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CHANGES IN SPINATION PATTERNS OVER THE COURSE OF METACERCARIAL DEVELOPMENT OF *DIPLOSTOMUM PSEUDOSPATHACEUM* NIEWIADOMSKA, 1984 (TREMATODA, DIPLOSTOMIDAE)

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Metacercarial development of most *Diplostomum* species including *D. pseudospathaceum* occurs in the eye lenses of their fish hosts and is accompanied by radical morphological changes often referred to as metamorphosis. One of the structures undergoing substantial changes in *D. pseudospathaceum* are tegumental spines. The present study used phalloidin staining and confocal microscopy to examine these changes in *D. pseudospathaceum* over the course of development from 3-day-old to infective metacercariae. Although the general pattern of spination remained essentially unchanged, most larval spines continued growing in size until late in metacercarial development. From day 10 of development, larval spination was gradually replaced by small incipient definitive spines and the infective metacercariae had only definitive spination. The possible adaptive role of spines in developing metacercariae is discussed.

Key words: Trematoda, *Diplostomum*, metacercariae, spination, sensilla, development.

ИЗМЕНЕНИЯ ОРГАНИЗАЦИИ ВООРУЖЕНИЯ ПОКРОВОВ В ХОДЕ РАЗВИТИЯ МЕТАЦЕРКАРИЙ *DIPLOSTOMUM PSEUDOSPATHACEUM* NIEWIADOMSKA, 1984 (TREMATODA, DIPLOSTOMIDAE)

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Развитие метацеркарий большинства видов *Diplostomum*, включая *D. pseudospathaceum*, происходит в глазном хрусталике рыб и сопровождается глубокими морфологическими трансформациями, которые часто называют метаморфозом. Одной из структур, которая претерпевает существенные изменения у *D. pseudospathaceum*, являются шипики тегумента. В настоящем исследовании эти изменения изучались у

D. pseudospathaceum при помощи окраски фаллоидином и конфокальной микроскопии на последовательных стадиях развития от трехдневных до инвазионных метацеркарий. Хотя общая организация вооружения покровов оставалась в целом неизменной, большинство личиночных шипиков продолжало расти до самых поздних стадий развития метацеркарий. Начиная с 10-го дня развития у метацеркарий происходила постепенная замена личиночных шипиков на маленькие зачаточные дефинитивные шипики, и инвазионные метацеркарии были покрыты только дефинитивными шипиками. В статье обсуждается возможная адаптивная роль шипиков у развивающихся метацеркарий.

Ключевые слова: Trematoda, *Diplostomum*, метацеркарии, шипики, сенсиллы, развитие.

Metacercariae of the genus *Diplostomum* are common pathogens of fishes infecting the brain and eyes of over 100 fish species (Chappell, 1995) including those of importance for commercial fisheries. Metacercariae of most *Diplostomum* species reside in the eye lenses, often causing severe vision impairment (various types of cataract) in their hosts. When a cercaria of a lens form enters the host, it migrates as diplostomulum through the host body and eventually establishes itself in the eye lens, where it continues its development as metacercaria. The development of *Diplostomum* from cercariae to infective metacercariae is accompanied by radical morphological changes often referred to as metamorphosis (Shigin, 1986; Ginetsinskaya, 1988; Galaktionov, Dobrovolskij, 2003). Recent electron microscopic (Podvyaznaya, Dobrovolsky, 2001; Podvyaznaya, 2013) and confocal studies (Petrov, Podvyaznaya, 2015) of *D. pseudospathaceum*, one of the most common and pathogenic species of *Diplostomum*, gave further evidence to the metamorphic nature of changes in some cercarial organs: the most profound of those occur in the midgut, body-wall musculature and anterior organ as it transforms into the oral sucker. One of the structures in *D. pseudospathaceum* that undergo substantial changes during metacercarial development are tegumental spines: larval spination is being lost in developing metacercariae and is replaced by small incipient definitive spines (Shigin, 1986; Podvyaznaya, 1999). Since actin is known to be the primary component of spines in trematodes (Cohen et al., 1982; Abbas, Cain, 1987), the spines can be stained with actin-specific probes, such as phalloidin, with subsequent visualization under a laser scanning confocal microscope. The aim of this study was to use this staining method in conjunction with confocal microscopy to examine changes in spination patterns during the course of metacercarial development of *D. pseudospathaceum*. Body armature of infective cercariae of this species is not described in this study, because it has been recently studied in detail by Selbach et al. (2015), and their results are in good agreement with our own observations.

