining to the purchasing department why I need cases of toilet paper together with 50-lb bags of Epsom salts!

III. Removing Testes and Making Preparations

There are two main things to learn in removing testes from crane fly larvae or pupae: (1) choosing animals of the proper age and sex; and (2) keeping the cells alive during and after dissection. I deal with each procedure in turn.

A. Choosing Animals

The various stages of spermiogenesis can be timed with respect to two occurrences, start of the last (IV) larval stage and start of the pupal period. Different species are somewhat different in their timing, but—to generalize (at least over the 4 *Nephrotoma* species I have worked with)—when larvae are reared at 22°–24°C, meiotic S-period occurs near the start of the IV-stage, and prometaphase I–telophase II occurs around 8–9 days later. From this time on, the testes contain spermatids that become progressively more and more mature until pupation (5–6 days later) and throughout the 6-day pupal period. Mature sperm are present in the adult.

Male pupae can be distinguished from female pupae using external morphological characteristics (Fig. 4), so it is a simple matter to choose male pupae with 100% accuracy. Thus in order to study stages of spermiogenesis from

FIG. 4. Male and female *N. abbreviata* pupae in bottom and in side view. (These were killed with 70° water just before photographing them.) The two male pupae (on the right) have more or less flat rear ends (uppermost), whereas the two female pupae (on the left) have "points" at the rear ends (uppermost). The scale is 1 mm between vertical lines. (Printed at about 2.6× magnification.)

pupa to adult, one would time pupae by removing them daily from dishes, then choose males of the desired age.

Male larvae cannot be distinguished from female larvae using external markers, however, so it is impossible to choose male larvae with 100% accuracy. With experience, though, one can choose male larvae reasonably reliably (say, 8 or 9 out of 10) by taking account of two factors. First, within any group of sibling larvae the male larvae tend to become IV-instar before the female larvae do. This is illustrated in a specific example (Fig. 5). In general, siblings become IV-instar over a 6- to 12-day period; in the one example in question the siblings became IV-instar over a 12-day period. For these siblings the sex of the larvae was determined at the pupal stage, where identification is unambiguous. In this particular case, which is completely unexceptional, 80% of the males had become IV-instar when only 62% of the total had become IV-instar. Thus, in general, even if there were *no* way to distinguish male from female crane fly larvae, one increases one's chances of choosing males by restricting the choice to the first 50–60% of the group to become IV-instar.

Second—and even better—there *is* a difference between males and females that one learns to recognize subjectively, and that is a difference in size (e.g., Hadley, 1971b; Pritchard and Hall, 1971). The IV-instar male larvae tend to be shorter than female larvae; in addition, even if they are near the same length, they tend to be *thinner* than female larvae.

Before describing the procedures for dissection, it is relevant to state explicitly