MATERIAL AND METHODS

Metacercariae of *D. pseudospathaceum* of various ages were obtained from experimentally infected guppies (*Poecilia reticulata*). The snails *Stagnicola palustris* infected by *D. pseudospathaceum* were collected from Lake Pertozero in the outskirts of Petrozavodsk (62°10' N, 33°58' E). Each guppy was placed for 1.5—2 h in a Petri dish (80 × 35 mm) filled with water containing 25—30 freshly emerged cercariae. All fish were then maintained in a common tank

at 22—23 °C and dissected 3, 6, 7, 8, 10 and 20 days after infection; 20-day-old larvae were found to have all features of mature metacercariae. In addition, metacercariae of later stages of development (pre-infective metacercariae) and infective (mature) metacercariae were extracted from eye lenses of *Rutilus rutilus* caught in Lake Pertozero near the habitat of infected *S. palustris*. The worms were sorted, processed and stained as described in Petrov and Podvyaznaya (2015). Confocal microscopy was performed on a Leica TCS SP5 microscope at the Taxon Resource Research Centre (Zoological Institute RAS, Saint-Petersburg, Russia).

RESULTS

Three-day-old metacercariae still have all the essential features of cercariae in their spination patterns. In these metacercariae, the entire surface of the protruding region of the anterior organ (fig. 1, *A*, *B*, *ra*) is covered with pre-oral and post-oral spines. The pre-oral spines are located at the tip of the anterior organ and are usually partially or completely retracted into it; only the large spines (2—3.5 μm in length) comprising the medial group of pre-oral spines are clearly seen in most individuals (fig. 1, *C*, *pr*). Posterior to the pre-oral spines are 8—9 closely spaced circumferential rows of post-oral spines, with the length of spines decreasing posteriorly (fig. 1, *A—C*, *pt*). Behind the protruding region of the anterior organ is a belt of scarce spines about 1.5 μm in length (area of dispersed post-oral spines, fig. 1, *A*, *C*, *ds*); this belt is somewhat wider on the ventral side and laterally. The body spination between the post-oral spines and acetabulum (ventral sucker) is arranged in 8 complete rings (fig. 1, *D—F*, *r1-8*) positioned more or less at equal distances (5—10 μm), with the posterior-most situated immediately in front of the acetabulum. The next 3 rings (9 to 11th) (fig. 1, *D—F*, *r9-11*) are located at the level of the acetabulum and are interrupted medially on the ventral and dorsal sides each forming 2 lateral fragments. The spines in these 11 rings are 1.3—1.7 μm long and aligned in a single row, except for the first 2 rings that have ventrally 2 rows of spines. Some of the rings may also have additional spines laterally.

The opening of the acetabulum in 3-day-old metacercariae is surrounded by 2 rows of spines, with the spines in the rows arranged in an alternating pattern (fig. 1, *G*). The spines in the outer row are much longer (2—2.2 μm, fig. 1, *G*, *as2*) than those in the inner row (0.7—1 μm, fig. 1, *G*, *as1*). Just outside the outer row of spines lies a ring of 6 sensilla (fig. 1, *G*, *arrows*), which are roughly equidistant from one another and arranged in a regular hexagon. In some metacercariae only 3—5 sensilla are clearly visible in this ring; however, a more careful examination shows that the ring consists invariably of 6 sensilla and apparent variability is likely due to a low concentration of actin in some sensilla. Another, inner ring of somewhat smaller sensilla is located to the inside of the spines in the wall of the acetabular cavity (fig. 1, *G*, *arrowhead*); the number of sensilla in this ring appears to vary from 1 to 6.