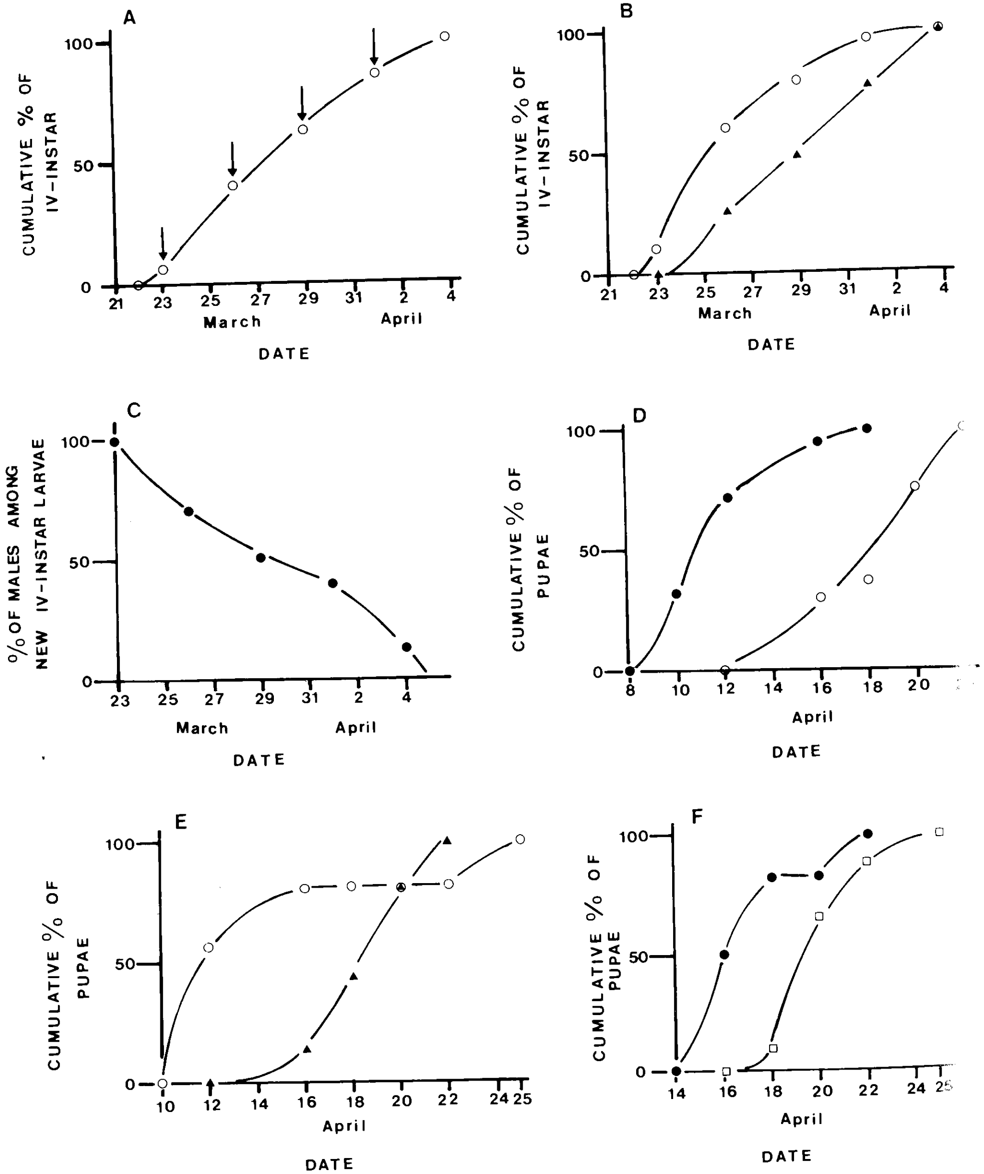

FIG. 5. Timing of larvae molting to IV-instar (A–C) and becoming pupae (D–F). (A) Cumulative percentage of larvae that are IV-instar (ordinate) versus time (abscissa), out of a total population of 86 *N. suturalis* larvae. The entire population became IV-instar in a 12-day period. (B) Same data for the same population, but with separate curves for the male and female populations (the sexes were determined at pupation). ○, Males; ▲, females. (C) Percentage of male larvae found among the new IV-instar larvae on that given day of sorting (ordinate) versus the day on which the IV-instar larvae

a point inherent in the data of Fig. 5: siblings in the same dish become less and less synchronous as they grow. Eggs are laid over a period of a few *hours*, yet IV-stage starts over a period of 6–12 *days*. Those animals that entered IV-stage in a 3-day period became pupae in a 12-day period. Even if one considers males and females separately, those that became IV-instar over a 3-day period became pupae over at least a 6-day period. Therefore, if one waits for 8–9 days after the start of the IV-stage to choose testes that contain cells undergoing cell division, the animals in the dish are not all at exactly the same stage, even if they are siblings: *some* spread has occurred. This is usually not important, but nonetheless it would help to have some additional way to recognize animals of the desired age. One way that seems to work is to look at the development of fat bodies in the larvae. Early in the IV-stage there is no internal fat, so one can see the entire gastrointestinal tract through the body wall; as the IV-stage proceeds there is more and more internal fat, which more and more obscures the gastrointestinal tract. Just before pupation the animals are almost completely white. In a relatively short time one learns to recognize the desired stage by the amount of fat in the animal.

It is relevant to point out that different species are different with respect to the synchrony between testis maturation and fat proliferation. For example, a male *N. ferruginea* larva with maximal spermatocyte cell division is much whiter (i.e., has much more fat) than a male *N. suturalis* larva with maximal spermatocyte cell division: choosing *N. suturalis* larvae using *N. ferruginea* criteria results in *N. suturalis* testes that contain only spermatids rather than cells in division!

It is conceivable that better synchrony might be obtained by keeping the animals at constant density and at constant humidity in a constant-temperature incubator. However, I have not tried to maintain constant conditions other than temperature, and I know of no work along these lines.

B. Dissections

There are two main points to keep in mind when removing testes from larvae or pupae. First evaporation must be prevented. Testes are $\leq 1\ \mu l$ in volume and evaporation occurs rapidly: cells die within seconds if testes are left uncovered on the stage of the dissection microscope. To prevent evaporation during the

were sorted from III-instar (abscissa). Those that molt first are male, and progressively smaller and smaller percentages of those that molt later are males. (D–F) Subsequent molting of the same larvae into pupae. (D) Animals started the IV stage between March 23 and 26—that is, between the first and second arrows in (A). ●, Males; ○, females. (E) Animals started the IV stage between March 26 and 29—that is, between the second and third arrows in (A). ○, Males; ▲, females. (F) Animals started the IV stage between March 29 and April 1—that is, between the two furthest right arrows in (A). ●, Males; □, females. These curves show that male larvae have a shorter IV-stage than do female larvae, and that synchrony is gradually lost as the animals progress through the IV-stage.

dissection, I cover larvae or pupae with nontoxic fluorocarbon oil, and the testes remain under oil throughout the dissection procedure. Further, there must be a heat filter on the lamp of the dissection microscope—even with testes under oil—or else the cells are irreparably damaged during the dissection. Second, in order to keep spermatogenic cells alive the preparations should be bacteria free. Thus I use clean dissecting tools (and rinse them in 70% ethanol between dissections, if necessary), and I make sure that the bacteria-laden gastrointestinal tract is not punctured during the dissection.