Immediately behind the acetabulum is a transverse belt of 5—6 rows of short sparse spines (fig. 1, *D*, *tb*). The 11th ring of spines is often merged laterally into this belt. Two wide ventrolateral longitudinal bands of spines (fig. 1, *D*, *vlb*) extend from the transverse belt towards the posterior end of the body where they merge with the posterior collar of spines (fig. 1, *D*, *E*, *pc*). The latter is
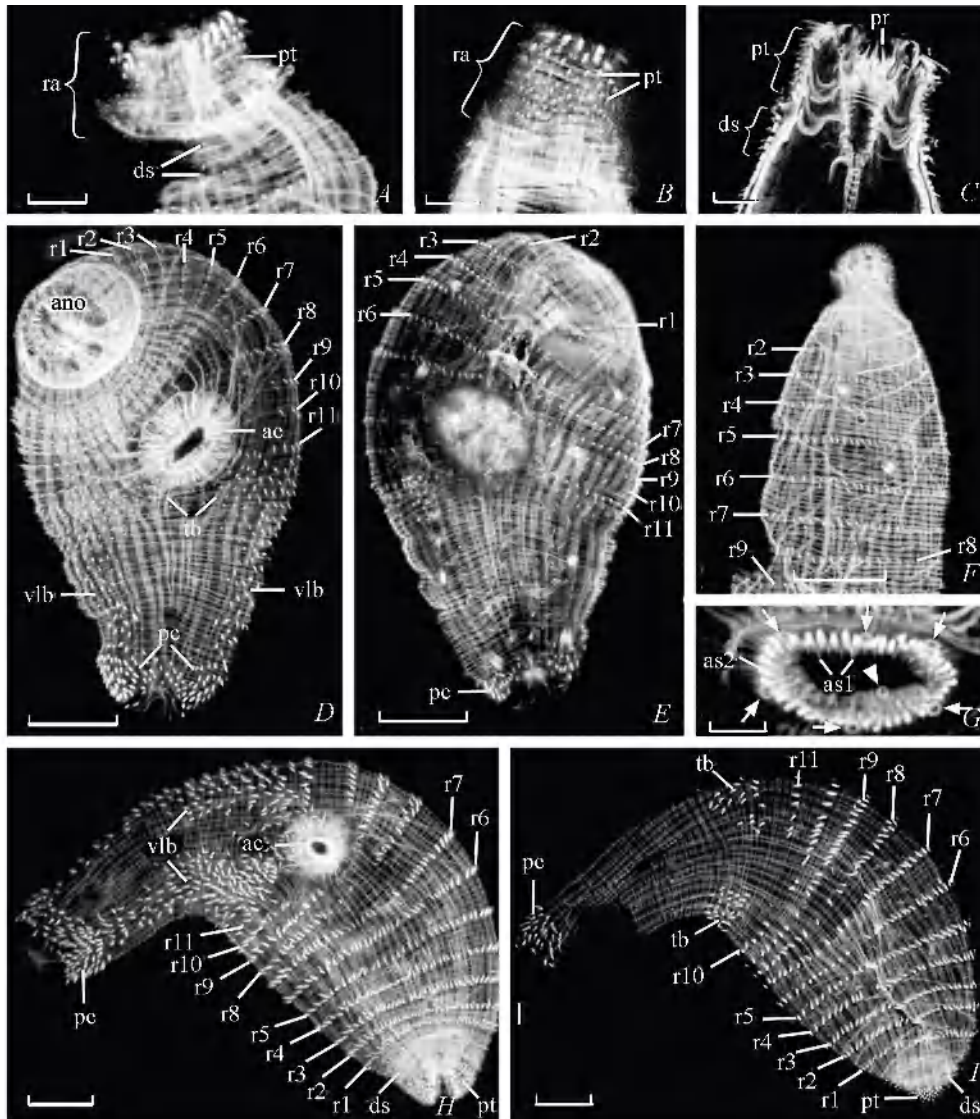
Fig. 1. Spination of early *D. pseudospathaceum* metacercariae.

A—C — anterior end of 3-day-old metacercariae showing spination of the anterior organ; *D* — 3-day-old metacercaria from the ventral side; *E* — 3-day-old metacercaria from the dorsal side; *F* — anterior body region of a 3-day-old metacercaria showing the preacetabular rings of spines; *G* — acetabular spination of a 3-day-old metacercaria with sensilla of the outer (*arrows*) and inner (*arrowhead*) rings; *H* — 6-day-old metacercaria from the ventral side; *I* — 6-day-old metacercaria from the dorsal side. Scale bars: *A—C, G* — 5 μm; *D, E, H, I* — 20 μm. ac — acetabulum, ano — anterior organ, as1-2 — rows of acetabular spines, ds — dispersed post-oral spines, pc — posterior collar of spines, pr — pre-oral spines, pt — post-oral spines, r1—11 — rings of spines, ra — rostral region of anterior organ, tb — transverse belt of spines, vlb — ventrolateral bands of spines.