Larval testes are removed using the following procedure. A drop of Voltalef oil, on a clean microscope slide, is put on the stage of the dissection microscope, out of the field of view. Then a larva is rinsed in tap water, dried on a tissue, and placed on the stage of the dissection microscope *in* the field of view. I then press on the head capsule with one finger. This builds up the "turgor pressure" in the inside of the larva, so when I cover the rear of the animal with Halocarbon oil and then make a slit in the animal's body wall, the body contents are pushed out through the slit in the animal's body wall. I immediately add more Halocarbon oil on top of the "popped-out" internal organs (in case oil has run off from the organs), find the testes, and remove them (covered with oil) and place them in the drop of Voltalef oil on the clean slide. The procedure is illustrated in Fig. 6. Further details and rationale for some of these steps follow.

I cut the slit in the animal's body wall in the direction perpendicular to the animal's long axis, but only a small way across the animal (say one-quarter or less across the width); this is to prevent the gastrointestinal tract from being punctured, which would occur if the larva were cut in two. Similarly, if the larva defecates when I push on the head capsule, the larva is recleaned so that bacteria will not get into the preparation.

Both oils that we use—Voltalef oil 10S and Halocarbon oil 10-25—are high-viscosity polytetrafluoroethylene oils. The Halocarbon oil sometimes has a "scum" on the surface that is seen when one makes open-to-the-air preparations. Thus the Voltalef oil is preferred for some purposes. Regardless of which oil the cells finally are put into, Halocarbon oil, the less expensive of the two, is used during dissection to cover the animals; the testes are removed under Halocarbon oil and then transferred to either Halocarbon or Voltalef oil, usually the latter. (Voltalef oil is Huile 10S from Ugine Kuhlmann, Division Plastiques, 11 Boulevard Pershing, F-75017 Paris, France; and the Halocarbon oil is series 10-25 oil, from Halocarbon Products Corporation, Burlews Court, Hackensak, New Jersey 07601.)

Testes are distinguishable from ovaries throughout the IV-stage (Fig. 7). At the start of the IV-stage the testes and ovaries are round and are about the same size; however, for the species we rear, when they are under the fluorocarbon oil the testes are somewhat translucent, whereas the ovaries are transparent. (If there is doubt about which is ovary and which is testis at this early stage, the techniques described below are used to study the cells contained within.) One or two

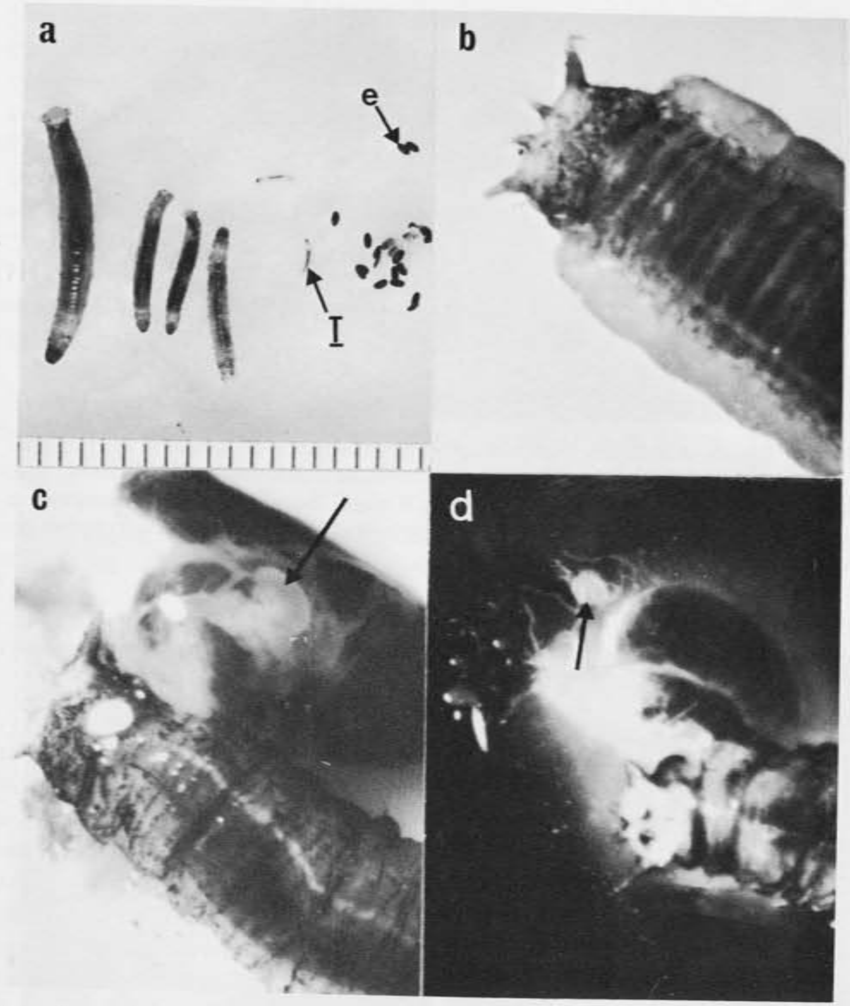

FIG. 6. (a) Relative sizes of *N. abbreviata* eggs (e), first-instar larvae (I), and other larvae, including an early IV-stage larva (at the far left). The vertical lines are 1 mm apart. (b, c) A dissection from a larva that is 8 days past the molt to the IV-stage. The rear end of the larva is illustrated in (b); this is before the larva was slit, but after I pressed on its head. (c) Same larva after I slit the body wall: the insides have "popped out" and the larva is of much smaller width. One testis (arrow) is seen on top of the GI tract. (d) Similar "popped out" insides, but this time from a larva that was only a few hours into the IV-stage. One testis (arrow) is visible, which is much smaller than that in (c). (b–d) Magnification ×11.