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composed of 7—8 rows of spines on the ventral side and 3—4 rows on the dorsal side and encircles the posterior blunt end of the body, where in cercariae the body transitions into the tail. The spines of the lateral bands and posterior collar are about twice as wide as those on the rest of the body.

Throughout the metacercarial development up to 10-day-old metacercariae (intensively growing metacercariae with a distinct holdfast primordium), the overall spination pattern remains essentially the same as in 3-day-old metacercariae. All specimens had 11 rings of spines (figs. 1, H, I, 2, A, B, r1-11), with 2 or 3 posterior rings interrupted ventrally by the acetabulum, and postacetabular spination composed of the transverse belt (figs. 1, I, 2, A, E, F, tb), 2 ventrolateral longitudinal bands (figs. 1, H, 2, B, vlb) and the posterior collar (figs. 1, H, I, 2, B, pc). Two anterior rings are usually arranged ventrally into 2 rows and some rings may have additional spines laterally. In most 10-day-old individuals, the 10th ring consists ventrally of 2 or 3 rows. The transverse belt can be interrupted medially on the dorsal side (figs. 1, I, 2, A, tb) or continuous around the body.

As metacercarial development proceeds, most spines on the body become progressively longer. On the anterior end of the body, only the dispersed post-oral spines increase in length to any significant degree; they start growing in 6-day-old metacercariae and reach 2.5 μm by day 10, after which time they stop growing. The spines of the rings and postacetabular spines grow more or less evenly up to day 10 of development reaching the lengths of 3.5—4.5 and 2.5—3.5 μm, respectively. After that, their growth stops or slows down significantly. In some 8- to 10-day-old individuals, small, irregularly distributed spines become visible between the rings of preacetabular spines (fig. 2, D). The acetabular spines also continue to grow (fig. 2, E, F): by day 6, the spines in the inner row around the acetabular opening increase in size and become comparable in length to the spines in the outer row (ca. 2.3 μm). In some specimens, a third incomplete row of acetabular spines are readily seen between the other two rows (fig. 2, F, as3).

From day 10 of development, the larval spines begin to resorb. The pre-oral spines are already absent in most 10-day-old individuals and disappear completely in pre-infective metacercariae (large metacercariae with distinct primordia of holdfast, lappets and hindbody). Acetabular and post-oral spines are still present in most pre-infective metacercariae, but some individuals lack them entirely. The spines of the rings and postacetabular area and dispersed post-oral spines persist to pre-infective metacercariae, but their number becomes reduced and the remaining spines are often very thin and have distinctly irregular shapes (fig. 2, G).