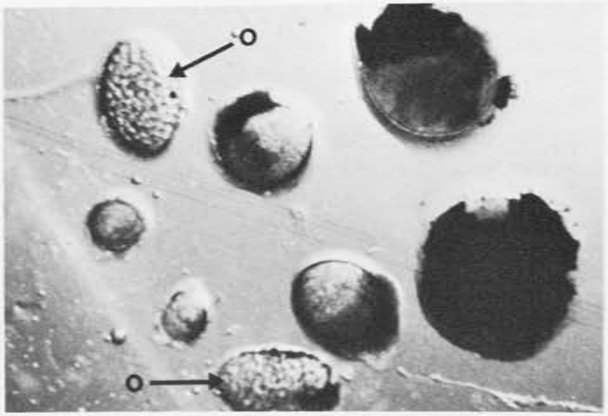

FIG. 7. Various-sized *N. abbreviata* testes plus a pair of ovaries (o). The smallest testes are from an animal just molted to IV-stage; the medium-sized testes are from an animal 8 days into IV-stage; and the largest testes are from an animal 15 days into IV-stage. The ovaries are from an animal about 15 days into IV-stage; large oocytes can be seen in the ovaries, even using a dissection microscope. Magnification ×15.

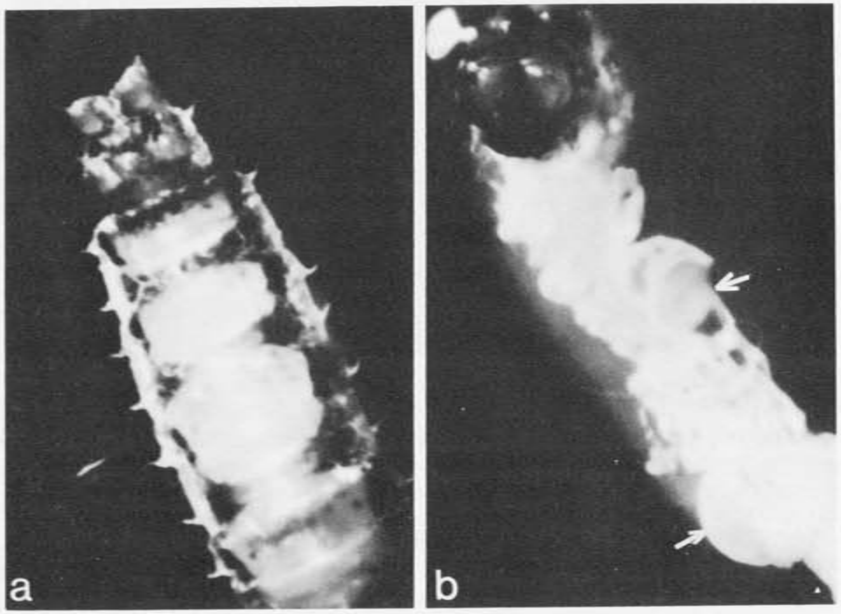

FIG. 8. Dissection of a *N. abbreviata* pupa. (a) Rear end of a pupa before the dissection; (b) appearance after the dissection, with the pupal insides spread more-or-less linearly. The two testes are indicated by arrows. Magnification ×11.3.

days after the start of the IV-stage the testes and ovaries still are round, but the translucent testes are much larger than the transparent ovaries. The testes remain round and much larger than the round ovaries until near the end of the IV-stage, about which time the ovaries grow to be as large as the testes, and even larger. The large ovary is very transparent, and contains large transparent cells, the outlines of which are visible using the dissection microscope, whereas the testes are translucent, with the spermatids identifiable in the dissection microscope as "lines." In some species the ovaries and testes are of different shapes [e.g., Pritchard and Hall, 1971; A. Forer, unpublished data, on *Limonia* (*Metalimnobia*) *annulus cinctipes* (Say)], but this is not true for the four *Nephrotoma* species that we rear.

Pupal testes are removed using procedures similar to those used in obtaining larval testes. I push on the head and cover the rear end with oil, as with larvae; but rather than cutting a slit in the body wall, as with larvae, I cut completely across the pupa. I cut at the junction to the last segment: the blade is across the whole body, and as I cut I pull the rearmost segment away from the head. I pull in this way to get the insides spread out in a more-or-less linear array. This is the easiest and most reliable way for me to find the testes (Fig. 8).

C. Living Cell Preparations

What does one do after the testes are placed in the Voltalef oil? If one wants to treat the cells with some agent (say colchicine), or to incorporate radioactive nucleotides or amino acids (for example), and if one is not interested in observing the cells at the same time, the easiest way is to transfer the testes to a Ringer's solution containing the agent (or precursor) in question—or to a glycerol or other solution. The easiest method I have found to do this is to suck up testes (and surrounding oil) into a Pasteur pipet, release them into the solution in question, and then make sure that the oil has not remained around the testes. (If oil *did* remain around the testes, the oil would seal off the testes from the solution.) To free testes from surrounding oil (should this happen), I suck the testes up and back in a Pasteur pipet until the testes are freed from the oil.