The definitive spines begin to emerge in 10-day-old metacercariae. The growth of these spines proceeds from anterior to posterior: they first appear immediately behind the oral sucker (fig. 2, C, df) and then emerge on the rest of the body. In infective metacercariae, all cercarial spines are completely replaced by small incipient definitive spines about 0.5 μm in length (fig. 2, H). The definitive spines are evenly spaced in a checkerboard pattern and cover most of the body including the area around the holdfast, which was previously devoid of spines, but absent on the hindbody (posterior body region containing primordia of the reproductive system) and dorsally over an area about 60—70 μm in front of the hindbody. The definitive spines are also absent around the mouth and on
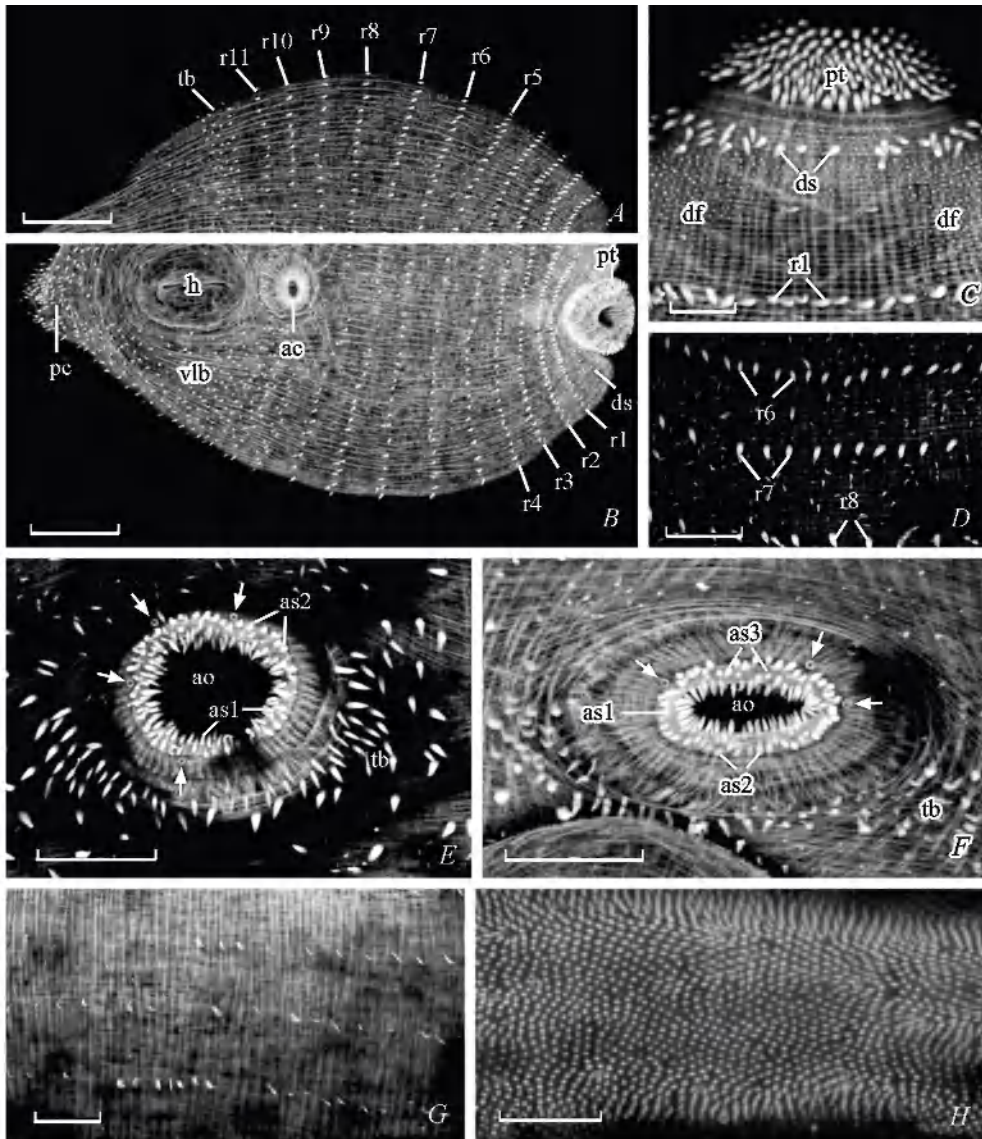
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Fig. 2. Spination of late and infective metacercariae of *D. pseudospathaceum*.

A — 10-day-old metacercaria from the dorsal side; *B* — 10-day-old metacercaria from the ventral side; *C* — anterior end of a 10-day-old metacercaria showing spination around the oral sucker and emerging definitive spines; *D* — region of the dorsal body surface of a 10-day-old metacercaria showing small irregular spines between the preacetabular rings; *E* — acetabular spination of a 10-day-old metacercaria with the outer ring of sensilla (*arrows*); *F* — acetabular spination of a pre-infective metacercaria with the outer ring of sensilla (*arrows*); *G* — region of the dorsal body surface of a pre-infective metacercaria showing irregular resorptive spines of the preacetabular rings; *H* — definitive spination on the ventral side of an infective metacercaria. Scale bars: *A*, *B* — 50 μm; *C*, *H* — 10 μm; *D*, *E*, *F* — 20 μm. ac — acetabulum, ao — acetabular opening, as1-3 — rows of acetabular spines, df — definitive spines, ds — dispersed post-oral spines, h — holdfast, pc — posterior collar of spines, pt — post-oral spines, r1—11 — rings of spines, tb — transverse belt of spines, vlb — ventrolateral bands of spines.