If one wants to observe the cells and is not interested in adding experimental agents while the cells are under observation, the easiest method is to transfer the testis to a drop of Voltalef or Halocarbon oil on a coverslip, and to make a smear of cells. I do this by piercing the testis with a fine needle and then moving the testis around under the oil, against the coverslip; the cells flow out into the "channels" (paths) made by the needle (Fig. 9). (I generally pierce the testis with a needle held in my left hand, and move the testis around with forceps held in my right hand.) If the cells all flow out into one big drop, they are not at all flattened, and internal details are often not easy to see; if, on the other hand, the

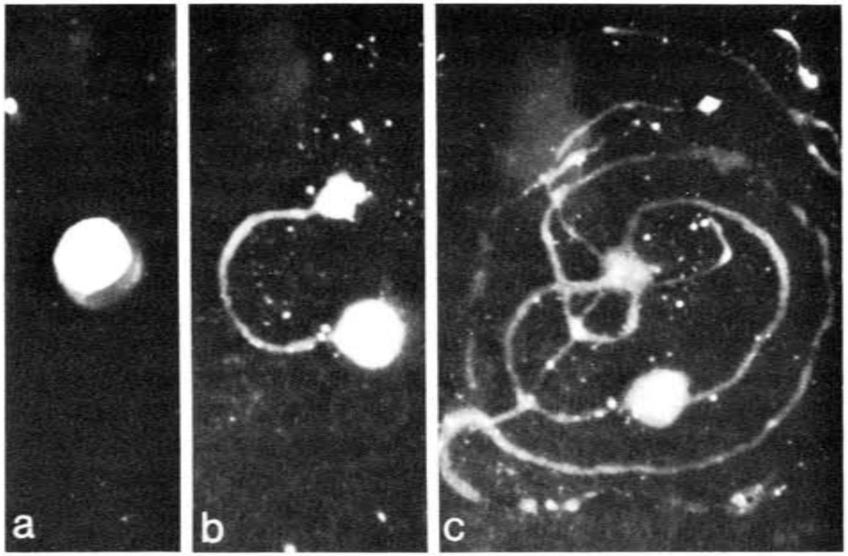

FIG. 9. Method for smearing cells. As seen in reflected light (which gives an appearance similar to that of dark-field illumination): (a) a testis, under oil; (b) the same testis after some cells have been spread into a narrow "channel"; (c) the cells from the same testis spread around in many "channels." Magnification ×11.

cells flow into channels that are only two or three cells wide, then they become flattened and internal details are quite easy to see. Bacteria-free spermatocyte smears made in this way live and divide for at least 5–8 hours. I have even seen telophase-II spermatocytes develop into spermatids when left overnight.

In order to treat cells with some experimental solution, one can transfer the testis to the experimental solution, as previously described, and then transfer the testis from the solution to a drop of Voltalef oil on a coverslip and make a smear preparation, as usual. This is not suitable, though, if one wants to observe cells while they are being perfused. Nor can one perfuse a smear preparation (say with colchicine–Ringer's), because the cells flow away when the solution is introduced under the oil. One method of observing a cell while it is being perfused is to hold the cells in place using a fibrin clot. To do this, the testis is transferred from Voltalef oil into a drop of fibrinogen (in Ringer's solution) that is on a coverslip under oil. Then one pierces the testis to release the cells, mixes the cells with fibrinogen, pulls the fibrinogen into channels, and then—when the cells are flattened—adds thrombin to cause the fibrinogen to clot (Forer, 1972). Once the cells are held in place in the clot, the oil can be removed and the cells placed in whatever aqueous medium is desired.

The Ringer's solution that I use is Ephrussi–Beadle insect Ringer's solution

(Ephrussi and Beadle, 1936), to which is added phosphate buffer at pH 6.8; the final solution contains 0.13 *M* NaCl, 0.005 *M* KCl, 0.003 *M* $CaCl_2$, and 0.01 *M* Sørensen's phosphate buffer, final pH 6.8. This Ringer's solution has been used to keep cells alive for hours in a clot (Forer, 1972), to treat testes in various ways (Behnke and Forer, 1967, 1972; Forer and Behnke, 1972c), and to keep cells alive for incorporation of tritiated thymidine during overnight incubations (A. Forer, unpublished). Begg and Ellis (1979b) have used a more complicated Ringer's solution, but in our hands the simpler Ephrussi–Beadle Ringer's solution plus buffer has been sufficient.

I have run into unexpected problems from "rubber bulbs" and from plastic tubing. We keep the Voltalef oil and the Halocarbon oil in glass bottles. The bottles have glass droppers that fit to the bottles with a ground-glass seal, and in order to get oil from the bottles we put "rubber" bulbs onto the ends of the glass droppers. If the bulbs are kept permanently on the glass droppers some vapor collects in the oil and kills the cells. This once cost me several months of work, so we now keep the bulbs on the ends of the droppers only when we need to remove oil. When the droppers are not in use we remove the rubber bulbs and cover the open ends of the droppers with Parafilm. Similarly, rubber tubing and soft plastic tubing leading from our distilled water bottle introduced into the water a contaminant that is toxic to the cells, and this plastic had to be replaced by Teflon.

All dissection tools are made from stainless steel; this is to avoid heavy-metal contamination during the dissection. We do not use special cleaning procedures for coverslips: they are simply taken new from the box, flamed, and used. For preparations using an inverted microscope we do not use wax to seal the coverslips over holes in glass slides, as we have previously done (e.g., Forer and Koch, 1973; Forer and Jackson, 1979). Rather, we tape coverslips over holes in glass or metal slides. The tape makes a leakproof seal, and is easily peeled off, especially if the end of the tape is bent over, sticky side against sticky side (Sillers and Forer, 1981a). [To affix coverslips over holes, we use "magic transparent tape" (3M Co.) for preparations kept under oil, and we use either No. 850 polyester film tape (3M Co.) or silicone vacuum grease (Dow) for preparations in which the oil is replaced by aqueous solution.]

IV. Other Relevant Data, and Hints on Rearing the Animals

Males can be distinguished from females both as adults and as pupae: the males have a cylindrical abdomen and the rear end is more or less flat, with "claspers" on the sides, whereas the female abdomen tapers to "points" at the rear end (Figs. 4 and 10). The pointed ovipositor at the rear end of females

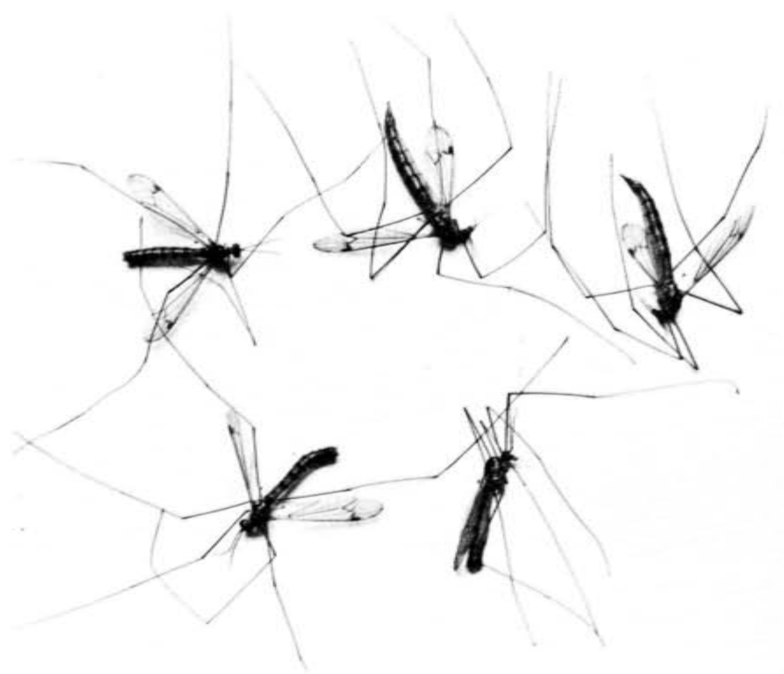

FIG. 10. Male and female *N. abbreviata* adults. The females (the two furthest right in the upper row) have pointed rear ends, whereas the males have more or less flat rear ends. Magnification about ×0.9.

appears double as viewed from the side, but usually single when viewed from the top or bottom (e.g., Fig. 4).

Males remain fertile after mating with single females; that is to say, individual males can be used to mate with at least four females and still give fertilized eggs. Females, too, can mate with several males.

Inbreeding (brother-sister mating) causes reduced numbers of progeny compared with outbreeding. I do not take precautions to avoid inbreeding, however, for the following reason. We generally have several pairs that mate every day, and we consequently need to throw away animals at about the time we transfer them into large dishes. So, rather than *pre*select against brother-sister matings, we *post*select: we discard those dishes in which the animals eat poorly and we keep those in which the animals eat well. (In the former, much of the nettle remains uneaten after 2–3 days, and the paper is not eaten; in the latter, the nettle is gone, and the paper is eaten.) This "postselection" method is easier for us than trying to keep track of siblings.

I keep stocks of four species: *Nephrotoma suturalis* (Loew), *N. ferruginea* (Fabricius), *N. abbreviata* (Loew), and *N. wulpiana* (Bergroth). The methods described above have also been used to rear *N. macrocera* (Say), *N. maculata* (Meig), *N. flavipalpis* (Meig), *N. flavescens* (Linneaus), *N. breviorcornis* (Doane), *Tipula* (*Yamatotipula*) *furca* (Walker), and *Limonia* (*Metalimnobia*) *annulus cinctipes* (Say), but I no longer keep these stocks.

There are no mutant stocks of any species I rear (or of any others that I know of). Dietz has had stocks of *N. ferruginea* that contained chromosomal rearrangements (e.g., Dietz, 1969, 1972a,b; Fuge, 1975), but I do not know if he still keeps these. Before describing methods for getting chromosomal rearrangements, it is relevant to point out that Dietz and Fuge work with one of the same crane flies that I do, but they use the name *Pales ferruginea* (e.g., Dietz, 1969; Fuge, 1980a) whereas I use the name *Nephrotoma ferruginea!* According to Dr. George Byers (Entomology Department, University of Kansas), the two names arose as follows. Meigen named the genus *Pales* in 1800, and renamed it *Nephrotoma* in 1803. Macquart named it *Pachyrhina* in 1834. From 1840 to 1900 the genus was called both *Nephrotoma* and *Pachyrhina*. In the early 1900s Herdel discovered the rare 1800 paper, and hence, by "priority," the name should be *Pales*. Because of possible "taxonomic instability," however, the naming of this genus was taken to the International Commission on Zoological Nomenclature; the decision (in 1963) was that the original 1800 paper is considered "suppressed," and that the names in it (including *Pales*) should be excluded from use. [This is also described in Hemmingsen (1965), in a note on p. 104, citing *Bull. Zool. Nomencl.* **20,** 321–400 (1963).] My usage complies with this advice, and with the official decision on nomenclature.