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the lappets. The two rings of sensilla in the acetabulum persist over the course of metacercarial development, but in infective metacercariae there is also a third, outermost ring of 26—28 sensilla located in the acetabular fold.

DISCUSSION

Diplostomid cercariae have well-developed body spination (Blair, 1977; Niewiadomska, 1986; Niewiadomska, Kiseliene, 1994; Selbach et al., 2015), which is used by larvae for penetration and migration in the second intermediate host and during site-establishment process (for details, see Höglund, 1991; Conn et al., 2008). In metacercariae of *Diplostomum phoxini, D. pseudospathaceum* and *Ornithodiplostomum ptychocheilus*, these larval spines were shown to be replaced by incipient definitive spines (Bibby, Rees, 1971; Podvyaznaya, 1999; Goater et al., 2005; Conn et al., 2008, this study) and it is very likely that this spination replacement occurs in the majority of diplostomid species. Conn et al. (2008) have demonstrated in an experimental study that in *O. ptychocheilus* the cercarial spines disappear at a relatively early stage of development in the second intermediate host, after the larvae have finished the site-establishment process in the brain. Our study shows that in contrast with *O. ptychocheilus*, a major part of cercarial spination in *D. pseudospathaceum* persists until very late in metacercarial development, and during this period the spines increase in length by approximately a factor of 2.5 to 4. The most intensively growing spines are those comprising the rings, ventrolateral bands and the area of dispersed post-oral spines. These spines cover the most rapidly growing regions of the body and they are also the last to be resorbed; the spines involved in host penetration (pre-oral, post-oral and acetabular spines) disappear earlier during metacercarial development. A comparison of spination from different developmental stages also indicates that some spines may be so small in cercariae and early metacercariae that they appear almost indistinct, but as they grow in size, they become readily seen in later metacercariae. This may give a false impression that larval spines increase in number in developing metacercariae.

The continued growth of larval spines raises the possibility that in *D. pseudospathaceum* cercarial spination can have some adaptive significance not only for infective cercariae and diplostomula, but also for developing metacercariae. Shigin (1986) has noted that young metacercariae are able to move in the cortical layer of the eye lens and it is likely that spines help them wade through this relatively dense medium. The spines may also participate in mechanical breakdown of crystalline lens tissue, on which these parasites feed. Podvyaznaya (1999) has demonstrated that cercarial spines are resorbed in the tegument, rather than shed, as was suggested by Shigin (1986). It is, therefore, quite possible that the material of the resorbed spines (filamentous actin and associated proteins) is used in some way to build new spination.

The acetabular muscles have been studied in detail in two diplostomoid species: *Cotylurus cornutus* (Rudolphi, 1808) (Krupenko, 2014a) and *D. pseudospathaceum* (Petrov, Podvyaznaya, 2015) and have been shown to be arranged in 3- or 6-fold radial symmetry. The arrangement of outer acetabular sensilla in *D. pseudospathaceum* also conforms to this 6-fold symmetry. The acetabular sensilla in the inner ring appear to vary in number; this number, however, never

exceeds 6 and it is very likely that this ring also has 6 sensilla, but some sensilla are invisible due to a low concentration of actin. Of the 13 trematode species from 11 different families studied for acetabular musculature (Krupenko, 2010, 2011, 2014a, b; Petrov, Podvyaznaya, 2015), 3- or 6-fold symmetry is evident only in diplostomoid species and may constitute a synapomorphy of this super-family.

Phalloidin staining for spines holds the potential to become a valuable taxonomic tool, because spination is widely used as a source of diagnostic characters in trematode taxonomy. For instance, in *Diplostomum*, differences in spination, such as the arrangement of preacetabular rings and spines of the anterior organ, have been shown to be instrumental in species identification (Blair, 1977; Niewiadomska, Kiseliene, 1994; Selbach et al., 2015, and others). Because phalloidin staining is highly specific for filamentous actin and confocal microscopy provides better resolution than conventional light microscopy, these methods are less susceptible to errors and can be especially useful in differentiating closely related species.

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