Chromosomal rearrangements can be induced by X-irradiating adult males, single-pair mating the F1, and brother-sister mating the F2 (Rohloff, 1970). I have used this method to obtain translocations in *N. suturalis*. Adult males were X-irradiated for a total of 4000 roentgens (100 R per minute for 40 minutes), and were then mated with virgin females. Any given rearrangement in any given sperm will appear in only *one* of the F1 animals, and at that only as a heterozygote. In order to get "clones" with altered chromosomes, the F1 are single-pair mated with nonirradiated (wild-type) adults. If the F1 animal contained no chromosomal rearrangements, then all F2 animals should have no chromosomal rearrangements. If, on the other hand, the F1 animal was heterozygous for a rearrangement, then half the F2 animals will be heterozygous for the rearrangement and half will not have the rearrangement at all. To see which animals contain chromosomal rearrangements, one observes cells at metaphase-anaphase of meiosis-I and looks for tetravalents instead of bivalents (caused by a heterozygous reciprocal translocation), or for trivalents (caused by autosomal translocation onto the X- or Y-chromosome), or for chromosomal bridges (caused by heterozygous inversions). Once the desired rearrangement is found, one can obtain homozygous stocks by brother-sister matings.

Which species of crane fly should one choose to work with—and why does A. Forer keep four species instead of just one? First, note that different species have different habitats, ranging from freshwater ponds and streams (Byers, 1978; Kavaliers, 1981), to marine habitats (e.g., Toye, 1967), to pasture, to moist soil, to desert habitats (e.g., Hemmingsen, 1958, 1959), to those in the Arctic, and those that emerge in the winter and are found on the snow. Consequently the life cycles vary: those I keep in the laboratory have generation times of about 7 weeks, at room temperature, while others can be one generation per year—or, in the Arctic, for example, 2 or 4 years per generation (e.g., Hofsvang, 1972; MacLean, 1973). Some species have a diapause (e.g., Butterfield, 1976a). Some species, though, have generation times of only 3–4 weeks, and these might be preferred for genetic experiments.

We keep different species because different ones have somewhat different cytological characteristics. For example, the sex chromosomes are different sizes in different species (e.g., Henderson and Parsons, 1963). When the sex chromosomes are small, as they are in *N. suturalis,* they can be lost in among cytoplasmic granules that invade the spindle region as the sex chromosomes start to move poleward. For following sex-chromosome movements, then, *large* sex chromosomes are preferable; that is why we keep *N. abbreviata* (Fig. 11). [One can even choose crane flies that do *not* have sex chromosomes and in which $2n = 6$ rather than the more usual $2n = 8$ (e.g., White, 1949; Dietz, 1956); *Limonia (Metalimnobia) annulus cinctipes,* for example, has $2n = 6$ and has no sex chromosomes (A. Forer, unpublished data).] As another example of different cytology, the spindle birefringence can be different in different species. When studied at room temperature, *N. suturalis* spermatocytes at metaphase and anaphase have very little continuous-fiber birefringence, whereas *N. ferruginea* spermatocytes at metaphase and anaphase have considerable continuous-fiber birefringence (e.g., Forer, 1976; A. Forer and P. Shirley, unpublished data). Finally, although *N. ferruginea* has more cells per testis than *N. suturalis,* the synchrony of the cells in *N. ferruginea* testes is not as good as the synchrony of cells in *N. wulpiana* testes, or the other two species we rear.

It is relevant to point out that in some crane flies the chromosomes are achiasmatic in male meiosis (e.g., Dietz, 1956). Thus one can directly compare chiasmatic versus achiasmatic meiosis by comparing related species. It is also relevant to note that crane flies have polytene chromosomes, which are useful for some kinds of experiments.

To collect crane flies in the wild, one uses standard methods. They can be caught with a net, or by using a night-light in conjunction with a net or trap. Larvae can be collected by spraying orthodichlorobenzene onto the surface of the ground. Crane fly larvae immediately come to the surface, unharmed, and can then be collected and reared (Barnes, 1937, 1941). One should be somewhat cautious in bringing animals into the laboratory from the wild, though, because

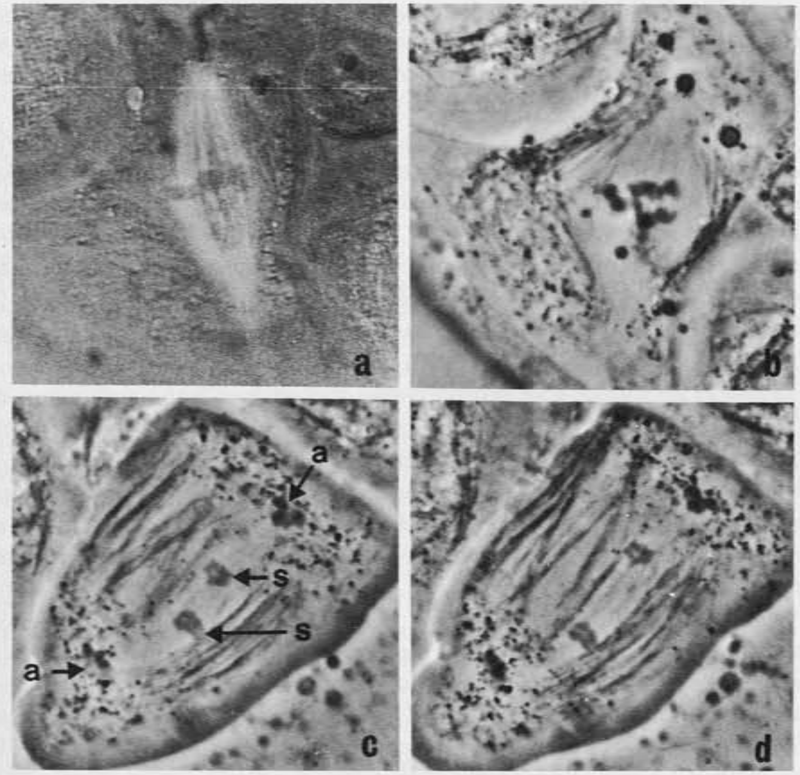

FIG. 11. *N. abbreviata* spermatocytes. (a) A metaphase cell as seen in a polarizing microscope; the spindle fibers appear bright against the gray background. (b) Same cell as (a), as seen in a phase-contrast microscope; the picture was taken shortly after the autosomal half-bivalents began to move poleward in anaphase. (c and d) Sex-chromosome movements—in a different cell than (a) and (b)—as seen in a phase-contrast microscope. The autosomal half-bivalents (a) are at the poles, and the sex chromosomes (s) then move poleward, as in (c) and (d). In this species the sex chromosomes are at least as large as, if not larger than, the autosomal half-bivalents (cf. Fig. 16c and 16d with Fig. 16b). Magnification ×980.

there are known pathogens that can kill them, such as viruses (e.g., Bird, 1961; Carter, 1973, 1974, 1975), microsporidians (e.g., Weissenberg, 1926; Carter, 1976), and coccidians (Beesley, 1977a,b). Mites also attack (and hang onto) crane flies; in fact, I lost my first laboratory stock of *N. abbreviata* because of infections by mites. (The only way I know of to fight a mite infection is to wash down table tops daily, and to remove larvae from dishes and place them in new paper daily.)

Other methods have been used for rearing crane flies in the laboratory, and these can be found in Barnes (1937), White (1951), Laughlin (1958, 1960), and

Butterfield (1976b). However, these do not seem as reliable or convenient as the method just described. Ricou (1967) has compared the nutritional value of various grains, in having crane fly larvae grow and develop; unfortunately, however, nettle leaves were not included in the list of those tried.

The temperature at which the stocks are reared affects the growth rate of the flies (A. Forer, unpublished data; Butterfield, 1976b). Our *N. suturalis* stocks do not tolerate temperatures above about 30°C, and our *N. ferruginea* stocks do not tolerate temperatures above about 27°C (see also Schaap and Forer, 1979)—but for each species the rate of development is faster at higher temperatures than at lower temperatures. [Butterfield (1976b) and Carter (1975) found the same for a different species.] For example, *N. suturalis* has a generation time of about 5.5–6 weeks at 27°C, but about 7–7.5 weeks at 22°C.

I know of no way to store animals frozen, as one does with tissue culture cells or *Dictyostelium,* for example. It would be convenient to be able just to thaw them out when they are needed, but I know no way of doing this. The growth rate does slow almost to zero when animals are kept at about 10°C, however, and I have kept larvae at 9°C for months while their siblings at room temperature went through several generations. Those at 9°C were moistened and fed when necessary (about every 1 or 2 weeks), but they stayed at about the same stage of development as when they were first put into the incubator. They developed normally when returned to room temperature. (This is the most useful way I know of to get an initial stock spread out so that one has all stages at all times: let some develop at 25°C, others at 20°C, and hold others back at 10°C.) Storing animals at 10°C is not as useful as deep-freezing tissue culture cells, but it is the only method I know of for keeping some "in reserve."

Acknowledgments

Roland Dietz first introduced me to growing crane flies, in 1961, and he gave me seven larvae of *N. suturalis,* from which my present stock derives. He and I have both modified our laboratory rearing methods, in divergent ways: My most recent methods are given here. I am grateful to Roland for introducing me to the flies and cells. I am grateful to Dr. Jim La Fountain, Jr., in Buffalo, New York, for help in replenishing our *N. suturalis* stock in our time of need, and to Dr. Donna Kubai for sending the eggs of *N. abbreviata,* from which our present laboratory stock (designated DK) derives. I continue to be grateful to Dr. George Byers (University of Kansas) for identification of stocks and for general encouragement, to Alexandra Engberg, and to F. B. T. Birkes. I thank Mrs. A. Adhihetty and Mrs. D. Gunning for excellent typing, through several versions of this chapter.

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